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Original Research Article

Cytotoxic Activity of the Leaf and Stem Extracts of *Hibiscus rosa sinensis (Malvaceae)* against Leukaemic Cell Line (K-562)

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Abstract

Purpose: To evaluate the cytotoxic activity of Hibiscus rosa sinensis against K-562 cancer cell line.
Methods: The crude petroleum ether, ethyl acetate and methanol extracts of the leaf and stem of Hibiscus rosa sinensis were prepared using cold extraction method. The in vitro cytotoxic activity of the extracts (20 - 100 µg/ml) was evaluated on leukaemic cancer cell line (K-562) and Mardin-Darby kidney cell line (MDBK) (control) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assays, followed by morphological detection with Hoeschst staining.
Results: The methanol leaf extracts showed higher activity (IC_{50} value: 30.9 ± 1.1 µg/ml) against K-562 cells than petroleum ether and ethyl acetate extracts which exhibited IC_{50} of 87.6 ± 0.91 and 57.6 ± 0.61 µg/ml ($p < 0.05$), respectively. Meanwhile, stem extracts from methanol showed IC_{50} of 79.80 µg/ml against K-562. MDBK cells (positive control) showed $IC_{50} > 100$ µg/ml for all the extracts. On treating K-562 cells with methanol leaf extract (30 µg/ml), the former were observed to undergo apoptosis with nuclear segmentation after 24 h incubation. The methanol leaf extract produced cell death on K-562 cells by apoptosis.
Conclusion: Hibiscus rosa sinensis extracts possess potentials as effective cytotoxic agents against K-562 cells.

Keywords: Hibiscus rosa sinensis, Cytotoxicity, Leukaemic cancer cell, Kidney cancer cell, Apoptosis

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INTRODUCTION

Natural products from plants are potent sources of potent anticancer agents. Previous studies had showed that *Hibiscus rosa sinensis* possesses anticomplementary, antidiarrhetic and antiphologistic activity [1]. It has been reported that the flower possesses antispermatogenic and androgenic [2], antitumour [3] and anticonvulsant activities [4]. The leaves and flowers have been observed to promote hair growth and aid in healing of ulcers [5,6].

In vitro cytotoxicity screening offer a suitable strategy to select plant extracts with potential of antineoplastic properties. Cytotoxic assay is based on the evaluation of a rare characteristic of most cytotoxic agents, commonly drugs, hormones, nutrients and irradiation. Cytotoxic assays have been used to measure the amount

of death resulting from treatment with compounds that can cause cancer [7].

The objective of this study was to evaluate the potential of *Hibiscus* species as anticancer agent in the continuing search for effective therapies for cancer.

EXPERIMENTAL

Plant material

Fresh *Hibiscus rosa sinensis* plant was collected in November 2003 at Jinjang, Kuala Lumpur, Malaysia. The plant was identified and authenticated by Mr Sabri, of Herbal Unit, Institute for Medical Research, Kuala Lumpur. A voucher specimen (no. 051103) was kept in the herbarium at the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The plant materials were dried in the oven (40 ºC), powdered to a coarse consistency and stored at -20 ºC.

Preparation of extracts

The powdered leaves (501.9 g) and stem (560.5 g) were extracted separately using petroleum ether, ethyl acetate and methanol by cold extraction technique for one week at room temperature. The extract was filtered through a nylon filter (0.45 µm) and concentrated using a rotary evaporator at 40 ºC under reduced pressure. The gummy extract was kept in a freezer (-20 ºC) (Haier, New Zealand) pending further studies.

Cell line culture

K-562 and MDBK cell lines were obtained from the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The cells were cultured in RPMI 1640 medium supplemented with 10 % foetal bovine serum (FBS) with 100 unit/ml penicillin/streptomycin at 37 ºC in 5 % CO_2 incubator. Cell viability was determined using trypan blue (Sigma, UK) and counted using haemocytometer.

