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Full Length Research Paper

Cytotoxic activity of leaf and rhizome extracts of *Alpinia scabra* (Blume) Náves, a wild ginger from Peninsular Malaysia

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The leaves and rhizomes of *Alpinia scabra* (Zingiberaceae) were investigated for their cytotoxic effect against selected human cancer cell lines, namely MCF7 (hormone-dependant breast carcinoma cell line), HT29 (colon carcinoma cell line) and SKOV-3 (ovarian cancer cell line) by using an *in vitro* neutral red cytotoxicity assay. The methanol extracts of both leaves and rhizomes did not show active cytotoxic activity against the selected cancer cell lines. The n-hexane extract of the leaves exhibited remarkable cytotoxic effect against SKOV-3 cells with IC_{50} value of 6.3 µg/ml while dichloromethane extract showed high cytotoxic effect against MCF7 and SKOV-3 with IC_{50} values of 6.7 and 5.9 µg/ml, respectively. The n-hexane and dichloromethane extracts of the rhizomes possessed high cytotoxic effect against SKOV-3 cells with IC_{50} values of 8.3 and 7.0 µg/ml, respectively. This is the first report of the cytotoxic activity of *A. scabra*.

Key words: Zingiberaceae, *Alpinia scabra*, cytotoxic activity, cancer cell lines.

INTRODUCTION

Recently, there is increasing interest in the search for plant based lead compounds for the development of new pharmaceuticals, along with the increase of deadly illness such as AIDS and cancer (Newman et al., 2003). There are approximately 250,000 plant species in the world and 60% of them are located in the tropical rainforests. The plant resources of Malaysia comprise about 15,000 species. It was estimated that about 1,000 species of the Malaysian flora have undergone simple chemical screening and much less have been subjected to thorough chemical or pharmacological studies (Goh et al., 1993). Plants in the Zingiberaceae family are widely distributed throughout the tropics, but concentrated mainly in Southeast Asia. Current work indicates that there are about 18 genera with over 160 species of Zingiberaceae in Peninsular Malaysia (Larsen et al., 1999). Several species from the genera *Alpinia*, *Amomum,* *Curcuma*, *Kaempferia* and *Zingiber* are reported to have medicinal values and have been used for generations in various traditional health care systems.

Alpinia scabra (Blume) Náves belonging to the botanical family Zingiberaceae, is an aromatic, perennial and rhizomatous herb, which is sometimes known by its vernacular name 'Lengkuas raya' among the locals. It is a wild species and grows mainly on mountains at moderate elevations in Peninsular Malaysia. However, it can also survive in the lowlands as this has been recorded in the states of Terengganu and Northern Johor. *A. scabra* is closely related to *A. galanga* (alternatively called greater galangal), an edible species which is utilized in traditional medicine preparations. Apart from having black ripe fruits, *A. scabra* differs slightly from *A. galanga* in its inflorescence and floral details. Recent researches reported that *A. galanga* has been categorized as anticancer, antimicrobial, carminative, anti-rheumatic, anti-flatulent and anti-itching agents (Matsuda et al., 2003; Lee and Houghton, 2005). There are extensive investigations on *A. galanga.* However, there is little information available in the literature about *A. scabra*.

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In view of the traditional use of *A. galanga* in cancer treatment, the investigation of cytotoxicity of *A. scabra* was thus pursued in the present study and the findings are reported in this article. This study aims to investigate the cytotoxic activity of *A. scabra* and identify the active fractions that contribute to the cytotoxic activity before further study on the isolation of active ingredients. The findings of the current study may support the claims regarding the use of Alpinia species for treatment of cancer.

In the present study, the leaves and rhizomes of *A. scabra* were used to perform preliminary cytotoxic tests against selected human cancer cell lines, namely MCF7 (hormone-dependant breast carcinoma cell line), HT29 (colon carcinoma cell line) and SKOV-3 (ovarian cancer cell line) by using an *in vitro* neutral red cytotoxicity assay. To our knowledge, this is the first report on the cytotoxic effect of *A. scabra*.

MATERIALS AND METHODS

Plant sample collection and identification

The fresh leaves and rhizomes of *A. scabra* were collected from Genting Highland, Pahang, Malaysia. The samples were authentic-cated by Professor Dr. Halijah Ibrahim of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia and a voucher specimen (herbarium no. HI 1419) was deposited at the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

Preparation of extracts for cytotoxic investigation

Briefly, the fresh leaves and rhizomes of *A. scabra* were washed, dried in the oven and ground to fine powder by using a blender. The dried, ground leaves and rhizomes were soaked in n-hexane (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decantered and filtered. The extraction of the ground leaves and rhizomes were further repeated (2x) with n-hexane (1.5 L each time). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Rotavapor, Switzerland) to give n-hexane extracts. The n-hexane-insoluble residue of leaves and rhizomes were further extracted with dichloromethane (CH_2Cl_2) to give CH_2Cl_2-soluble extracts and CH_2Cl_2-insoluble residue. The CH_2Cl_2-insoluble residue was further extracted with methanol to give methanol extract. The weights of all the extracts were measured after solvent evaporation. All the extracts were dissolved in dimethylsulfoxide (DMSO) to form stock solutions 20 mg/ml before testing. The final concentration of DMSO in test wells was not in excess of 1 v/v%.

