Research Article

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INVESTIGATING THE CYTOTOXIC EFFECT OF COMBINED RHIZOME EXTRACT (*ALPINIA CALCARATA* AND *ALPINIA SPECIOSA*) AND GOLD NANOPARTICLE IN THE BREAST CANCER MCF-7 CELL LINES

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Received on: 10/04/17 Accepted on: 18/05/17

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DOI: 10.7897/2277-4343.082114

ABSTRACT

This study was carried out to investigate the cytotoxicity of human breast carcinoma cell lines (MCF-7) using *Alpinia calcarata* and *Alpinia speciosa* (Family: *Zingiberaceae*) rhizomes and rhizome extracted gold nanoparticles (green synthesis). Cells were cultured in DMEM medium and treated with rhizomes (RR2282) and gold nanoparticles (RR2283) for consecutive days. Cell proliferation assay, DNA fragmentation assay and LDH leakage assays were performed to evaluate and investigate the cytotoxicity in MCF-7 cells. It can be seen that inhibition of cell proliferation induced by the drug was dependant on concentration of plant extracts and gold nano particles. At the highest concentration of RR2282 tested (1000µg/ml), the proliferation of MCF-7 decreased by 90.69±2.0% after 12h. On the other hand, the proliferation of MCF-7 decreased by 77.05±1.5% at the highest concentration (1000µg/ml) of RR2283 tested. When the concentration of RR2282 and RR2283 test samples were decreased, cell cytotoxicity percentage was found decreased. LDH leakage assay showed RR2282 and RR2283 were capable of killing MCF-7 cells more effectively by increasing the cell permeability leading to increased cell death. Electrophoretic analysis of DNA extracted from untreated and treated cells showed significant differences in DNA fragmentation. The DNA from untreated cells was not fragmented, but all other samples resulted in DNA fragmentation. The study concluded that the extracts of *Alpinia* spp could be considered as a promising chemotherapeutic agent in breast cancer treatment. Gold nanoparticles anticipated that nanoparticle-mediated targeted delivery of drugs might significantly reduce the dosage of anti-cancer drugs with better specificity, enhanced efficacy and lower toxicities.

Keywords *Alpinia calcarata*, *Alpinia speciosa*, Cytotoxicity, DNA fragmentation, Anticancer.

INTRODUCTION

Over 80,000 breast cancer cases were reported in India every year. Breast cancer is the second common cancer and also reported as the leading causes of death in women. This implies that there is a continuing need for the development of new anticancer drugs to effectively treat breast cancer patients. Plant-based natural products after systematic screening provided good hope for effective treatment of cancer.[1] Plants have played an important role as a source of effective anticancer agents, and it is significant that 60% of currently used anticancer agents are derived from natural sources including plants, marine organisms, and microorganisms.[2] The plant-based natural products like the aqueous and solvent extracts showed significant anti-tumor, anti-cancer and anti-proliferative effects on cultured human tumor cell lines.[3,4,5] The rhizomes of Zingiberaceae family are widely used in many ancient countries as traditional medicine for the treatment of disease and disorder.[6] The plants *Alpinia calcarata* and *Alpinia speciosa* belongs to this family, Zingiberaceae has a widespread occurrence in Bangaldesh, India, Sri Lanka and Malaysia. Researchers have reported the anti-inflammatory effect[7], antioxidant, antifungal[8] and anticancer activity[9] of the plant extracts of *Alpinia calcarata* and *Alpinia speciosa*. The plants are slender aromatic herb used in the traditional systems of medicine to treat diabetes, rheumatism, fever and stomachache[10]. Based on this medical significance of *Alpinia calcarata* and *Alpinia speciosa*, the anticancer properties were investigated in the present study.