MTS and MTT assays

Cancer cells (K-562) and normal cells (MDBK, control) at a concentration of 1 X 10^5 cells/ml were seeded separately in 96-well microliter plate (Nunc, Denmark) and exposed to various concentrations of the extracts (20 to 100 µg/ml). Cell survival fraction was determined using MTS and MTT assays. After treatment for 24, 48 and 72 h, MTS solution was added into each well containing K-562 cells; while MTT (5 mg/ml) was added to MDBK cells. Cells were further cultured for 4 h at 37 ºC. For MTT, the formazan crystals formed were dissolved with DMSO. The amount of coloured formazan formed was determined by measuring the absorbance at 490 nm for MTS assay and 540 nm for MTT assay using ELISA reader (Dynex MRX, USA). The cytotoxic effect of *Hibiscus rosa sinensis* on K-562 and MDBK cells were determined by measuring the dose that inhibits 50 % of the cancer cell population *vis a vis* the untreated control. The percentage of cell viability was obtained by dividing the absorbance value of the samples treated with different concentrations of extract with the absorbance value of control and then multiplied by 100 % [8]. Dose - response curves of % cell viability versus extract concentration were constructed and IC_{50} determined from the plots by interpolation [9].

Hoeschst (HO) 33258 staining

A stock of Hoeschst stain (100 µg/ml) was prepared by dissolving 100 µg Hoeschst powder in 1 mL of phosphate buffer saline (PBS) (Merck, Germany). Further serial dilution was done to prepare a solution of 1:10 ratio using PBS. This test was conducted using 6-well microtiter plate with 1 X 10^5 cells/well. After incubation, a wet count was done using microscopic slides for visualisation.

Statistical analysis

Cytotoxic assay was repeated thrice for both cell lines and the mean value and standard deviation (mean ± SD) of the data were obtained using SPSS software (version 11). $P < 0.05$ was considered statistically significant.

RESULTS

Table 1 shows the IC_{50} values for K-562 and MDBK cells after 72 h incubation. The strongest cytotoxic activities were found for the methanol leaf extract, as it lowered IC_{50} to 30.9 ± 1.1 µg/ml against K-562 cells after 72 h incubation (Fig 1). Petroleum ether and ethyl acetate leaf extracts showed higher IC_{50} of 87.6 ± 0.91 and 57.6 ± 0.61 µg/ml, respectively. No cytotoxic activities were found for petroleum ether and ethyl acetate stem extracts since they exhibited IC_{50} > 100 µg/ml. On the other hand, methanol stem extracted displayed IC_{50} of 79.8 ± 0.31µg/ml against K-562 cells.

IC_{50} values and % inhibition of K-562 and MDBK cells were compared since MDBK is a normal cell which show no inhibition after treatment with extracts. The IC_{50} values of all extracts was >

100 µg/ml after 72 h incubation (Table 1). Since, methanol leaf extract showed the strongest cytotoxic activities against K-562 cells, it was used to determine the mode of cell death. Fig 2(a) shows morphology of healthy K-562 cell line at 80 % confluency and used in staining. K-562 cells were treated with the methanol leaf extact for 48 h using Hoeschst staining and the results are shown in Fig 2(b). After 24 h incubation, the K-562 cells underwent nuclear segmentation, thus indicating apoptosis as shown in Fig 2(c) .

Table 1: IC_{50} values of crude extracts of *Hibiscus rosa sinensis* in K-562 and MDBK cells after 72 h incubation.

