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In-Vitro Cytotoxicity of Gandhaga Rasayanam (GR) on Human Breast Cancer (MCF-7) Cells

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ABSTRACT: Clinically speaking, cancer is characterized by unchecked cell proliferation and a gene-level deficiency in regulating cell division and tissue growth. According to a alarming WHO statistic, breast cancer has overtaken ovarian cancer as the second most frequent malignancy in women. In 2020, there were more than 2.3 million new cases and 6,85,000 fatalities. The current situation, which includes more adverse reactions to conventional chemotherapy and repeated desensitization of tumor cells by exposure to radiation, prompt researchers in the field of cancer biology to look into potential alternatives to current treatments that might not have as many side effects. *Gandhaga Rasayanam* (GR), a siddha formulated medication, recommended for treating leucoderma, piles, psoriasis, skin infections as per Siddha text *"Siddha Vaithiya Thirattu"* present to possess anti cancer properties. Using the MCF-7 breast cancer cell line and an in-vitro MTT assay, the current study examined the assessment of cell viability and cytotoxicity of this unique formulation.

Keywords: Siddha medicine, *Gandhaga Rasayanam*, Breast cancer, Anti-cancer, MTT assay.

INTRODUCTION

Cancer is clinically characterized by uncontrolled cell proliferation with gene level deficit in controlling cell multiplication and tissue growth. Alarming statistic by WHO, breast cancer become the second most commonly diagnosed cancer in females. Over 2.3 million new cases and 6,85,000 deaths occurred in 2020. Currently, conventional chemo-therapy and repeated desensitization of tumor cells towards radiation has many adverse effects and often delays therapeutic benefits. This piques the interest of researchers studying cancer biology, who may then look for other medications that might not have any side effects. Therefore, to lessen the Financial and psychological burden it's essential to research and explorate alternate medicines (Gunduz and Gunduz 2011). It has been clear in recent years that breast cancer is not a single disease but rather a group of tumors with distinct molecular makeup that arise from the breast's epithelial cells9 (Done, 2011). Cell lines appear to be an essential component for the molecular detection of breast cancer since they may be widely used in many areas of laboratory research and are particularly helpful in vitro models in cancer research (Brudall *et al.,* 2003).

A popular breast cancer cell line called MCF-7 has been produced over many years by several groups (Baguley and Leung 2011). It serves as a useful model cell line for research on breast cancer everywhere, including studies including anti-cancer medications. It is ER (oestrogen receptor)-positive and PR (progesterone receptor)-positive and belongs to Luminal-A molecular subtypes (Shirazi *et al.,* 2011) MCF-7 cells studies has given abundant practical knowledge data for patient care than any other cell line studies (Sweeney *et al.,* 2012).

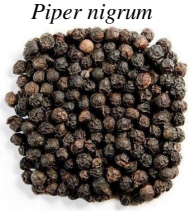
AIM: Present study aimed at exploring the assessment of cell viability and cytotoxicity of this novel formulation using MCF-7 breast cancer cell line by in-vitro MTT assay.

MATERIALS AND METHODS

Study drug selection: Ingredients of Gandhaga Rasayanam (*Therayar vaatha kaviyam*) are mentioned below in the Table 1.

Table 1.

Sr. No.	Botanical name	Tamil Name	Vernacular Name	Parts Used	Quantity
1.	Purified Sulphur 	Gandhagam	-	-	300grams
2.	*Withania somnifera* 	Amukkara Kizhangu	Wintercherry	Roottuber	150grams
3.	*Smilax china* 	Parangichakkai	China root	Root	60grams
4.	*Terminalia chebula* 	Kadukkai	Inknut	Fruit	30grams
5.	*Phyllanthus emblica* 	Nellikai	Indian gooseberry	Fruit	30grams
6.	*Terminalia bellerica* 	Thandrikkai	Belleric myrobalans	Fruit	30grams
7.	*Zingiber officinale* 	Chukku	Driedginger	Rhizome	30grams

8.	*Piper longum* 	Thippilimoolam	Longpepper	Root	30grams
9.	*Piper nigrum* 	Milagu	Blackpepper	Fruit	30grams
10.	*Embelia ribes* 	Vaividangam	Embelia	Seeds	30grams
11.	*Syzygium aromaticum* 	Kirambu	Cloves	Inflorescence	30grams
12.	*Santalum album* 	Chandhanam	Sandalwood	Wood	30grams
13.	*Plumbago zeylanica* 	Chithiramoolam	Ceylonleadwort	Rootbark	30grams
14.	*Cicer arietinum* 	Kadalai	Bengal gram, chick pea	Seeds	30grams
15.	*Semecarpus anacardium* 	Senkottai	Markingnut	Nut	30grams

16.	*Elataria cardamomum* 	Elam	Cardamom seeds	Seeds	30grams
17.	Palmsugar 	Nattuchakkarai	-	-	800grams
18.	Honey 	-	-	-	400grams
19.	Ghee 	-	-	-	400grams

Preparation of study drug: The drugs required for the preparation of GR were purchased from Ramasamy chettiyar herbal shop. After proper authentication of the drugs GR was prepared as per the reference of the siddha classical text book *"Therayar vaidhya kaaviyam 1500"*.

