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EVALUATION OF CYTOTOXICITY OF LEAF AND RHIZOME EXTRACTS OF *Alpinia calcarata* ROSC. AGAINST HUMAN LUNG NCI-H460 AND CERVICAL HELA CANCER CELL LINES

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Abstract

The cytotoxicty of *Alpinia calcarata* rhizome and leaf extracts, fractions and essential oils were evaluated *in vitro* against human lung NCI-H460 and cervical HeLa cancer cell lines using sulphorhodamine-B assay. Although an array of bioactivities of *A. calcarata* rhizome have been reported previously, no attempt has been made to study the cytotoxicity of rhizome in human lung NCI-H460 and cervical HeLa cancer cell lines. In the present study, both the leaf and rhizome extracts (ethanolic and water) along with their fractions (hexane, dichloromethane, ethylacetate, butanol and water) and essential oils against human lung (NCI-H460) and human cervical (HeLa) cancer cell lines were investigated. Fresh rhizomes and leaves of *Alpinia calcarata*, collected from Western Province of Sri Lanka were used to obtain the extracts. The essential oils were obtained by hydro-distillation. All the samples were stored at 4 °C. The extracts, fractions and essential oils demonstrated a varying degree of growth inhibition against NCI-H460 cell line. Several fractions showed high growth inhibitory activity (4-94%). The growth inhibition order was, rhizome water extract (107.7%) < rhizome ethanolic extract (68.2%) < leaf ethanolic extract (-25.1%) < leaf water extract (-72.3%) < rhizome oil (-93.0%) < leaf oil (-94.3%). Dichloromethane fraction of leaf ethanolic extract (-33.8%) showed the most promising inhibition at GI_{50}: 30.6 µg/mL on NCI-H460. For HeLa cells, the ethyl acetate (-0.5%), butanol (-5.0%) and aqueous fractions (-18.1%) of rhizome water extract showed high growth inhibitions. The potency of the growth inhibition was, rhizome ethanolic extract (76.56%) < rhizome water extract (16.65%) < leaf oil (15.88%) < rhizome oil (-49.34%). Efficacy and mechanisms of action in various normal and cancer cell models coupled with bioassay-guided purification to identify active anticancer compound(s) from the crude extract will be useful.

Key words: *Alpinia calcarata*, sulforhodamine-B, HeLa, NCI-H460, cytotoxicity

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1. Introduction

Cancer is a complex group of diseases incorporating physical, environmental, metabolic, chemical and genetic factors. It is one of the leading causes of morbidity and mortality worldwide, accounting for 14 million new cases and over 8 million deaths in 2012. The number of new cases of cancer is expected to reach to 22 million in two decades (WHO, 2014). Due to adverse effects, increasing resistance and unaffordable prices of cancer chemotherapy agents, it is necessary to discover non toxic potential drugs from natural resources for the treatment and prevention of cancer (Demain*et al.,* 2011).