Solvent	Parts	IC_{50} value (µg/ml)	
		K-562	MDBK
Petroleum ether	Leaf	87.60 ± 0.91	>100
	Stem	> 100	>100
Ethyl acetate	Leaf	57.60 ± 0.61	>100
	Stem	> 100	>100
Methanol	Leaf	30.90 ± 1.10	>100
	Stem	79.80 ± 0.31	>100

DISCUSSION

The findings of this study suggest that there may be cytotoxic compounds in *Hibiscus rosa sinensis* extract that can induce the cytotoxic action against cancer cells and initiate antiproliferation effect leading to cancer cell death [10]. Cytotoxic assays are an important approach for drug discovery from natural products. The mode of cell death is important for determining the effectiveness of cytotoxic agents. Apoptosis is a cell suicide program through a tightly regulated process resulting in the removal of damaged or unwanted tissue [11]. Recently, much effort has been directed toward the search for compounds or herbs that influence apoptosis and their mechanism of action [12]. Cells undergoing apoptosis show characteristic morphological and biochemical features, including chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasmic and nucleus into membrane-bound vesicles [13].

Methanol leaf extract showed the strongest cytotoxic activity and also showed cell death by apoptosis with its nuclear segmentation after incubation. *Hibiscus rosa sinensis* possesses various secondary metabolites that are responsible for its cytotoxic activity. The bioactive components of the leaves and stems are β-sitosterol, stigmasterol, taraxeryl acetate and cyclopropane, as well as their derivatives [14].

These bioactive compounds are polar in nature which can be extracted by polar solvents such as

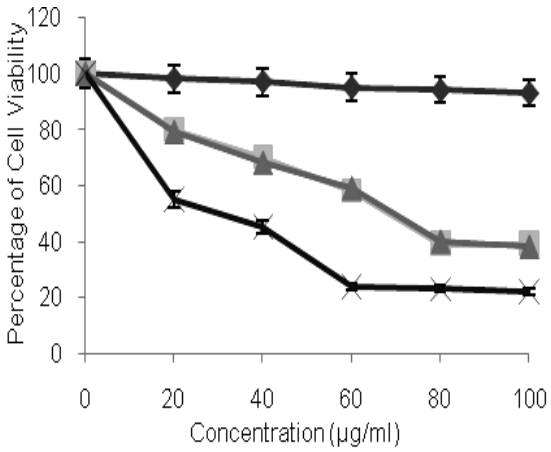

Fig 1: Cell viability (%) of K-562 cancer cell line after 72 h treatment with various concentrations of methanol leaf extracts of *Hibiscus rosa sinensis*. Key: ● 0 hour, —■— 24 hours, ▲—48 hours, x 72 hours

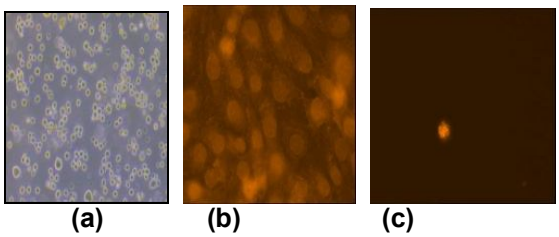

Fig 1: (a) Healthy K-562 cells at 80 % confluency after 48 h incubation in RPMI 1640 medium supplemented with 10 % FBS (100x); **(b)** Effect of methanol leaf extract (30 µg/ml) on K-562 using Hoeschst staining at 0 h (100 x), and It indicates there was no effect on the cells were; **(c)** Effect of methanol extract (30 µg/ml) on K-562 using Hoeschst staining after 24 h (100x), and it shows nuclear segmentation after treatment

methanol. Thus, the hgh cytotoxic activity of the methanol extract could be due to the presence of these bioactive components. A previous investigation showed that the polyphenolics in the methanol flower extract of *Hibiscus rosa sinensis* exhibited significantly higher inhibitory effect on phosphatase enzyme activity *in vitro*. This finding suggests that the crude extracts caused cytotoxic activity via apoptotic mechanism, due to its secondary metabolites. Further fractionation and isolation need to be carried out to obtain pure compounds.

CONCLUSION

Crude extracts of *Hibiscus rosa sinensis*, in particular the methanol leaf extract, exhibit significant cytotoxic activity against K-562 cell line and therefore, further investigations including bioassay guided fractionation to isolate its active constituents and molecular studies are required for the elucidation of its activity.

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