Nanoparticles with biological molecules are the promising tools for the development of diagnostic devices and cancer therapy. Recent advancement in technology has introduced gold nanoparticles into the medical field for the chemotherapy, treatment and diagnosis of cancer in human beings. The anti-cancer activity of gold nanoparticles was reported. The anticancer abilities of gold nanoparticles were studied with MCF 7 breast cancer cell lines with reference of standard anticancer drugs like Tamoxifen and Letroz.[11] Gold is used internally in the tissues of human due to their chemical inertness past 50 years. Gold nanoparticles were highly significant nanoparticles due to their biocompatibility and non-cytotoxicity.[12] Gold nanoparticles accumulate in the tumour cells and show optical scattering. Therefore, these can act as the probe for the microscopic study of cancer cells.[13]

Green synthesis of gold nanoparticles was reported from the plant extracts of *Camellia sinensis*, *Coriandrum sativum*, *Mentha arvensis*, *Phyllanthus amarus*, *Artabotrys hexapetalus*, *Mimusops elengi*, *Syzygium aromaticum*.[14, 15, 16, 17]

Hence, based on this medicinal significance the *in vitro* anticancer activity of rhizomes extract and green synthesized gold nanoparticles in combination against MCF-7 breast cancer cell lines were investigated for the first time in the present study.

MATERIALS AND METHODS

Collection of plant material and Identification

The rhizomes of *Alpinia speciosa* (Herbarium voucher specimen numbers AUT/NGP/53) and *Alpinia calcarata* (Herbarium voucher specimen numbers AUT/NGP/54) of Family *Zingiberaceae* were collected from ABS Botanical garden Kaaripatti-636 106, Salem, Tamil Nadu, India. The rhizomes were authenticated by Dr.A.Balsubramanian, Department of Botany, Consultant Central Siddha Research, Salem, Tamil Nadu, India. Research work was carried out from September 2016 to February 2017 in the Department of Biochemistry, Bharathiar University, Coimbatore, Tamil Nadu, India.

Plant Preparation

Fresh rhizomes *Alpinia calcarata* and *Alpinia speciose* of Family *Zingiberaceae* were washed thoroughly under running tap water followed by sterile distilled water, were cut into small pieces and air dried for 12 - 15 days in the shade. The shade dried rhizomes were pounded to coarse powder in a low speed blender and stored in air tight container at room temperature till future use.

Preparation of Rhizome extract[18]

About 500g of each *Alpinia calcarata* and *Alpinia speciosa* powdered rhizomes were extracted with 1.5L of ethanol separately using Soxhlet extraction apparatus for 3days. The extract was evaporated to dryness using rotary flash evaporator. About 5.42g of *Alpinia calcarata* and 5.65g of *Alpinia speciosa* was obtained after the extraction procedures. The extraction was dried and weighed in electronic balance (d = 0.02g accuracy). The dried extract was stored for further testing in the refrigerator. The combined rhizomes of ethanolic extract *Alpinia calcarata* and *Alpinia speciosa* (1:1) was subjected to the cultured human breast carcinoma, MCF-7 cells was investigated.

Extraction for Synthesis of Gold Nanoparticle using Rhizomes extract[19]

About 15ml of aqueous combined rhizomes extract were carefully added 10ml of 1mM $HAuCl_4$ solution in 250ml Erlenmeyer flasks. The flasks containing extract were incubated at room temperature in dark conditions. After kept in dark conditions for 24hrs.The gold nanoparticles synthesized from the rhizome and they were centrifuged at 1600 rpm for 15min in order to obtain the pellet. The pellets were dried and analysed for further experiments.

Cell culture and harvesting[20]

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS) was used for culturing the MCF-7 (Human breast carcinoma) cell lines. MCF-7 (Human Breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Medium was supplemented with penicillin (100 IU/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml). Stock cells were cultured in humidified atmosphere of 5% CO_2 at 37°C until become confluent. The stock cultures were grown in 25cm^2 culture flasks (Tarsons India Pvt. Ltd., Kolkata, India). Cells were harvested from 80 to 90% confluent culture by trypsinization (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS).

