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Cytotoxic Effect of *Cayratia carnos* Leaves on Human Breast Cancer Cell Lines

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Abstract: Leaves of *Cayratia carnos* have been ethnomedically claimed to possess a wide array of biological activities including anticancer activity. To verify the folklore claim, this study was performed in a Human breast carcinoma cell lines, MCF-7 and MDA-MB-231. Methanol and aqueous extracts of the leaves of *C. carnos* showed cytotoxic effect on MCF-7 and MDA-MB-231 cell line, as determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) microculture tetrazolium viability assay. Subcellular alterations were evaluated by using normal inverted microscope. Cells treated with methanol extract showed degeneration of cytoplasmic organelles, profound shrinkage of cells and apoptotic characteristics. The results showed that the methanol extract possesses cytotoxic effect which was greater than aqueous extract when compared to that of the control.

Key words: *Cayratia carnos*, methanol extract, aqueous extract, breast cancer, folk medicine

INTRODUCTION

Cancer is one of the most life-threatening diseases of modern times. It is a group of disease characterized by uncontrolled growth and the spread of abnormal cells. If the spread is not controlled, it can result to death. The disease is caused by a number of external factors (tobacco, chemicals, radiations and external organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). It is now the fourth leading cause of death among medically certified deaths in Malaysia. Conditions may worsen if necessary measures are not taken (Lim, 2002). Frequently occurred cancer diseases are life style dependent with offending factors such as tobacco and nicotine usage, poor socio-economic factors, multiple pregnancies and poor sexual hygiene (Hunter *et al.*, 2002; Cairns, 2006). Among all cancers in women, breast cancer still remains the leading cause of cancer related mortality (30%) in females (Canfeza *et al.*, 2002). So, we have selected breast cancer cell lines (MCF-7 and MDA-MB-231) for present study.

Cancer research is increasingly being drawn towards the investigation of plant derived anti- cancer compounds many of which have been used traditionally as herbal remedies for centuries. Paclitaxel an effective tool for the treatment of ovarian and breast cancer which is isolated from the bark of Pacific yew tree is the best examples to benchmark the importance

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of medicinal plants for curing cancer (Yaniv and Uriel, 2005). Recently, Fumoleau *et al.* (1995) and Mantle *et al.* (2000) suggested several studies demonstrating that medicinal plants could bring to the identification of antitumor compounds. *C. carnososa* (Vitaceae) is a perennial climber with stems woody at base widely distributed in Africa, Australia, Asia and Pacific Islands. It is otherwise called as Foxgrape (Purohit *et al.*, 2003). The whole plants have been traditionally used in vitiated conditions of *Vata* and *Kapha*, tumors, fever, neuralgia and splenopathy (Warrier *et al.*, 1996). It is also used traditionally for the treatment of ulcers, wounds, hemorrhoids (Khare, 2008) and as CNS depressants (Rashtra, 2008). The phytochemical review reported the presence of hydrocyanic acid, delphinidin and cyanidin (http://www.indian-herbs-exporters.com/_cayratia_carnososa.html). As per the traditional claim the leaves and tubers of *C. carnososa* is taken orally for the treatment of malignancy.

There was no literature available on this plant for this claim, so to scientifically investigate these claims, the present study, investigated the effects of methanol and aqueous extract from the leaves of *C. carnososa* on Human breast carcinoma cell lines MCF-7 and MDA-MB-231. Here, we wish to report that the extracts exhibited cytotoxic effects with intense alterations in cellular mechanism and virtues further studies as a potential therapeutic agent for cancer treatment.

MATERIALS AND METHODS

Cell Culture

Human breast carcinoma cell lines MCF-7 and MDA-MB-231 were obtained from ATTC and maintained in RPMI 1640 supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin at 37°C in 5% CO₂ incubated in a humidified incubator (Greeve *et al.*, 2004). The flask containing cells were incubated in a humidified incubator with 5% CO₂ at 37°C. Cultures were frequently examined under inverted microscope (Micros, Austria). Once the cells reached 80% confluency, media was removed and the cells were washed 3 times with 7 mL of PBS (Phosphate Buffer Saline). Two milliliter of trypsin was added to the cells and was incubated for 5 min. The flask was tapped gently to detach the cells from the wall of the flask to appear as single cells. Ten milliliter of RPMI 1640 with 10% Fetal Bovine Serum (FBS) was added to the flask and the content of the flask was resuspended to allow the cells to disperse. About 6 mL of cell suspension was transferred into a 75 cm³ flask. Ten milliliter of RPMI 1640 with 10% FBS was then added and incubated in 5% CO₂ at 37°C. The cells were frequently examined under an inverted microscope for confluency and viability.

