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Chemical Composition and Anticancer Properties of *Alpinia calcarata* Rosc. Rhizome

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Abstract
Alpinia calcarata, a member of Zingiberaceae family, is commonly found in Bangladesh and has been used for many years in traditional medicine systems for the prevention of various disease conditions and EEACR showed antiproliferative activity against Ehrlich ascites carcinoma cells [1]. In the present study, EEACR induced Ehrlich ascites carcinoma (EAC) cell death in a dose dependent manner (at a range of concentration 6.25-100µg/mL). EEACR induced nuclear condensation and fragmentation which are notable features of apoptosis as observed by fluorescence microscopy after staining EAC cells with Hoechst 33342. In addition, GC–MS analysis of EEACR confirmed the presence of carotol, 3a(1H)-azulenol, 2,3,4,5,8,8a-hexahydro-6,8a-dimethyl-3-(1-methylethyl) (2.305%), hexadecanoic acid or palmitic acid (11.448%), oleic acid (7.603%), arachidic acid (3.199%), palmitic acid 2-(tetradecayloxy)ethyl ester (1..696%), 1-phenylcyclohexanecarboxylic acid (18.477%) and phthalic acid, 6-ethyloct-3-yl 2ethylhexyl ester (55.271%). The overall findings of this study suggest that EEACR may provide a natural source of antineoplastic activity.

Keywords: *Alpinia calcarata*, Anticancer, Apoptosis, GC-MS analysis.

1. Introduction
The growing incidence of cancer, a fatal disease claiming 6 million lives each year, along with tremendous adverse effects of the existing drugs has increased the demand for effective chemotherapeutic agents with availability and least or no unfavorable effects to our body [1, 2]. Recently, use of plant secondary metabolites has been indicated to be a promising approach for the prevention of human cancer due to their scavenging ability of reactive oxygen species, a contributing factor for cancer development, as well as apoptotic death inducing ability of cancer cell [3, 4].
Alpinia calcarata Roscoe, a member of Zingiberaceae family, is widely distributed in Sri Lanka, India, and Malaysia and traditionally used in medicinal systems in Sri Lanka [5]. Rhizome is the most important part of this plant and is used to treat cough, respiratory ailments, bronchitis asthma, arthritis and diabetes [6-9]. Several studies conducted on this part have reported important biological properties including antibacterial, antifungal, antihelminthic, anti-inflammatory, antinociceptive, aphrodisiac, gastroprotective, and antidiabetic activities [10-17]. A study previously conducted by our group using Ehrlich ascites carcinoma (EAC) tumor bearing Swiss Albino mice has disclosed the cytotoxic properties of ethanolic extract of *Alpinia calcarata* rhizome [1]. In this present study, we have concentrated towards cell growth inhibition, cell morphological studies of EAC cells. The study also analyzed the chemical composition of ethanol extract of *Alpinia calcarata* rhizome.

2. Materials and methods
2.1. Collection of plant materials and authentication
Rhizome of *Alpinia calcarata* Rosc (Family: Zingiberaceae) were collected from Rajshahi University area, Rajshahi, Bangladesh, in December, 2015 and were authenticated by a taxonomist at the Department of Botany, University of Rajshahi, where a voucher specimen (No. 02) of this collection was deposited for further reference.

2.2. Preparation of extract
The dried powdered Rhizome of *Alpinia calcarata* Rosc was extracted with ethanol (250g powder in 500 ml ethanol) at room temperature and after filtration, filtrates were evaporated under reduced pressure at 40ºC using a rotary evaporator to have ethanol extract (about 9% yield) and it was designated as EEACR (Ethanol extract of *Alpinia calcarata* rhizome).

2.3. Chemicals and reagents
Hoechst 33342, RPMI-1640 medium, and MTT were purchased from Sigma (USA). Penicillin-streptomycin and fetal calf serum from Invitrogen (USA). Trypan blue and all other chemicals were of analytical grade obtained commercially.

2.4. Animals and ethical clearance
Swiss albino mice of 3-4 weeks old, weighting 28±32 grams, were collected from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B), Mohakhali, Dhaka. The mice were grouped and housed in iron cages and sustained under standard laboratory conditions (temperature 25±2^{o}C; humidity 55±5%) with 12:12 h light–dark cycle. All the animals were permissible free access to standard dry pellet diet (Collected from ICDDR'B, Dhaka) and water ad libitum. Protocol used in this study for the use of mice as a animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources, (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.5. Tumor cells
EAC cells required for this study were collected from the Indian Institute for Chemical Biology (IICB), Kolkata, India and maintained by weekly i.p. inoculation of 10^6cells/mouse under laboratory conditions.