Assessment of cell proliferation[21]

MTT assay was used to assess the cytotoxicity based on the reduction of MTT by mitochondrial dehydrogenase enzyme of the viable cells to purple formazon product as described by Mosmann, (1983).[20)] Briefly, cells were diluted in the growth medium and seeded in 96 well plates at a concentration of 1×10^5 cells/well. After 12 hours, the growth medium was replaced with exposure medium (DMEM without FBS) containing the different concentrations (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml) of rhizome extracts [Combined rhizomes of *Alpinia calcarata* and *Alpinia speciosa* (RR2282) and rhizome synthesized gold nanoparticles (RR2283)]. After incubation, the spent medium was discarded and cells were washed once with DMEM and resuspended in fresh medium (200 ml/well). MTT (0.1 mg/well) was added to cells followed by incubation for 4 h at 37°C (+5% CO2). The formazan crystals formed were solubilized by incubating the cells with 10% SDS overnight. The absorbance of the solution was measured at 570nm, using a microplate reader (Bruken, Germany). Cell survival was expressed as percentage of viable cells of treated sample to control samples. The test was performed in triplicates and the experiments were repeated at least three times.

DNA fragmentation studies[22]

MCF – 7 (3 x 10^6 /ml) were seeded into 60mm Petri dishes and incubated at 37°C with 5% CO_2 atmosphere for 24 h. The cells were washed with medium and were treated with extract, standard drug and incubated at 37ºC, 5% CO_2 for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with G Biosciences, USA apoptotic DNA ladder kit. The recovered DNA was loaded onto 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed.

LDH leakage assay[23]

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37º C for 3 days in 5% CO_2 atmosphere. After 72 h, the drug solutions in the wells were pooled separately and LDH levels were estimated as per manufacture instructions (ERBA diagnostics).

RESULTS AND DISCUSSION

Assessment of cell proliferation

MCF-7 cells were treated with increasing concentration of RR2282 and RR2283 (62.5µg/ml - 1000 µg/ml) and their loss of viability was assessed by the MTT assay (Figure 1 and 2). It was found that rapid loss of cell proliferation occurred with the increasing concentration. It can be seen that inhibition of cell proliferation induced by the drug was dependant on concentration of plant extracts and gold nano particles. Comparison of results has shown that MCF-7 cells were sensitive by three folds to RR2282 than RR2283 [Figure 1 and 2 respectively]. At the highest concentration of RR2282 tested (1000µg/ml), the proliferation of MCF-7 decreased by

90.69±2.0% after 12h. On the other hand, the proliferation of MCF-7 decreased by 77.05±1.5% at the highest concentration (1000µg/ml) of RR2283 tested. During this study, it was found interesting that when the concentration of RR2282 and RR2283 test samples were decreased, the cell cytotoxicity percentage was found decreased. For each concentration of RR2282 and RR2283, more cell cytotoxicity percentage was observed for the former than the latter one. This was evident from Figure 1 and 2.

The doses that caused 50 percent inhibitory effect (CTC_{50}) of RR2282 and RR2283 are shown in Table 1. In MCF 7 cells treated with RR2282, the CTC_{50} values after 12 h of incubation were 196.67±2.9µg/ml. Compared to RR2282, the RR2283 treated MCF-7 cells exhibited 730.00±5.0µg/ml of CTC_{50}.

The obtained results have shown that RR2282 and RR2283 remarkably reduced proliferation of wild-type MCF-7 cells in a dose dependant manner. The effect of the drug in these cells was significantly perceptible within 12h of incubation. Treatment of cells with low drug concentrations (5–10mg/ml) showed decrease in proliferation by 50% indicating the intense sensitivity of these cells to plant extracts. This cell line showed considerable tolerance to the drug till doses around 250µg/ml for incubation up to 12h. RR2283 (gold nanoparticles) treatment, however, produced anti-proliferative effect in MCF-7 cells at lower concentrations (3.51±1.3% cytotoxicity at 62.5µg/ml; and 7.69±2.3% cytotoxicity at 125 µg/ml) thus proving that the effect was purely concentration and time dependent. This resistance of MCF-7 cells to lower concentration of gold nanoparticles was a function of drug concentrations and incubation time, which may, partly be ascribed, to the absence of p53 dependant signalling process in these cells. According to them the chemotherapeutic drugs are known to induce cytotoxicity in tumour cells through diverse mechanisms, in which signalling events play an important role depending upon the cell type and stimulus. This study aiming to investigate the effects of two different samples (RR2282 and RR2283) on MCF-7 breast cancer cell lines, differing in its p53 status was thus proved.