Plant Material

The leaves of *C. carnososa* were collected from Taman Kemacahaya, Batu-9, Cheras, Selangor, Malaysia in the month of May 2008. The plant material was authenticated by Dr. Sani Miran, Botanist, National University Malaysia, Selangor, Malaysia. A voucher specimen of the collected plant sample was kept in the herbarium of Masterskill University College of Health Sciences, Malaysia (MUCHSPHA/C1/M 005).

Preparation of *C. carnososa* Extracts

Fresh leaves collected were dried under sunshade. Methanol and aqueous extracts were prepared by maceration of coarsely powdered leaves in methanol and water separately for 7 days (Sunilson *et al.*, 2008). The extracts were concentrated under vacuum on rotary evaporator (Buchi, USA) and then dried in lyophilizer (Labconco, USA) under pressure to yield 8.6 and 32.6% of extract respectively. The crude plant extracts were redissolved in

dimethylsulfoxide (DMSO) and final concentration of DMSO was 0.1% (v/v). The stocks were serially diluted using the same solvent (0.1% DMSO). Control cells were exposed only to 0.1% DMSO.

Preliminary Phytochemical Screening

Phytochemical tests were carried out for the methanol and aqueous extracts of *C. carnososa* employing standard procedures described in Harborne (1998). Presence of secondary plant metabolites such as amino acids, carbohydrates, saponins, tannins, phytosterols, alkaloids, proteins, glycosides, flavanoids and phenolic compounds were tested.

Cytotoxic Assay

The cytotoxic profiles of the extracts were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide Microculture Tetrazolium (MTT) viability assay as described by Mosmann (1983). Cell suspensions were seeded into 96-well microtitre plates at a plating density of 0.1 million cells mL⁻¹. After 24 h incubation, cells were exposed to various concentration of plant extract and incubated for 68 h. MTT (5 mg mL⁻¹) was added to each well at appropriate time and further incubated for 4 h after which the media was removed. DMSO was added later into each well to solubilize the formazan crystals. The absorbance was read at a wavelength of 595 nm using a microtitre ELISA plate reader. Experiments for each extract were carried out in triplicate including untreated cell control and a blank cell-free control. The percentage cellular viability was calculated with the appropriate controls taken into account. The concentration which inhibited 50% of cellular growth (IC₅₀ value) was determined. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition} = \frac{\text{OD control} - \text{OD treated}}{\text{OD control}} \times 100$$

The cytotoxic effect of sample on cancer cells was expressed as IC₅₀ values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Morphological Studies of Cell Lines using Normal Inverted Microscope

Morphological studies by using the normal inverted microscope were carried out in order to observe the morphological changes of cell death in MDA-MB 231 and MCF-7 cells elicited by the methanol and aqueous extracts. The concentration of the IC₅₀ value of the respective crude extracts of plant was used for the morphological studies. Both the cells were treated with crude extracts for 72 h. The untreated cells served as the negative control. The morphological changes of the cells were observed under the normal inverted microscope after 72 h post-treatment (Jun *et al.*, 2007).

RESULTS

The preliminary phytochemical studies of *C. carnososa* are reported in Table 1. The methanol extract of *C. carnososa* showed cytotoxic effect against MCF-7 cells and MDA-MB 231 cell lines when compared to control (Fig. 1-4). The results obtained for cytotoxic activity suggest that highest inhibitory effect by the methanol extract of *C. carnososa* was shown

Table 1: Preliminary Phytochemical screening of *C. carnosia* extracts

Constituents	Aqueous	Methanol
Amino acids	+	+
Carbohydrates	+	+
Saponins	-	-
Tannins	-	-
Phytosterols	+	+
Alkaloids	+	+
Glycosides	+	+
Flavanoids	+	+
Phenolic compounds	+	+

+: Presence, -: Absence

Table 2: *In vitro* cytotoxic activity (IC_{50} $\mu\text{g mL}^{-1}$) of crude methanol and aqueous extracts of *C. carnosia* against MDA-MB 231 and MCF-7 cell lines