Alpinia calcarata Rosc. (Zingiberaceae) is a rhizomatous perennial herb that is cultivated in tropical countries, including Sri Lanka, India and Malaysia (Jayaweera, 2006). Also known as Heen-araththa (Sinhala); Amkolinji (Tamil); small galangal or snap ginger or cardamom ginger (English); Rasna (Sanskrit); and Kattuchena (Malayalam) (Arambewela *et al.,* 2006), the plant is used as a major constituent of many Ayurvedic formulations, such as Maharasnadhi Quathar (Thabrew *et al.,* 2001), as well as in Sri Lankan indigenous systems of medicine (Arambewela *et al.,* 2006). In Sri Lanka, the rhizomes are commonly used to treat a variety of conditions, such as rheumatoid arthritis (WHO, 2014; Thabrew *et al.,* 2001), polyuria, diabetes, asthma, fungal infections, respiratory ailments, skin diseases, stomach disorders and heart diseases (WHO, 2014). Scientific studies (*in vivo*) have shown that the rhizomes of *A. calcarata* possess antinociceptive (Arambewela *et al.,* 2004), antioxidant (Arambewela *et al.,* 2005), anti-inflammatory (Arambewela *et al.,* 2006; Arambewela *et al.,* 2012), analgesic, ameliorative effects in alloxan-induced diabetic rats (Navinraj *et al*., 2011), reproductive competence in male rats (Ratasooriya, W.D., and Jayakody, J.R.A.C, 2006), antifungal (Arambewela *et al.,* 2005; Arambewela *et al.,* 2010), antihelminthic, aphrodisiac, gastroprotective (Arambewela *et al.,* 2009), hypoglycemic, and antihyperglycemic (Arambewela *et al.,* 2009), hypolipidimic and antidiabetic properties (Arambewela *et al.,* 2006). An anti-inflammatory study on carrageenan-induced paw oedema model in rats using 250, 500, 750, and 1000 mg/kg doses of hot water and hot ethanolic extracts of *A. calcarata* showed that 500 mg/kg hot ethanolic extract induced a higher anti-inflammatory effect than the reference drug indomethacin at 4 h (Arambewela *et al.,* 2006). *Alpinia calcarata* rhizome contains various elements such as Cr (1.28 ppm), Mn (2.02 ppm), Fe (55.48 ppm), Ni (0.13 ppm), Cu (0.96 ppm), Zn (2.03 ppm), Mg (516.4 ppm), Ca (899.7 ppm), Na (417.4 ppm) and K (868.8 ppm). *Alpinia calcarata* rhizome contains crude fat (1.68%), crude protein (6.39%), carbohydrate (75.0%), crude fiber (7.25%) and nutritive value (340.8 cal/100 g) (Indrayan *et al.,* 2009).

Gas Chromatographic Mass Spectrometric (GC-MS) analysis of rhizome oil of *Alpinia calcarata* confirm the presence of α-pinene, β-pinene, ρ-cymene, 1,8-cineol, limonene, camphene, camphor, α-terpineol, γ-muurolene, caratol, fenchyl acetate, α-eudesmol and trace amount of fenchol, fenchone, linalool, α-cadinene and β-caryophyllene. The leaf oil is reported to have α-pinene, β-pinene, ρ-cymene, 1,8-cineol, limonene, camphene, camphor, α-terpineol, γ-muurolene, caratol, α-eudesmol, α-cadinene and β-caryophyllene and trace amounts of

fenchone. Root oil also contains similar compounds as rhizome and leaf oils (Kathirgamanathar, 2011).

Two bis-labdanic diterpenoids, named calcaratarins D & E, isolated from the rhizome of *Alpinia calcarata* grown in China, were found to have cytotoxic activity against human KB cells (Ling-Yi LY Kong *et al.,* 2002). The ethanol extract of rhizome at 8 mg/kg/day significantly reduced Ehrlich ascites carcinoma in Swiss Albino mice (Perveen *et al.,* 2012).

(K562) (Chauhan *et al.,* 2014) and gastric (AGS) (Hadjzadeh *et al.,* 2014) cancer cell lines, while galangin, isolated from *A. galanga*, showed strong anticancer, antioxidant, antimutagenic and anti-inflammatory activities (Pandey Govind, 2011). Pinostrobin chalcone, isolated from the ethylacetate extract of *A. mutica*, showed a remarkable cytotoxic activity against the human cancer cells named as KB, MCF-7 and Caski cells with IC_{50} values of 6.2, 7.3 and 7.7 µg/mL, respectively (Sri Nurestri *et al.,* 2011).