2.6. Cell Culture
EAC cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum, and 1 % (v/v) penicillin–streptomycin, in a humidified atmosphere of 5 % CO_2 at 37 °C.

2.7. *In vitro* cell viability test by MTT colorimetric assay
Viability of EAC cells was tested by MTT assay [18]. Cells (2.5×10^5 in 200µl RPMI 1640 media) plated in a flat bottom culture plate containing extract at different concentrations (6.25-100 µg/ml) were kept in CO_2 incubator at 37 ^{0}C for 24 h, followed by the removal of the supernatant. Then 180 µl of PBS along with 20 µl of MTT was added and incubated for the second time at 37°C for 8 h. After clearing away the supernatant, 200 µl of acidic isopropanol was put into each well of the culture plate and finally incubation was carried out at 37°C for 1 h. Titer plate reader was used to read the absorbance at 570 nm. Cell proliferation inhibition ratio was calculated by the following equation:

$$\text{Proliferation inhibition ratio (\%)} = (A - B) \times \frac{100}{A}$$

Where A is the OD_{570} nm of the cellular homogenate (control) without EEACR and B is the OD_{570} nm of the cellular homogenate with EEACR.

2.8. Cell Morphologic Change and Nuclear Damage
The nuclear morphological changes of EAC cells in EEACR - treated (8 mg/kg/mouse/day) and untreated control mice were investigated using a fluorescence microscope (Olympus iX71, Korea). Briefly, after washing with PBS for several times, EAC cells (both EEACR-treated and untreated) were stained with Hoechst 33342 for 10 min at 37 °C in the dark and again washed with PBS. Finally, a fluorescent microscope was used to examine the extract-induced apoptosis morphologically [18].

2.9. GC-MS analysis of bioactive molecule
Separation and identification of the components of ethanol extract were performed by GC–MS agilent 6890 N gas chromatography hooked to agilent 5973 N mass selective detector. They equipped with a flame ionization detector and capillary column with HP-5MS (30 m × 0.25 mm × 0.25 µm). In GC settings: the initial oven temperature was set at 60°C for 1 min and ramped at 10° C min^{-1}to 180° C for 1 min and then ramped at 20°C min^{-1} to 280 °C for 15 min. The temperature of the injector was controlled at 270°C. The samples (1 µl) were injected neat, with a split ratio of 1: 10. Helium was used as the carrier gas at a flow rate of 1.0 ml min $^{-1}$. Spectra were scanned from 20 to 550 *m/z* at 2 scans s^{-1}. Identification of most constituents by gas chromatography was done by comparing their retention indices with those reported in the literature or with those of authentic components available in database.

2.10. Statistical analysis
All values were expressed as mean ± SD (Standard Deviation). Graph Pad Prism software was used to find the IC_{50} values. Data have been analyzed by one way ANOVA followed by Dunnett 't' test using SPSS software of 16 version.

3. Results
3.1. Inhibition of cancer cell growth with EECZ *in vitro*
MTT assay was used to investigate the effect of EEACR on Ehrlich ascites carcinoma (EAC) cells growth. EEACR induced EAC cell death in a dose dependent manner (Fig 1A). A reduced cell growth was observed with EEACR at a concentration as low as 6.25µg/mL which markedly increased with increasing concentration of EEACR compared to control. A strong inhibition (60.87%) of EAC cell growth was observed at concentration of 50µg/mL which is further increased (79.89%) at concentration 100µg/mL of EEACR. The IC_{50} value of the EEACR was determined as 25.78µg/mL against EAC cell (Fig 1B).