Extracts	IC_{50} of MCF-7 ($\mu\text{g mL}^{-1}$)	IC_{50} of MDA-MB 231 ($\mu\text{g mL}^{-1}$)
Methanol	170	425
Aqueous	>500	>500

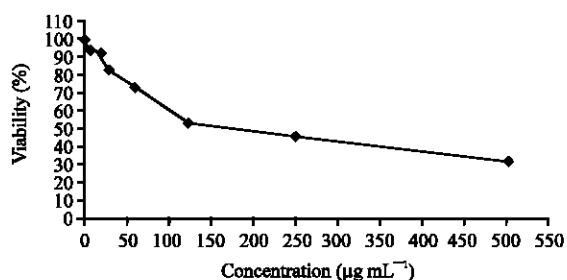


Fig. 1: MTT% inhibition of methanol extract on MCF 7

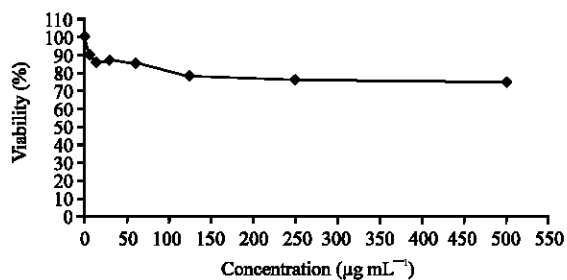


Fig. 2: MTT% inhibition of aqueous extract on MCF

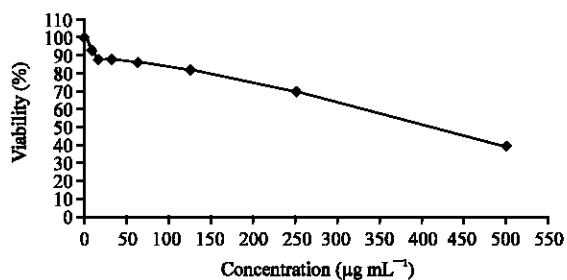


Fig. 3: MTT% inhibition of methanol extract on MDA-MB-231

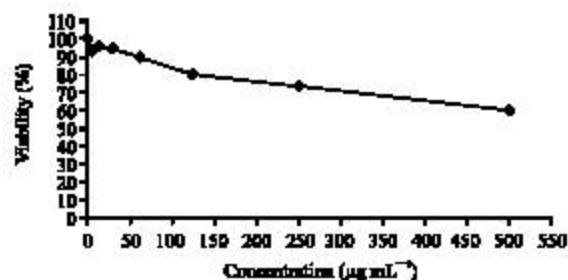


Fig. 4: MTT% inhibition of aqueous extract on MDA-MB-231

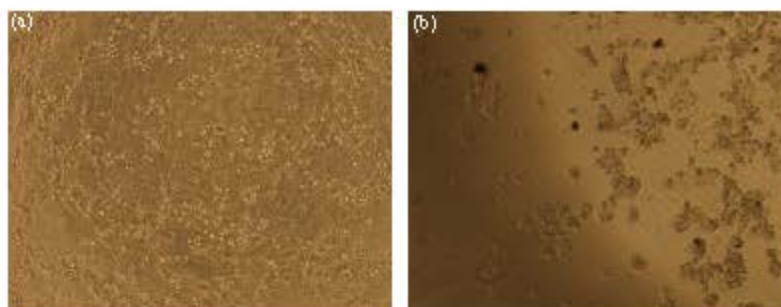


Fig. 5: (a) Morphological features of MCF-7 before treatment and (b) the changes observed after 72 h treatment at IC_{30} concentration with methanol extract of *C. carnososa* (100x Magnification)