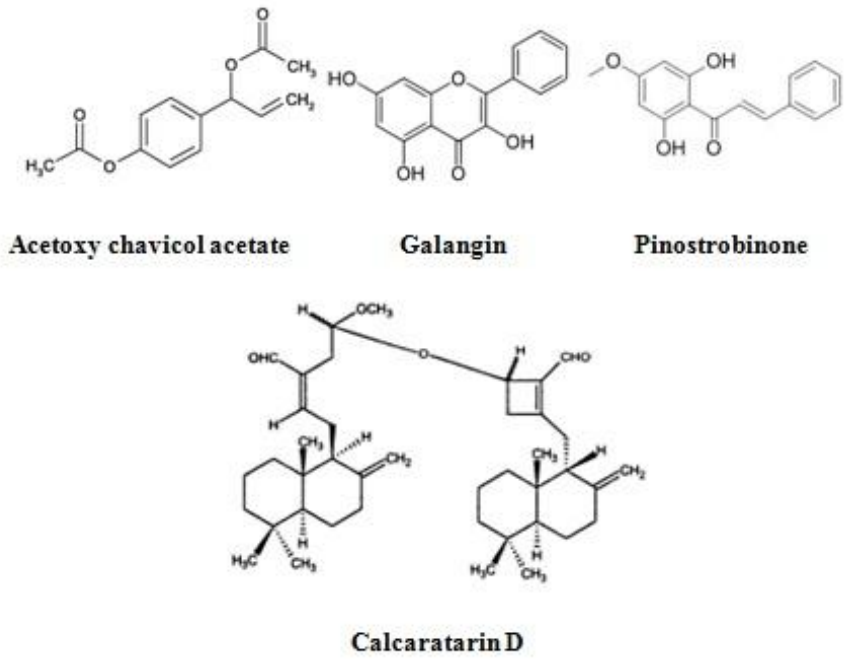

Figure 1. Anticancer active compounds from *Alpinia* species (Sri Nurestri *et al.,* 2011)

Acetoxy chavicol acetate, isolated from the rhizomes of *A. galanga*, showed significant anticancer activities against breast (MCF-7 and MCF-7/ADR) (Samarghandian *et al.,* 2014), lung (H522), prostate (DU145), leukemia

n-Hexane and dichloromethane extracts of leaves and rhizomes of *A. scabra* grown in Malaysia were screened for cytotoxic effect against SKOV-3 and MCF-7 cells. The n-hexane and dichloromethane extracts of rhizome showed a high

cytotoxic effect against both cells with IC_{50} values of 8.3 and 7.0 µg/mL, respectively (Ibrahim *et al.,* 2010).

Although an array of bioactivities of *A. calcarata* rhizome have been reported previously (Arambewela *et al.,* 2004), no attempt has been made to study the cytotoxic activity of the rhizome and leaf in human lung NCI-H460 and cervical HeLa cancer cell lines. Additionally, in previous studies, only the rhizome extracts were studied. However, in present study, *A. calcarata* rhizome and leaf extracts (aqueous and ethanolic) along with their fractions (hexane, dichloromethane, ethyl acetate, butanol and water) and essential oils against human lung (NCI-H460) and human cervical (HeLa) cancer cell lines were investigated.

2. Material and Methods

2.1 Plant collection

A. calcarata rhizomes and leaves were collected from a home garden in Western Province, Sri Lanka, during July 2011. The plant was identified by comparing with an authenticated sample that was grown in Industrial Technology Institute and deposited in Royal Botanical Gardens, Peradeniya, Sri Lanka (Voucher specimen number AC 01).

2.2 Chemicals and cancer cell lines

Human lung NCI-H460 and human cervical HeLa cancer cell lines were obtained from the National Cancer Institute, USA. FBS was purchased from Gibco and sulforhodamine-B from MP Biomedicals, USA. All the other chemicals were purchased from Sigma, USA.

2.3 Extraction, fractionation and distillation of oil

The collected plant material was washed under running water, cut into small pieces and shade dried for 10 days. The dried samples were extracted with water (refluxing for 5 h) and 70% ethanol (Soxhlet extraction for 6 h) separately. The water extract was evaporated under reduced pressure (150 mbar at <50 °C) in a rotary evaporator, and freeze dried. The dried crude extracts were fractionated with hexane, dichloromethane, ethyl acetate, butanol and water. For the extraction of oils, dried rhizomes and leaves (500 g each) were subjected to hydro-distillation using Clevenger apparatus for 6 h. The oils were stored at 4 °C in air-tight containers until used for the cytotoxicity test.