The purified form of sulphur were finely powdered, followed by this the other drugs were also finely powdered and mixed with purified powdered sulphur. Equal ratio of palm jaggery powder and added. Followed by this required amount of honey and ghee was added.

RATIONALE BEHIND SELECTING STUDY DRUG

Purified sulphur: Treatment of MDA-MB-231 cells with inorganic sulphur inhibited cell growth via the ErbB-Akt pathway. By preventing EGFR expression and activation, it prevents cell division. Cancer cell proliferation was unaffected by treatment for the first 24 hours, whereas 72 hours of treatment significantly and dose-dependently reduced cell proliferation (P0.05) (Ha *et al.,* 2013).

***(a) Withania somnifera* (WS):** A C28 steroid lactone known as withaferin A, 5, 20-Dihydroxy-6, 7-epoxy-1-oxo-with a-2, 24-dienolid, was discovered in the root extract of WS in 1973. Its structure was discovered to be comparable to a with anolide isolated from the roots of Withania coagulants (Menssen and Stapel 1973). Are porton the involvement of WS in malignancies dates back to 1992 (Devi *et al.,* 1992). When WFA (Withaferin A)'s proapoptotic response was investigated, it was discovered that the phytochemical decreased the expression of the oestrogen receptor (ER) protein in MCF-7 cells. In the presence of 17-estradiol (E2), this effect was reversed. As a result, WFA has an anti-estrogenic impact, and p53 knock down lessens some of the proapoptotic effects of WF (Hahm *et al.,* 2011). The MCF-7 cell line can undergo apoptosis when WFA binds to vimentin intermediate filaments, causing them to collect in the cytoplasm (Mohan and Bargagna-Mohan 2016).

Through the suppression of vimentin, the root extract also prevents the spread of breast cancer and the transition of epithelial to mesenchymal tissue (Yang *et al.,* 2013). Inhibition of oxidative phosphorylation and complex III activity, as well as the release of apoptotic histone-associated DNA fragments in the cytosol, were the causes of WFA-induced ROS-mediated apoptosis (Antony *et al.,* 2014). Amukra can be employed for effective and secure cancer therapy since it mediates the selective destruction of cancer cells by inducing ROS generation and mitochondrial damage (Widodo *et al*., 2007; Ghosh *et al.,* 2016; Sehrawat *et al.,* 2019; Hahm and Singh 2013).

Smilax china.

According to the study, Smilax china substantially reduces invasiveness in the human breast cancer cell lineMDA-MB-231 by utilising the uPA, uPAR, and TIMP expression modulators (Nho *et al.,* 2015).

Triphala (*Terminalia chebula, Phyllanthus emblica, Terminalia bellerica*):

Similar to ascorbic acid, triphala may be able to scavenge free radicals. (Parveen *et al.,* 2018). It eliminates free radicals like superoxide and DPPH (Naik *et al.,* 2005). Studies showed that triphala's pro-oxidants raises ROS levels and causes apoptosis in MCF-7 and barcl-95 breast cancer cells (Sandhya and Mishra 2006).

As a result, it made the treated MCF-7 breast cancer cells less viable. Triphala administration was discovered to cause MCF-7 to undergo in vitro apoptosis in addition to cytotoxicity. Additional mechanistic studies of breast cancer cells using single-cell gel electrophoresis revealed that the factor raised intracellular ROS and produced DNA damage, which are characteristics of death. Because the addition of antioxidants decreases the anti-proliferative activity, triphala-induced ROS production plays a significant role in apoptosis (Sandhya *et al.,* 2006).

Chebulinic acid, one of its active constituents, selectively prevents VEGF-induced angiogenesis by preventing the phosphorylation of VEGF receptor 2 (VEGFR-2), which slows the growth of tumours. decrease metastases, etc. (Lu *et al.,* 2012). Western blotting was used to examine the anti-cancer impact, and the results showed that it prevented the phosphorylation of EGFR, AKT, and ERK. (Tsering and Hu 2018). It has pro-oxidant effects on cancer cells, which causes it to function as an anti-cancer agent. Its dual nature makes it more effective as both a chemo-preventive and a chemotherapeutic agents inceit acts as an antioxidant in healthy cells and as a pro-oxidant in cancer cells.