In vitro cytotoxicity assay

Cell lines and culture medium

MCF7 (hormone-dependant breast carcinoma cell line), HT29 (colon carcinoma cell line) and SKOV-3 (ovarian cancer cell line) were purchased from the American Tissue Culture Collection (ATCC, USA). The viability of the cells was checked before and after treatment by the trypan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196°C) prior to use.

The MCF7 and HT29 cells were maintained in RPMI 1640 medium (Sigma) and SKOV-3 cells in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% foetal bovine serum (FBS, PAA Lab, Austria), 100 µg/ml penicillin or streptomycin (PAA Lab, Austria) and 50 µg/ml of fungizone (PAA Lab, Austria). The cells were cultured in a 5% CO_2 incubator (Shel Lab water-jacketed) kept at 37°C in a humidified atmosphere. The culture was subcultured every two to three days as needed and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination.

In vitro neutral red cytotoxicity assay

The neutral red cytotoxicity assay was carried out as previously described (Sri Nurestri et al., 2009). Briefly, cells were detached from the tissue culture flask with 0.25% trypsin-ethylene diamine tetra acetic acid (EDTA) solution and phosphate buffered saline (PBS) solution and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 min. The density of the viable cells was counted by 0.4% of tryphan blue exclusion in a haemocytometer. The cells (1×10^4/well) were then plated in a 96-well microtiter plate (Nunc) in a volume of 190 µl. The plate was incubated in a CO_2 incubator at 37°C for 24 h to allow the cells to adhere and achieve 60 - 70% confluence at the time of the addition of the test agents (that is, test extracts and positive control). After 24 h of incubation, the cells were treated with different concentrations (1, 10, 50 and 100 µg/ml) of each test agents in three replicate tests. The plates were incubated for 72 h at 37°C in a 5% CO_2 incubator. DMSO was used to dilute the test agents and the final concentration of DMSO in test wells and control wells used was not in excess of 1% (v/v). No effect due to the DMSO was observed. *Cis*-platin (Aldrich) was used as the positive control. The well containing untreated cells is the negative control.

At the end of the incubation period, the media was replaced with medium containing 50 µg/ml neutral red. The plates were incubated for another 3 h to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After the incubation period, the media was removed and cells were washed with the neutral red washing solution. The dye was eluted from the cells by adding 200 µl of neutral red resorb solution and further incubated for 30 min at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). Dye absorbance was measured at 540 nm using ELISA reader (Molecular Devices EMax). Three replicate plates were used to determine the cytotoxicity of each test agent. The cytotoxic effect of each test agent was evaluated based on percentage inhibition values. The percentage of inhibition (%) was calculated according to the following formula:

$$\text{Percentage of inhibition (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100\%$$

The average of three replicates was then obtained. Cytotoxicity of each test agent is expressed as IC_{50} value. The IC_{50} value is the concentration of test agents that cause 50% inhibition or cell death, averaged from the three experiments, and was obtained by plotting the percentage inhibition versus concentration of test agents. According to US NCI (United States National Cancer Institute) plant screening program, a plant extract is generally considered to have active cytotoxic effect if the IC_{50} value, following incubation between 48 to 72 h, is 20 µg/ml or less, while it is 4 µg/ml or less for pure compounds (Lee and Houghton, 2005; Geran et al., 1972).

RESULTS AND DISCUSSION

Extraction yield of leaves and rhizomes of *A. scabra*

Solvent extraction is the most popular method used in

Table 1. Yield of extracts of leaves and rhizomes of *A. scabra*.

Parts	Samples /Extracts	Weight (g) (%)
Leaves	Dried and ground plant material	332 .00
	n-hexane extract	3.45 (1.04)
	CH_2Cl_2 extract	9.82 (2.96)
	Methanol extract	8.18 (2.46)
Rhizomes	Dried and ground plant material	522.00
	n-hexane extract	3.11 (0.60)
	CH_2Cl_2 extract	4.98 (0.95)
	Methanol extract	14.53 (2.78)

sample preparation (Sim et al., 2010). The percentage yield of the extracts was based on the weight of dried and ground plant materials. As indicated by Yan et al. (1999), a single solvent may not be enough to identify certain extracts responsible for the activity. Thus, three extraction solvents were used in the present study, namely n-hexane, CH_2Cl_2 and methanol. The yield of extracts of leaves and rhizomes is shown in Table 1.