LDH Leakage Assay

The effect of RR2282 test concentrates (100 µg/ml and 200 µg/ml) and RR2283 (350µg/ml and 700 µg/ml) on cell permeability of cell lines are presented in Table-2. The amount of LDH in the media was quantified as enzyme units.

LDH leakage is considered as a marker of cytotoxicity. LDH leakage assay monitors the integrity of the plasma membrane and is sensitive and easy to perform. This assay was used to determine the effect of the extract on the integrity of the plasma membrane of cultured MCF – 7 cells. This is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The extracts of RR2282 and RR2283 were capable of killing MCF – 7 cells more effectively, by increasing the cell permeability leading to increased cell death. When compared to RR2283, RR2282 test concentrates even at lower concentrations found more lactate dehydrogenase enzyme units. The release of LDH to the media was in a concentration dependent manner. This observation also suggests the lesser damage done by RR2283 extract to the cell lines. It has been well documented that lactate dehydrogenase levels, as a marker of necrosis in the cell medium, elevate when cells are exposed to high concentration of anticancer agents. The decreased release of LDH by RR2283 is a result of its anticytotoxic activity, while an increased amount of LDH in the media is suggestive of the anti-cancerous potency of RR2283 extracts against MCF – 7 cells.

DNA fragmentation studies

Electrophoretic analysis of DNA extracted from untreated and treated cells [RR 2282 (400 µg/ml), RR 2282 (200 µg/ml), MCF – 7 (Untreated), Doxorubicin (5 µg/ml), RR 2283 (1400 µg/ml), RR 2283 (700 µg/ml)] are shown in Figure 3. The DNA from untreated cells was unfragmented (MCF – 7), but all the other samples resulted DNA fragmentation. The DNA migrated as discrete bands which was compared to DNA markers, gave a ladder. Such DNA ladders are considered to be a hall mark of apoptosis, continues smears may also indicate DNA fragmentation due to apoptosis. The ladder from DNA fragmentation catalyzed by an endogenous endonuclease that cleaves inter-nucleosomal DNA to form ladder like bands of oligonucleo some fragments. From the obtained results, it was revealed that all the treated test samples of RR2282 and RR2283 when compared with the control (Doxorubicin - 5µg/ml) have a potential anticancer activity on selected MCF - 7 cell lines.

Several studies have shown that a number of herbal medicine or mixtures have anticancer potential *in vitro* and *in vivo*. According to Lee and Houghton[24] herbal medicines were considered to be good candidate for the development of anti-cancer drugs. Most chemo-preventive agents suppress the various stages of signalling events of carcinogen-induced transformation of normal cells, with striking inhibition of diverse cellular events related to cancer development. Inhibition of cancer cell proliferation, growth factor signalling, induction of apoptosis in cells with malignant potential, contributes for chemo-preventive properties.

Ozoren et al.[25] described that increasing evidences suggest that most chemopreventive agents are capable of inducing apoptosis in the target cancer cells either with mitochondrial or by an uncharacterized mechanism in certain cases. Search for novel agents from plants with promising features of apoptosis induction in cancer cells is an attractive strategy. In this present research, such strategy was followed using the extracts of *Alpinia calcarata* and *Alpinia speciosa* and synthesized gold nanoparticles against breast cancer MCF-7 cell lines.