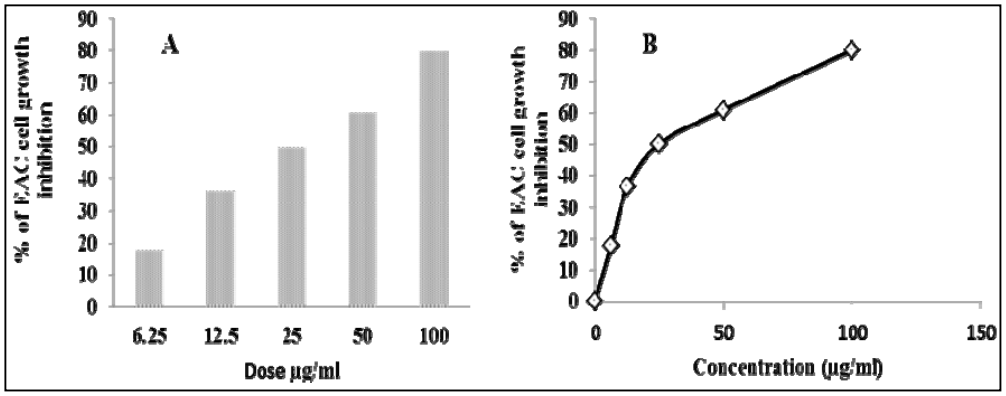


Fig 1: (A) Growth inhibition of EAC cells by EEACR when EAC cells were treated with various doses of EEACR for 24 h. The inhibition ratios were measured by the MTT assay (n = 3, mean ± S.D.). (B) IC_{50} value of EEACR was calculated from the dose-response curve.

3.2. Effect of EEACR on Cell Morphological Change

Hoechst 33342 staining was performed to confirm the ethanol extract induced apoptosis of EAC cells and the results were presented in Fig 3. In this morphological examination, the control cells were round, regular, and homogeneously stained with Hoechst 33342 (Fig 2A) while treated EAC cells exhibited membrane and nuclear fragmentation a hall mark of apoptosis (Fig 2B).

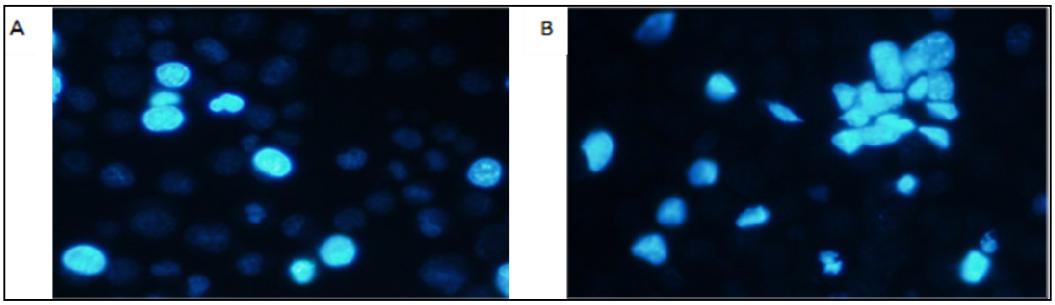
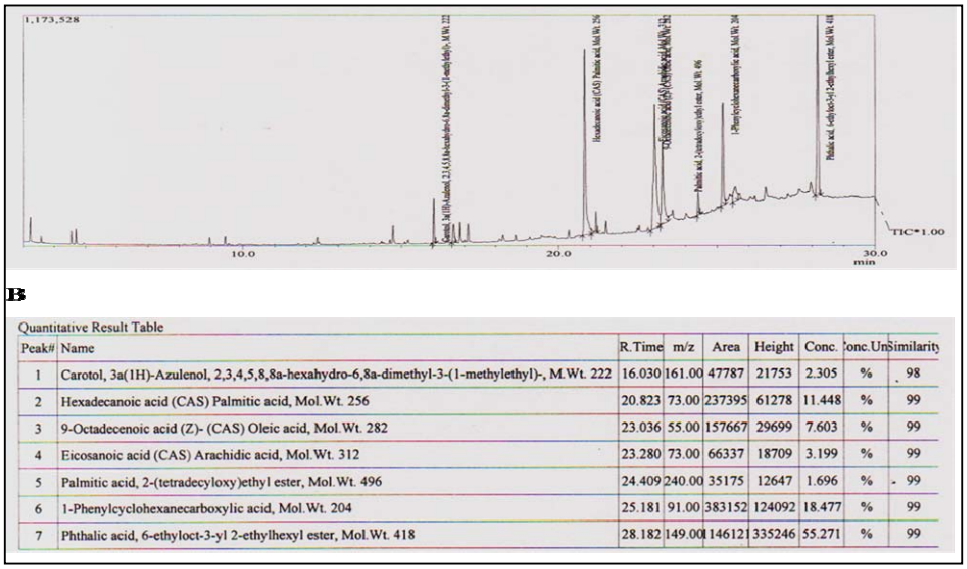


Fig 2: EEACR induced apoptosis is in Ehrlich ascites carcinoma (EAC) cell. EAC cells were treated for 24 h then cells were collected from the treated and non-treated EAC-bearing mice and stained with Hoechst 33342 and observed by fluorescence microscopy. Left panel (A) indicates control and right panel (B) indicates EEACR (8 mg/kg/day) noted that apoptotic characteristics e.g. nuclear condensation and fragmentation are seen in figure B.