Thirikadugu (*Zingiber officinale, Piperlongum, Pipernigrum*): The breast cancer cell line MCF-7's proliferation and colony formation were reduced by ginger treatment against breast cancer cell proliferation. Reduced cell viability, chromatin condensation, DNA fragmentation, activation of caspase-3,and apoptosis with cleavage of poly(ADP-ribose) polymerase were some of the effects it had at the Molecular level, Bax over expression and Bcl-2 protein down regulation may contribute to ginger-mediated apoptotic cell death. It reduced the expression of cell cycle regulators including cyclin D1 and cyclin-dependent kinase4 (CDK-4)as well as pro-survival genes like NF-B, Bcl-X, Mcl-1, and Survivin rice pad dock. On the other hand, it boosted p21's expression, which is a CDK inhibitor. Additionally, it reduced the expression of the well-known cancer molecular targets c-Myc and human telomerase reverse transcriptase (hTERT). These finding simply that ginger might be an effective therapy option for breast cancer (Elkady *et al*., 2012). *Piper nigrum* had cancer preventive and anti cancer effects on mammary tumerogenesis (Sriwiriyajan *et al*., 2016). *Piper longum* showed potential of anti-bacterial activity and anti-inflammatory activity (Meena and Ramaswamy 2015).

Embelia ribes:

By using the MTT and apoptotic assays, isolated embelin was evaluated in the MCF-7 breast cancer cell line. Different levels of embelin were employed, and it was shown that the IC50 value, which triggers apoptosis in a dose-dependent manner, is 80 g/ml (Kaur *et al.,* 2015).

Syzygium aromaticum:

In MTT assays, the essential oil of clove had the strongest cytotoxic effect, with IC50 values of 36.43g/mL and 17.6g/mL for the 24 and 48-hour time periods, respectively. As a result, they offer potential sources for the creation of anti-cancer drugs and exhibit excellent cytotoxicity when used on MCF-7 cells (Kumar *et al.,* 2014).

Santalum album:

Sandalwood is well-known for having a range of medicinal benefits, such as anti-inflammatory, antioxidant, antiviral, and antibacterial qualities. Sandalwood oil and -santalol have been shown to have chemopreventive properties in cell line and animal experiments. The capacity of -santalol to cause cell-cycle arrest and apoptosisin cancer cells is its mode of action against cancer. Regardless of the oestrogen receptor or p53 status, researchon MCF-7 (p53 wild-type) and MDA-MB-231 (p53 mutant) breast cancer cells demonstrated that -santalol-induced G2/Mphase cell-cycle arrest (Santha and Dwivedi 2015).

Plumbago zeylanica:

In MCF-7 cells, plumbagin (5-hydroxy-2-methyl-1,4napthoquinone) increased the expression of p21CIP1/WAF1 and decreased the level of cyclin B1, resulting in a G2/M cell cycle arrest. The Bax/Bcl-2 ratio and the release of cytochrome c were both dramatically elevated by plumbagin ,which caused the MCF-7 cells to undergo apoptosis. In addition, plumbag in markedly elevated intracellular ROS levels, but pre-treatment with the ROS scavenger N-acetyl cysteine shielded cells against the cytotoxicity caused by plumbagin. As a result, it is possible that ROS production is crucial to the anticanceraction (De *et al.,* 2019). Studies have demonstrated the considerable anti oxidant properties of *P. zeylanica* extracts and plumbagin, the active component (Tilak *et al.,* 2004).

Cicer arietinum:

Iso flavones in chickpeas may promote the apoptosis of MCF-7 cells by inhibiting the expression of HNRNP family genes mediated by lnc RNAs. The isoflavones have positive effects on improving the survival time of breast cancer patients by up-regulation and down-regulation of hub genes in the PPI network (Wang *et al.,* 2020).

***Semecarpous anacardium*:**
SA is well known for its antitumour property. the flavonoids present in SA has the ability to prevent various cancers. Hence it has promising anti-oxidant, anti-tumour, anti-neoplastic cytotoxic and cytostatic activity (Premalatha and Sachdanandam 1998; Premalatha and Sachdanandam 1999; Francis *et al.,*1989; Gothoskar *et al.,*1971; Vad, 1973).