Significant bioactive compounds like quercetin, cinnamate and protocatechinic acid were found to be present in the leaves, roots and rhizomes extracts of *Alpinia calcarata*.[26] Kong et al.[27] screened other biochemical diterpene compounds like sesquiterpenes, Calcaratarins and herniarin.

Similarly, essential oil from *Alpinia speciosa*, mostly extracted from rhizomes contains terpenoids and phenylpropanoids. This includes monoterpenoids such as α- and β-pinene, geraniol, borneol, citronellol, linalool, 1,8-cineole and camphor, sesquiterpenoids including eudesmol, β-sesquiphellandrene and curcumene, and phenylpropanoids such as methyl eugenol.[28]

In our previous study[29] the phytochemical analysis of different solvent extacts of *Alpinina calcarata* and *Alpinia speciosa* were investigated. During this analysis, all the significant phytochemical constituents emphasizing the anticancer properties of *Alpinina calcarata* and *Alpinia speciosa* extracts were reported. *Alpinina calcarata* ethanol extracts revealed the presence of alkaloids, flavonoids, phenols, tanins, carbohydrates, proteins and steroids. Similarly, *Alpinia speciosa* ethanol extracts revealed the presence of alkaloids, flavonoids, phenols, tanins, carbohydrates, proteins, steroids and thiols. It

was reported that flavonoids and phenolic constituents of the ethanolic extracts contains the anticancer properties.

The above reported bioactive compounds of *Alpinina calcarata* and *Alpinia speciosa* extracts (RR2282) were reported to induce cytotoxicity and apoptosis on MCF-7 cells. The toxic response of gold nanoparticle samples (RR2283) to human breast epithelial MCF-7 cells was compared to the result of plant extract samples (RR2282).

Gold nanoparticles exhibited significant cytotoxicity to MCF-7 cells in dose and concentration dependent manner when analyzed by MTT assay. The present research revealed that exposure of gold nanoparticle samples to MCF-7 cells cause cytotoxicity which is comparatively better than caused by the plant extract samples (RR2282). The dose and/or concentration range from 62.5 to 1000 µg/mL. This anti-proliferative effect was due to the induction of apoptosis. The obtained results are evident in Figure 1 and Figure 2; the explained apoptosis in this study thus partially contributed in the cell toxicity.

It was reported that induced toxicity may also leads to non-apoptotic cell death. Apoptosis is explained by different morphological features like cell and nuclear shrinkage, membrane blabbing and chromatic condensation[30]. Gold nanoparticles in the field of cancer therapy have the potential to provide a target based delivery in a localized way. Recently, several groups have reported the use of gold nanoparticles for the successful delivery of drugs like doxorubicin for overcoming drug resistance in cancer[31] as well as peptide functionalized gold nanoparticles for targeting tumor.[32]

Table 1: Concentrations of rhizomes and gold nanoparticles (RR2282 and RR2283) causing cytotoxicity and 50% inhibitory effect (CTC50) in MCF-7 cell proliferation

S. No.	Samples	Concentration (µg/ml)	Cytotoxicity (%)	CTC_{50} (µg/ml)
1	RR 2282	1000	90.69 ± 2.0	196.67 ± 2.9
		500	82.09 ± 1.9	
		250	73.69 ± 0.4	
		125	20.51 ± 1.9	
		62.5	13.58 ± 1.1	
2	RR 2283	1000	77.05±1.5	730.00 ± 5.0
		500	25.96±2.2	
		250	12.00±2.2	
		125	7.69±2.3	
		62.5	3.51±1.3	

RR2282: Concentrations of rhizomes, RR2283: Concentrations of gold nanoparticles

Table 2: Effect of rhizomes and gold nanoparticles (RR2282 and RR2283) on cell permeability of MCF-7 cell lines (LDH leakage assay)

S. No.	Test Samples	Test Conc. (µg/ml)	LDH IU/ml
1	RR2282	100	99.6
		200	171.6
2	RR2283	350	99.6
		700	171.6
3	Standard	1.5	1044.2
4	Control	-	61.7