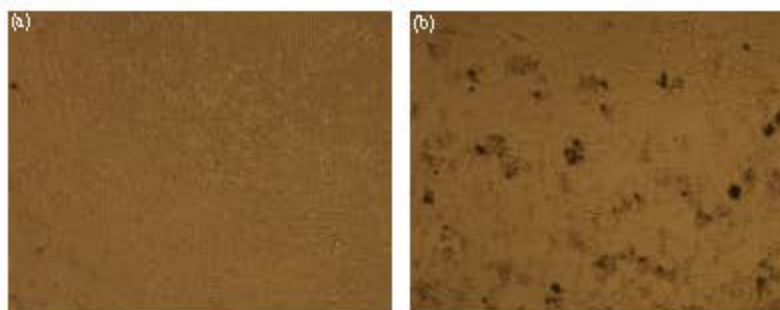


Fig. 6: (a) Morphological features of MDA-MB 231 before treatment and (b) the changes observed after 72 h treatment at IC_{30} concentration with methanol extract of *C. carnososa* (100x Magnification)

against MCF-7 with an IC_{30} $170 \mu\text{g mL}^{-1}$ when compared to MDA-MB 231 cell line [IC_{30} $425 \mu\text{g mL}^{-1}$ (Table 2)]. The inhibitory effect on MCF-7 cells was dose-dependent and the difference was remarkable starting from dose $120 \mu\text{g mL}^{-1}$.

As for the aqueous extract, the cytotoxic effect was not apparent even at higher concentrations. The extract exhibited lower cytotoxicity against both MCF7 and MDA-MB 231 cell lines ($\text{IC}_{50} > 500 \mu\text{g mL}^{-1}$) when compared to the methanol extract.

The morphological changes observed using the normal inverted microscope show slight alterations in cellular components after 72 h post-treatment under 100x magnification. The cell lines treated with IC_{30} of methanol extract revealed morphological changes (Fig. 5b, 6b) as

compared to non-treated cells (Fig. 5a, 6a). Treated cells showed a more prominent growth inhibition and shrinkage of the cells. To add on, the activity of alcohol extract on both MDA-MB 231 and MCF-7 was more compared with aqueous extract. On the contrary, untreated cells remained confluent throughout the incubation period.

DISCUSSION

A large variety of phytochemicals that have been reported from natural product research has been proven successful as anticancerous agents (Androutsopoulos *et al.*, 2008). This study was undertaken to scientifically prove the traditional claim of *C. carnososa*, possessing anti-neoplastic property. The findings from this study reveals that methanol extract is more potent than aqueous extract in exerting antineoplastic effect in both cell lines as evident by a dose dependent decrease in cell growth. The effect was analyzed at different concentration level ranging from 50 to 500 $\mu\text{g mL}^{-1}$. However, the decrease in cell growth upon treatment with aqueous extract on both MDA-MB 231 and MCF-7 cell lines did not prove to be as promising as the methanol extract. Though the inhibitory effect was dose dependent, the effect among different concentration levels was not very apparent. No studies have been reported for the cytotoxic effects of this plant. Therefore, comparison of this study was made based on previously reported chemical constituents of this plant such as, delphinidin and cyanidin which are anthocyanins and showed antiproliferative and proapoptotic properties in gastric adenocarcinoma and were also found to be protective against esophageal cancer in rodents (Lavanya *et al.*, 2007). Delphinidin is also known to inhibit constitutive and epidermal growth factor-induced phosphorylation and activation of breast tumor kinase signaling mediated through epidermal growth factor receptor. In addition, breast cancer cells treated with delphinidin, inhibited cell growth and induced apoptosis (Jung-Mi *et al.*, 2008). Hence, it is possible that these active components might have been responsible for the observed cytotoxic activity even though the exact mechanism of action of these extracts on MDA-MB 231 and MCF-7 cells are unclear.

The absence of normal apoptosis accounts for uncontrolled cell multiplication and these neoplastic cells have undergone alterations that tend to resist their susceptibility to apoptosis. Hence, for a crude extract with cytotoxic activity to be formulated as a potent anti-cancer agent it should have a direct and selective action on these resilient cancer cells (Nagamine *et al.*, 2009). Further studies have to be carried out in order to investigate the exact mechanism of apoptosis by cellular death with acridine orange/ethidium bromide staining, cell proliferation with immunocytochemistry of bromodeoxyuridine and subcellular alterations using electron microscopy. Our results clearly states that methanol extract of *C. carnososa* has cytotoxic effect and shows profound cellular damage and hence scientifically substantiates the folklore claims.

CONCLUSION

Cayratia carnososa was selected for the present study based on its traditional claim. The methanol extract showed inhibitory activity against MCF-7 and MDA-MB 231 cell lines but the aqueous extract showed cytotoxic activity only at higher concentrations. The morphological study provided more convincing evidence. However, further research are required to isolate and identify the active cytotoxic agents from the plant as well as elucidating their possible mechanism of action.

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