Cytotoxic activity of leaves and rhizomes extracts

There are a lot of methods to test the cytotoxicity of chemicals, such as MTT, LDH and neutral red cytotoxicity assay. Different cytotoxicity assays might give different results depending on the test agent and the cytotoxicity used. Assays like MTT and LDH, which are dependent on enzymatic reactions, might be influenced by enzyme inhibitors like chloroquine (Weyermann et al., 2005). The neutral red cytotoxicity assay was chosen in this study to determine cytotoxic effect of leaf and rhizome extracts of *A. scabra* against selected human cancer cell lines, with cis-platin as the positive control. Fotakis and Timbrell (2006) reported that the neutral red assay and the MTT assay are the most sensitive cytotoxicity assay that show statistically significant difference between the treated cells and the controls, especially in detecting early toxicity. They also suggested that the neutral red assay is a useful tool to detect lysosomal damage when used in conjunction with other tests in order to distinguish between cytotoxicity and organelle damage. Furthermore, Weyermann et al. (2005) pointed out that inexpensive assay like the neutral red uptake assay is sufficient when a more expensive test kits fails.

The cytotoxicity (IC_{50} values in µg/ml) of leaf and rhizome extracts of *A. scabra* against the tested cancer cell lines are summarized in Table 2. The methanol extracts of both leaves and rhizomes did not show active cytotoxic activity against the selected cancer cell lines. The n-hexane extract of the leaves of *A. scabra* exhibited remarkable cytotoxic effect against SKOV-3 cells (IC_{50} 6.3 µg/ml) and moderate cytotoxic effect when screened against HT29 cells (IC_{50} 33 µg/ml). The CH_2Cl_2 extract of *A. scabra* leaves showed high cytotoxic effect against MCF7 and SKOV-3 with IC_{50} values of 6.7 and 5.9 µg/ml, respectively.

The hexane extract of the rhizomes of *A. scabra* displayed high cytotoxic effect against SKOV-3 cells (IC_{50} 8.3 µg/ml), some mild effect against HT29 cells (IC_{50} value 57.8 µg/ml) and no activity against the MCF7 cells (IC_{50} > 100 µg/ml). The CH_2Cl_2 extract of *A. scabra* rhizomes revealed high inhibitory effect against the SKOV-3 cells (IC_{50} 7.0 µg/ml) and exert moderate effect on MCF7 and HT29 cells (IC_{50} 32.5 and 24.5 µg/ml, respectively). From the results of the present study, it can thus be concluded that the hexane and CH_2Cl_2 extracts of both leaves and rhizomes were selectively toxic against the SKOV-3 cells, with exception of CH_2Cl_2 extract of the leaves which also displayed high inhibitory effect on the proliferation of the MCF7 cells.

Cis-platin which is a platinum containing chemotherapeutic drug widely used for the treatment of several human cancers was used as the positive reference in the present study. However, a study done by Eskilsson et al. (1988) showed a high incidence of cardiotoxic effect during induction chemotherapy treatment with cis-platin. Thus, the routine use of this drug could bring major adverse effect. Although the cytotoxicity of the cytotoxic active extracts (hexane and CH_2Cl_2 extracts of both leaves and rhizomes) are not as impressive as cis-platin, they however may have lower toxicity against the normal cells in comparison to cis-platin. The screening of cytotoxic extracts of both leaves and rhizomes of *A. scabra* against the normal cell lines are now underway and the chemical investigation directed to the cytotoxic active extracts is now being planned. The active ingredients in the cytotoxic active extracts may lead to valuable compounds that may have the ability to kill the cancer cells.

Conclusions

In this study, we focused to investigate the cytotoxic

Table 2. *In vitro* cytotoxic activity (IC_{50} µg/ml) of leaf and rhizome extracts of *A. scabra* against various cancer cell lines.

Parts	Extracts	IC_{50} values (µg/ml)		
		MCF7	HT29	SKOV-3
Leaves	n-hexane	> 100.00	33.00 ± 0.00	6.30 ± 0.20
	CH_2Cl_2	6.70 ± 0.00	21.20 ± 0.30	5.90 ± 0.00
	Methanol	> 100.00	> 100.00	58.30 ± 1.50
Rhizomes	n-hexane	> 100.00	57.80 ± 1.00	8.30 ± 0.20
	CH_2Cl_2	32.50 ± 0.00	24.50 ± 0.00	7.00 ± 0.10
	Methanol	> 100.00	> 100.00	> 100.00
cis-Platin[a]		2.40 ± 0.60	5.00 ± 0.00	1.40 ± 00

[a] *cis*-Platin was used as positive reference compound; the values expressed are mean ± standard deviation of triplicate measurements.

activity of *A. scabra* against selected human cancer cell lines. Based on the outcome of the present study, the hexane and CH_2Cl_2 extracts of both leaves and rhizomes of *A. scabra* displayed very good cytotoxic effect against SKOV-3 cells, with exception of CH_2Cl_2 extract of leaves which also showed high inhibitory effect against the MCF7 cells. Based on this preliminary data obtained by us, *A. scabra* is considered as an agent with potential anticancer activity, and therefore can be a good candidate for further stages of screening *in vivo and /or in vitro*.

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