RR2282: Concentrations of rhizomes, RR2283: Concentrations of gold nanoparticles

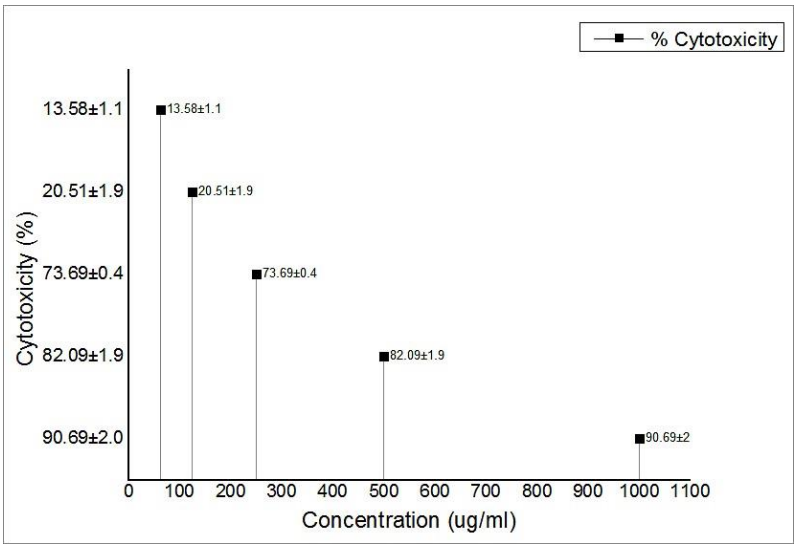


Figure 1: Concentration dependant effect of rhizomes (RR2282) on proliferation of human breast cancer cells (MCF-7)
Cells ($1X10^5$/well) were seeded in 96-well tissue culture plate followed by treatment with indicated concentrations of RR2282. Cell proliferation was determined by the MTT reduction assay after the specified incubation time of 12h. There was substantial decrease in proliferation of MCF-7 cells with respect to concentration (dose). Data are expressed as mean of results from three independent experiments.

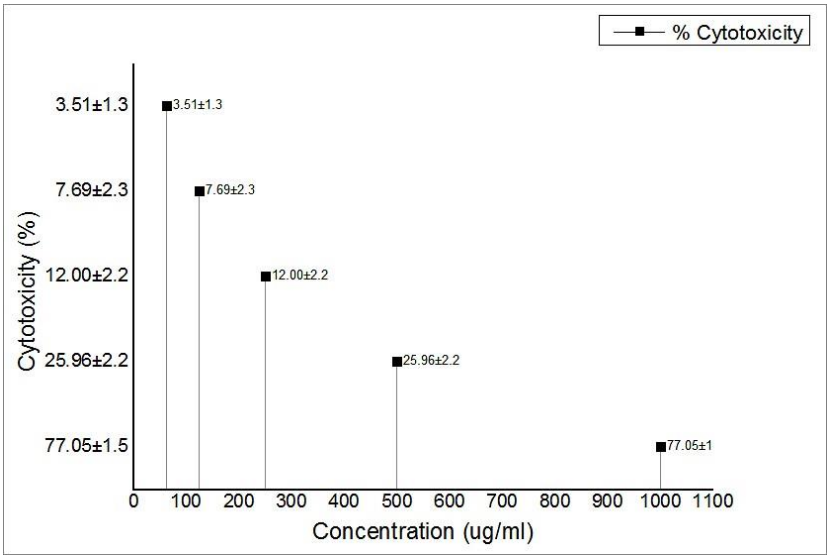
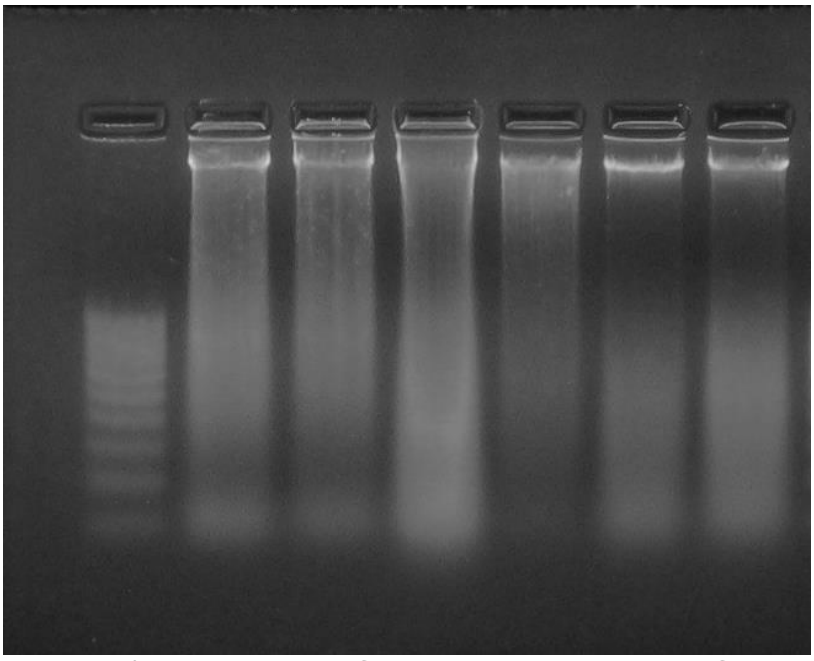