2.4 Cytotoxic activity test

The cytotoxic activity was evaluated by sulphorhodamine-B assay (Monk *et al.,* 1991). *Alpinia calcarata* extracts, oils and fractions, were evaluated against human lung NCI-H460 and human cervical HeLa cancer cell lines. Cells were plated in 96-microwell plates (NCI-H460: 7500 cells/well; HeLa: 10,000 cells/well) and incubated at 37 °C for 24 h in a humidified 5% CO_2 incubator. Doxorubicin, an anti-cancer drug was used as the standard. Stock solutions of ethanolic and water extracts of *A. calcarata* (40 mg/mL), fractions (20 mg/mL) and positive control doxorubicin (1 µM) were prepared in DMSO. For the initial screening, 100 µL of ethanolic and water extracts and the oils derived from rhizomes, and leaves (250 µg/mL) and the fractions (100 µg/mL) were added into each well and incubated. Fifty microliter of 50% cold trichloroacetic acid (TCA) was added in the wells and left for 30 min at room temperature. The wells were then washed with distilled

water and dried overnight. Sulforhodamine-B (SRB) solution (100 μL, 0.4% wt/vol in 1% acetic acid) was added. After 30 min, the unbound SRB was removed by washing with 1% acetic acid, and air-dried at room temperature. The protein bound stain was solubilized with 10 mM Tris-base (pH 10.2), the plates were shaken for 5 min by using a plate shaker and the absorbance was measured at 515 nm using SpectraMax Plus^{384} microplate reader. The absorbance of the appropriate blanks, including sample blanks, and control (without plant sample), was used to calculate the percentage net growth (%NG), and the cytotoxicity of the extracts and fractions. *A. calcarata* extracts and the fractions were further evaluated at various concentrations (extracts: 15.62, 31.25, 62.5, 125 and 250 μg/mL; fractions: 6.25, 12.5, 25, 50 and 100 μg/mL) to study the dose response. The growth inhibition and cytotoxicity of extracts and fractions was represented as GI_{50} (μg/mL) values. The standard drug doxorubicin demonstrated the GI_{50} (0.07 ± 0.00 μg/mL) and LC_{50} (0.86 ± 0.00 μg/mL). All cytotoxic activity was assessed at 12 h following the treatment. The sulforhodamine-B assay was used to quantify the cytotoxic effects.

2.5 Gas chromatography- Mass spectrometric analysis

Oil samples (rhizome oil, leaf oil and the hexane fraction of leaf ethanolic extract: 0.5 μL each) were injected into an Agilent 7890A/7000AGC triple quadrupole mass spectrometer system (GC/QQQ) for identification of compounds using 1:60 split injection ratio. A HP-5MS fused silica capillary column of 30 m length, 0.25 mm inner diameter and 0.25 μm film thickness (J&W Scientific, USA) was used. The initial oven temperature was 40 °C for 5 min ramped to 280 °C at 10 °C /min, and held there for 5 min. The linear velocity of the carrier gas (Helium) was constant at 40 cm/s. The inlet temperature was 250 °C, transfer line temperature was 250 °C and EI ionization source temperature was 230 °C. Mass spectra were acquired using full scan monitoring mode with a mass range of 50-500 m/z. Data Analysis was done by using Agilent Mass Hunter software version B.04.00 and National Institute of Standards and Technology (NIST) Data base Library 2010.

2.6 Statistical analysis

Results are presented as Mean±Standard Error. Significance of differences between groups was assessed with use of one way ANOVA.

3. Results

Hexane, dichloromethane, ethylacetate, butanol and aqueous fractions of *A. calcarata* ethanolic and water extracts as well as the oils derived from rhizomes and leaves demonstrated a varying level of growth inhibition (%NG) against lung cancer cell line NCI-H460 (Figure 1, Table 1 and 2).