Elataria cardamomum:
Cardamom has been shown to possess antioxidant properties and chemopreventive activity and suppression of breast cancer growth. Includes connection of DC(di-indolylmethane) and IC3 (indole-3-carbinol) compounds which inhibit proliferation of breast cancer cells. These compounds also boosts the host's immune system (Vutakuri *et al*., 2018).

Invitro Study OF GR:

Stock Solution:
10mg/ml concentration of the sample GR was prepared using n-hexane at the concentration of 10,50,100 and 200µg/ml. The diluted sample GR was transferred to the cuture plate. 500 µl of MCF7 cell at the 1×10^4 cells/well.

Cell lines and cultural conditions:
MCF7 breast cancer celline was obtained from National Centre for Cell Sciences (NCCS), Pune. MCF7 cell lines were cultured in Minimal Essential medium (MEM) with 10% FBS, Trypsin, EDTA, Glucose, 1% streptomycin, penicillin and amphotericin B under a fully humidified atmosphere 5% CO_2 at 37°C.

Cell Treatment Procedure:
To make single cell suspensions, the monolayer MCF7 breast cancer celline line were detached with trypsin ethylene diamine tetra acetic acid (EDTA) and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1 x 10^5 cells / ml. One hundred micro liters per well of cell suspension were seeded in to 96-well plates at plating density of 10,000 cells / well and incubated to allow for cell attachment at 37 degree Celsius, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentration 10, 50, 100 and 200µg/ml of the test formulation. They were initially dissolved in n-hexane and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, serial dilutions were made to provide four sample concentration. Following the treatment with test sample, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% air and 100% relative humidity. The medium without samples were used as control and triplicate was maintained for all concentrations.

MTT assay. The effect of test sample on the viability of MCF7 breast cancer celline were determined by MTT (3-[4,5-dimethylthiozole-2-yl]-2-5-di phenyl tetrazolium bromide) assay. 100µl of cells suspensions in growth medium were plated in 96-well micro titre plate at concentrations of 1×10^4 cells/well and incubated for 48 hrs at 37°C in a humidified incubator. After 48 hours incubation the cell reaches the junction. Then, cells were incubated in the presence of various concentrations of the samples in n-hexane for 72 h at 37°C. After removal of the sample solution and washing with phosphate buffered saline (pH 7.4), 20µL of MTT (5mg/mL) was added to each well of the plate. The plate was incubated for 4hrs at 37°C. The solution in each well along with MTT was aspirated, 100µL of buffered DMSO was added to dissolve formazone. The plates were shaken for 5min. Optical density was measured on a microplate ELISA readed at 540 nm with control. The cytotoxicity was observed by comparing the absorbance between the control and samples The percentage of inhibition was calculated as follows:

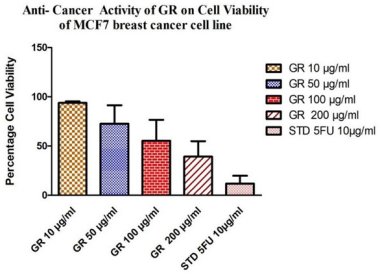
% cell viability = A540 of treated cells/A540 of control cells × 100% IC_{50} was calculated from dose-response curves (Zarei and Yaghoob 2019; Kis *et al.,* 2022)

RESULTS AND DISCUSSION

Effect of Test drug GR on Cell viability of MCF7 breast cancer cell line are given below in the Table 2 and Graphically represented in the Graph 1.

Table 2:

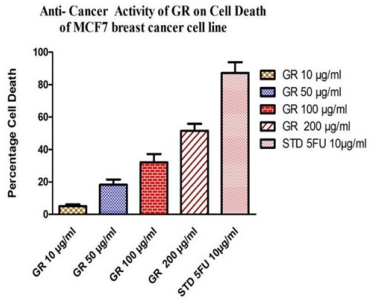
Sr. No.	Concentration µg/ml	%cell Viability
1.	10µg/ml	93.87 ± 1.536
2.	50µg/ml	72.61 ± 18.84
3.	100µg/ml	55.23 ± 21.58
4.	200µg/ml	39.23 ± 15.74
5.	STD(5-Fluorouracil 10µg/ml)	11.81 ± 7.93



Graph 1.

Effect of Test drug GR on Cell Death of MCF7 breast cancer cell line are given in the below Table 3 and Graphically represented in the Graph 2.

Sr. No.	Concentration in µg/ml	% cell Death
1.	10µg/ml	5.12 ± 1.126
2.	50µg/ml	18.28 ± 3.245
3.	100µg/ml	32.11 ± 5.13
4.	200µg/ml	51.66 ± 4.213
5.	STD(5-Fluorouracil10µg/ml)	87.2 ± 6.631



Graph 2.