Figure 2: Concentration dependant effect of gold nanoparticles (RR2283) on proliferation of human breast cancer cells (MCF-7)
Cells (1X10^5/well) were seeded in 96-well tissue culture plate followed by treatment with indicated concentrations of gold nanoparticles (RR2283). Cell proliferation was determined by the MTT reduction assay after the specified incubation time of 12h. There was substantial decrease in proliferation of MCF-7 cells with respect to concentration (dose). Data are expressed as mean of results from three independent experiments.



A B C D E F G

Figure 3: DNA fragments from untreated and treated cells [RR 2282 (400 µg/ml), RR 2282 (200 µg/ml), MCF – 7 (Untreated), Doxorubicin (5 µg/ml), RR 2283 (1400 µg/ml), RR 2283 (700 µg/ml)]
A: Marker, B: RR2282 (400 µg/ml), C: RR2282 (200 µg/ml), D: MCF – 7 (Untreated),
E: Doxorubicin (5 µg/ml), F: RR 2283 (1400 µg/ml), G:RR 2283 (700 µg/ml)

CONCLUSION

Overall, this study showed that the *Alpinia calcarata* and *Alpinia speciosa* extracts (RR2282) containing bioactive compounds and gold nanoparticles would inhibit the proliferation of breast cancer cell lines (MCF-7). The compounds were likely to be some benzenoids such as protocatechuic acid, alkaloids and different flavonoids isolated from leaves of *A. calcarata* and *Alpinia speciosa*. The extracts of *Alpinia calcarata* and *Alpinia speciosa* could be also considered as a promising chemotherapeutic agent in breast cancer treatment. Gold nanoparticles anticipated that nanoparticle-mediated targeted delivery of drugs might significantly reduce the dosage of anti-cancer drugs with better specificity, enhanced efficacy and lower toxicities. Further studies are needed to fully recognize the mechanism involved in cell death; and the present data showed that extracts inhibits significantly proliferation of the human breast carcinoma cells.

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Cite this article as:

Mohanasundari L and Suja S. Investigating the cytotoxic effect of combined rhizome extract (*Alpinia calcarata* and *Alpinia speciosa*) and gold nanoparticle in the breast cancer MCF-7 cell lines. Int. J. Res. Ayurveda Pharm. 2017;8(Suppl 2):207-212 http://dx.doi.org/10.7897/2277-4343.082114

Source of support: Nil, Conflict of interest: None Declared