The butanol fraction from both rhizome ethanolic extract and rhizome water extract, dichloromethane and ethylacetate fractions of the leaf ethanolic extract, and the hexane, dichloromethane, ethylacetate, butanol and aqueous fractions of *A. calcarata* ethanolic and water extracts, as well as the oils derived from rhizomes and leaves demonstrated a varying level of growth inhibition (%NG) against lung cancer cell line NCI-H460 (Fig. 1, Table1 and 2). The butanol

fraction from both rhizome ethanolic extract and rhizome water extract, dichloromethane and ethylacetate fractions of the leaf ethanolic extract and the hexane and dichloromethane fractions of the leaf water extract showed a higher inhibitory activity ranging between 4-94%. Results revealed differences among the extracts and fractions for growth inhibition. Leaf ethanolic extract showed a high growth inhibition as compared to the leaf water extract and the oils.

The growth inhibition potency order was rhizome water extract (107.7%) < rhizome ethanolic extract (68.2%) < leaf ethanolic extract (-25.1%) < leaf water extract (-72.3%) < rhizome oil (-93.0%) < leaf oil (-94.3%). Among all the fractions tested, the dichloromethane fraction of leaf ethanolic extract (%NG: -33.8%; cytotoxicity- GI_{50}: 30.6 µg/mL) was the most promising.

For the HeLa cell line, the ethylacetate and aqueous fractions of rhizome ethanolic extract showed significantly moderate growth inhibition while ethylacetate, butanol and aqueous fractions of rhizome water extract showed a high growth inhibition (Table 3). Rhizome oil showed highly potent growth inhibition on HeLa cells as compared to leaf oil. Among the extracts and oils tested, the potency of the growth inhibition was ranked as rhizome ethanolic extract < rhizome water extract < leaf oil < rhizome oil (Table 3).

4. Discussion

The major aim of this study was to identify potential anticancer extracts that were effective, even at low doses. In order to achieve this aim, the maximum test concentration was set at 100 µg/mL as the criteria for identifying extracts with potent activity within the range.

Table 1. Cytotoxicity dose response of *A. calcarata* extracts and oils on human lung cancer cell line NCI-H460

Extracts	Concentration (µg/mL)					GI_{50} (µg/mL)
	15.62	31.25	62.5	125	250	
Rhizome oil	103.68±0.98	111.74±0.75	102.33±8.53	5.46±2.43	-2.81±14.92	111.23±7.05
Leaf oil	108.9±4.89	101.44±1.88	107.11±8.57	64.74±8.09	-80.09±2.39	109.61±1.40
Leaf ethanolic extract	49.24±16.61	25.77±5.70	39.38±1.20	32.48±0.79	-65.01±3.84	22.82±10.91
Leaf water extract	105.60±1.84	106.83±8.02	111.13±6.02	105.50±4.50	35.76±1.52	239.47±1.50

represented as mean±S.E of three independent experiments in triplicate (n=3); GI_{50} – Growth inhibition at 50% concentration of extract or oil.