3.3. Chemical profile of EEACR analyzed by GC-MS

The chemical profile of this extract that was identified by GC-MS spectrum are summarized in Figure 3. A total of seven components as carotol, 3a(1H)-azulenol, 2,3,4,5,8,8a-hexahydro-6,8a-dimethyl-3-(1-methylethyl) (2.305%), hexadecanoic acid or palmitic acid (11.448%), oleic acid (7.603%), arachidic acid (3.199%), palmitic acid 2-(tetradecayloxy)ethyl ester (1..696%), 1-phenylcyclohexanecarboxylic acid (18.477%) and phthalic acid, 6-ethyloct-3-yl 2ethylhexyl ester (55.271%) were identified in the ethanol extract, accounting for 100% of the extract.



B

Quantitative Result Table

Peak#	Name	R.Time	m/z	Area	Height	Conc.	Conc.Un	Similarity
1	Carotol, 3a(1H)-Azulenol, 2,3,4,5,8,8a-hexahydro-6,8a-dimethyl-3-(1-methylethyl)-, M.Wt. 222	16.030	161.00	47787	21753	2.305	%	98
2	Hexadecanoic acid (CAS) Palmitic acid, Mol.Wt. 256	20.823	73.00	237395	61278	11.448	%	99
3	9-Octadecenoic acid (Z)- (CAS) Oleic acid, Mol.Wt. 282	23.036	55.00	157667	29699	7.603	%	99
4	Eicosanoic acid (CAS) Arachidic acid, Mol.Wt. 312	23.280	73.00	66337	18709	3.199	%	99
5	Palmitic acid, 2-(tetradecyloxy)ethyl ester, Mol.Wt. 496	24.409	240.00	35175	12647	1.696	%	99
6	1-Phenylcyclohexanecarboxylic acid, Mol.Wt. 204	25.181	91.00	383152	124092	18.477	%	99
7	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester, Mol.Wt. 418	28.182	149.00	146121	335246	55.271	%	99

Fig 3: (A) The results of the main chemical compositions of EEACR determined using GC-MS.
(B) GC-MS chromatogram of ethanol extract showing the peaks of the main active compounds.

4. Discussions

EAC cells counsel unique reimbursement for anticancer drug test attributable to their aptness to cram in almost any mouse host. Moreover, EAC cells lack H-2 histocompatibility antigen, which is the apparent reason for their quick proliferation [18]. In our previous study, EEACR at dose 8 mg/kg/day was injected i.p. in mice and found 85.7% growth inhibition against Ehrlich ascites carcinoma cells [1]. In the present investigation, MTT assay was performed in the present study and the result showed that EEACR was found to inhibit EAC cells proliferation in a dose-dependent manner.

An intrinsic cell-suicidal program known as apoptosis exists in multicellular organisms in which cells undergo destruction by showing characteristics changes including cell shrinkage, apoptotic body formation and condensation of chromatin [18]. Induction of apoptosis is a highly desired aspect of an anticancer drug since this process removes cancer or malignant cells without damaging normal cells. Fluorescence microscopic analysis of cells stained with Hoechst 33342, a blue fluorescing dye that stains chromatin DNA, is a rapid and convenient way to observe cell morphological features such as nuclear fragmentation, chromatin condensation etc [19]. In our study, we used Hoechst 33342 staining assay to confirm the proapoptotic effect of EEACR. Nuclear condensation, fragmentation and perinuclear apoptotic bodies were observed in EEACR treated EAC cells but not in control cells. These findings suggest that EEACR possesses antiproliferative activity on EAC cells which could be resulted from the induction of apoptosis or inhibition of cell growth. Induction of apoptosis in EAC cells by different plant extracts has been revealed by several previous studies [19].

In previous studies, oleic acid, Phthalic Acid and hexadecanoic acid or palmitic acid were found to possesses antitumor activity suggesting that the potent anticancer activity of EEACR may be responsible for the presence of these active components [20-22].

Conclusions

Our study demonstrated that ethanol extract of *Alpinia calcarata* rhizome exhibited strong anticancer activity by inducing apoptosis and could have great importance as therapeutic agent for the treatment of cancer.

Conflict of interest

The author declares no conflict of interest.

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