The results obtained from the study reveals that the percentage of cell viability ofMCF-7 breast cancer cell line viability decrease with increase in concentration of the test drug GR. Least viability of cell was observed at the concentration of 200µg/ml 39.23 ± 15.74%, followed by this at 100 µg and 50 µg shows 55.23 ± 21.58, 72.61 ±18.84, similarly 10 µg/ml shows 93.87±1.536% cell viability in MTT assay as shown in the below Fig. 1-6. The corresponding IC50 value of the sample GR was found to be188 ±20.44 µ.

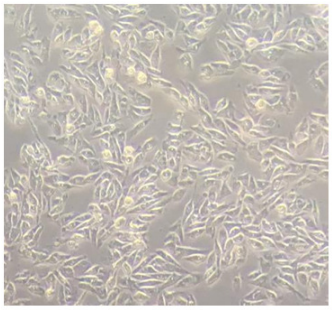


Fig. 1. MCF-7 Control Cells.

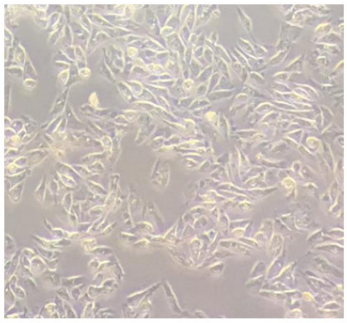


Fig. 2. Test Drug GR-10 µg/ml.

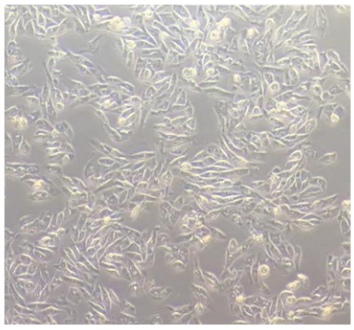


Fig. 3. Test drug GR -50µg/ml.

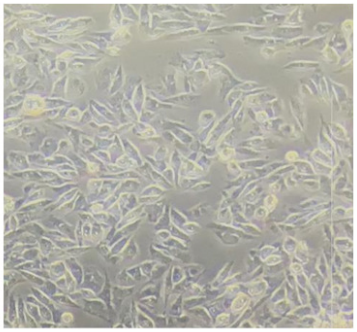


Fig. 4. Test drugGR -100 µg/ml.

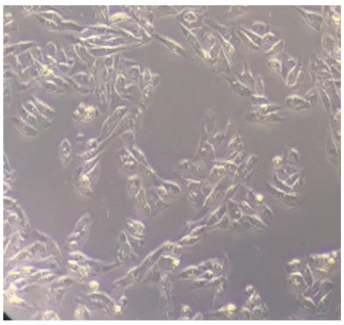


Fig. 5. Test drug GR -200µg/ml.

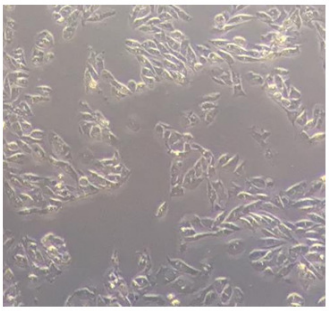


Fig. 6. STD Drug-10 µg/ml.

DISCUSSION

Since ancient times, herbal formulations have been practiced as remedies against several ailments. One of there requirements for anti-cancer drugs is that they must be cytotoxic compounds against cancer cells. It is a NCE or herbal substitute or biological compound for killing cancer cells. Many anti-cancer products on the market are in herently cytotoxic and are also approved by regulatory agencies. Anticancer agents are approved if they are found to be clinically effective despite cyto toxicity. Therefore GR is cytotoxic. As a result of this preliminary in vitro study of herbal drug, Gandhaga Rasayanam is one of the most important complementary, economic friendly and alternative medicine in breast cancer treatment. The study strongly suggest that when used in combination, natural compounds produce potential synergistic effects in-vitro against MCF-7 cancer cellline. Thus herbs in the GR presents a strong case for combined effect as well as additivism of the multiplicity of compounds, most of which have been scientifically claimed to fight and inhibit cancer in one or more aspects.

CONCLUSIONS

This in vitro study confirms that Fundamental anticancer properties of GR on dose dependent, pave the way for in vivo studies and support the benefits of *gandhaga rasayanam*, as an evidence-based alternative to breast cancer treatment.

FUTURE SCOPE

Anti-cancer property of Gandhaga Rasayanam (GR) should be further evaluated for pre-clinical and multi-centered clinical trials. Awareness of this drug is required for its world wide usage.

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Conflict of Interest. None.

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