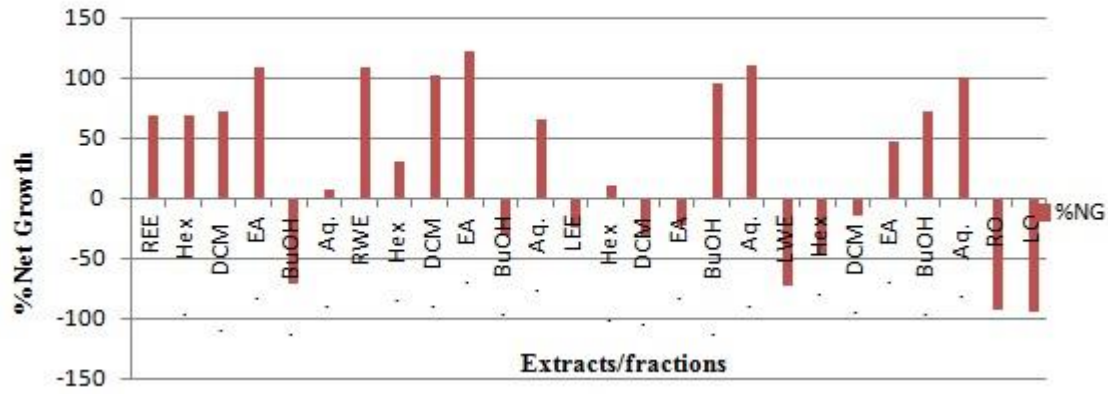

Figure 1. Cytotoxic effects of *A. calcarata* extracts, fractions and oils derived from rhizomes and leaves on lung cancer cell line NCI-H460. REE-Rhizome Ethanolic Extract; Hex–Hexane fraction; DCM–Dichloromethane fraction; EA–Ethylacetate fraction; BuOH–Butanol fraction; Aq.–Aqueous fraction; RWE-Rhizome Water Extract; LEE–Leaf Ethanolic extract; LWE–Leaf Water Extract; RO–Rhizome Oil; LO–Leaf Oil.

Table 2. Cytotoxicity dose response of solvent diluted fractions of extracts of *A. calcarata* on human lung cancer cell line NCI-H460

Fractions	Concentration (µg/mL)					GI_{50} (µg/mL)
	6.25	12.5	25	50	100	
DCM fraction of LEE	70.98±6.0	51.00±7.2	26.63±0.9	16.83±1.4	-5.66±12.7	30.61±2.9
EA fraction of LEE	66.01±3.5	39.32±3.4	13.01±5.5	15.62±0.8	-58.23±8.2	2351±1.9
BuOH fraction of REE	88.10±5.1	86.78±9.4	88.31±3.0	73.12±9.0	48.95±4.7	102.42±5.4
BuOH fraction of RWE	80.93±2.0	73.28±7.5	74.91±1.5	78.97±4.2	68.95±4.0	422.81±97.4

Data represented as mean±SE of three independent experiments in triplicate (n=3). GI_{50} – Growth inhibition at 50% concentration of extract or fraction
LEE-Leaf Ethanolic Extract; REE-Rhizome Ethanolic Extract; Hex–Hexane fraction; DCM-Dichloromethane fraction; EA–Ethylacetate fraction; BuOH–Butanol fraction; Aq.–Aqueous fraction; RWE-Rhizome Water Extract

Table 3. Effect of *A. calcarata* rhizome and leaf ethanolic and water extract, fractions and oils on cervical cancer cell line HeLa

Extracts and Fractions	Conc. (μg/mL)	%NG
Rhizome ethanolic extract	250	76.56±13.12[a]
Fractions		
• Ethyl acetate	100	10.36±4.46[b]
• Butanol	100	41.66±7.67[b]
• Aqueous	100	22.98±15.78[b]
Rhizome water extract		16.65±7.35[a]
• Ethyl acetate	100	-0.57±6.14[ba]
• Butanol	100	-5.04±8.14[ba]
• Aqueous	100	-18.14±1.85[b]
Rhizome oil	250	-49.34±16.04[b]
Leaf oil	250	15.88±6.33[a]

Data represented as mean±SE (n=3); %Net growth superscripted by different letters is significantly different at $p < 0.05$.

Using this criterion, extracts with less than 50% inhibitory activity within the test range were excluded from further screening.

Although such extracts may likely demonstrate cytotoxicity at higher concentrations, the focus in this study was limited to plant extracts that caused substantial growth inhibition in a given cell line within the test concentration of < 100 μg/mL. The assumption was that such activity in the plants crude nature would be indicative of even greater inhibitory effects in the purified state. For initial identification of extracts with activity, the effects were evaluated against human lung NCI-H460 and human cervical HeLa cancer cell lines.

5. Conclusion

This study has demonstrated the screening process of bioactive extracts with anticancer activity by eliminating the less active ones on the basis of cytotoxicity that takes effective dosage into consideration. Results obtained from extracts screened showed that all extracts exhibited a promising cytotoxic activity against human lung cancer cell line. Among the active extracts and fractions, the fraction with the highest anticancer activity was from the DCM fraction of leaf ethanolic extract, which was found to possess a potent anti-cancer activity with GI_{50}: 30.6 μg/mL. Efficacy and mechanisms of action in various normal and cancer cell models coupled with bioassay-guided purification to identify active anticancer compound(s) from the crude extract will be useful.

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