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IN VITRO EVALUATION OF ANTI-CANCER ACTIVITY OF KUKKUTNAKHI GUGGUL ON BREAST CANCER

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ABSTRACT

In the plant kingdom, there are number of plants that yield medicines useful to mankind. Some herbal products are a source of many traditional medicines. According to retrospective literary review, the *anubhut* herbal formulation *kukkutnakhi guggul* was prepared as per the reference of *Chikitsa pradeep* and due to its observed clinical efficacy in *arbuda*(cancer), the current in vitro anticancer study was conducted with an aim to check its anticancer effect on MCF-7, MDA-MB-435, MDA-MB-468, Zr-75-1, BT-474 breast cancer cell lines. The current in vitro study was conducted at ACTREC, Kharghar, Navi Mumbai. The selected cancer cell lines were procured from ATCC, USA and NCCS pune and cryopreserved in the liquid nitrogen vapours and DMSO (5-10%) in a liquid nitrogen container. The SRB assay protocol was followed to observe the activity of the study drug. The study drug was tested in 96 well plates with its 4 dilutions in triplicates at 4 dose levels. The growth curve graphs were plotted and LC50, GI50, TGI values were calculated. Adriamycin was used as positive control and solvent itself was used as vehicle control. As per SRB assay protocol, *Kukkutnakhi guggul* had shown moderate activity on MCF-7 breast cancer cell line and had shown negligible activity on MDA-MB-435, MDA-MB-468, Zr-75-1 and no activity had been noted on BT-474 breast cancer cell lines. *Kukkutnakhi guggul* has been definitely proved to be a safe for oral administration and non- toxic at cellular level as its LC50 values were > 160. To study its effect on targeted cancers, specific in vivo scientific studies and human clinical trials should be carried out by further researchers.

Key words: *Kukkutnakhi guggul*, breast cancer, MCF-7, MDA-MB-435, and SRB assay, n vitro study.

INTRODUCTION

According to *charakacharya* no product in the world is without any medical usage.[1]Fuelled by the worldwide demand for herbal medicines this laid the foundation for evolving proprietary medications. *Kukkutnakhi* (*Aspidiumcicutarium Sw*.)[2-6]is an herb found along the foot hills of *sahyadri* grown around the monsoon and rainy seasons. It is also known as *Kombadnakhi, waghchawdi, bichava* or *nirvishi.*[7-9]Several folklore claims are being subjected to scientific evaluation.[10]This

dravya is used to treat ailments especially of *mamsavahastrotas.*[11] Initially this plant was mentioned in the book named "*Gharguti aushadhe"*[11]and "Ferns of Bombay"[12]in year 1922. After its traditional use, in 1967 a scientific magazine named *"Ayurved patrika"*[13] published the medicinal use of this drug and the first time the formulation "*Kukkutnakhi guggul"* was mentioned in the famous book "*Chikitsa pradeep"*[14] by B.V. Gokhale and this gave a birth to the idea of combining *kukkutnakhi* and *guggul*. This way, if one sees the literary review of both drugs, both of them are used as an *arbudhar* drugs. So, by using suitable SOP, it was decided to prepare the formulation named*"Kukkutnakhi guggul"*.[15]Most of the pharmaceutical companies manufacture this drug with the objective of gaining commerce, but its therapeutic effects are not scientifically evident. So, in the current research it was decided to study efficacy of *kukkutnakhi guggul* preclinical before going for human trial.

As a single entity, cancer is the biggest cause of mortality worldwide. Overall, there were 14.1 million new cases and an estimated 8.2 million deaths from cancer in 2012. Breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed in 2012 (second most common cancer overall). This represents about 12% of all new cancer cases and 25% of all cancers in women.[16-22]Current approaches to combat cancer rely primarily on chemotherapy and radiation, which are themselves carcinogenic and may promote recurrences and the development of metastatic disease. Hence, most of the research work on cancer drugs is targeted on plants and plants derived natural products.

This formulation is expected to act on *Stanarbuda* (Breast cancer). Hence, before experimenting human trials it becomes mandatory to study its efficacy on breast cancer cell lines. Keeping this aim in mind 5 breast cancer cell lines i.e. MCF-7, MDA-MB-435, MDA-MB-468, Zr-75-1, BT-474 were selected for study to evaluate the in vitro anticancer activity of *kukkutnakhi guggul*.

2. Material and Methods:

2.1Material **:**MCF-7, MDA-MB-435, MDA-MB-468, Zr-75-1, BT-474 breast cancer cell lines were procured from American Type Culture Collection (ATCC) 10801 University Boulevard Manassas, VA 20110 USA and NCCS Pune. Srb colorimeter, 25 cm^2 tissue culture flasks, 15 ml centrifuge tubes, 96 well plate, CO_2 incubator, Haemocytometer, Drug dispensing machine, Multichannel automated pipette, Elisa reader etc. were used for in vitro study. The chemicals like liquid nitrogen, DMSO, SRB dye, 1% acetic acid Ethanol, 10%TCA were used in the current study.

2.2 Method: 2.2.1 Cancer cell line culture[23-25]**:** The selected cancer cell lines were checked for quality control and then cryopreserved in the liquid nitrogen vapours and DMSO (5-10%) in a liquid nitrogen container. The cell lines were grown in cell culture flasks containing RPMI 1640 medium. At the time of cell culture single cell suspension of the required cell line grown in tissue culture is made. Cell culture flask was then kept in a CO_2 incubator at 37.5^0C for 24-48 hrs for the purpose of cell division. Cells were counted with the help of haemocytometer

and cell count was adjusted according to the titration readings (approximately 1x 10^5 cells/ml) Cell culture was done under all aseptic condition inside the laminar flow hood to avoid bacterial contamination.

2.2.2 Preparation of solution of test drug[23]: The test substance name and container were confirmed before preparing drug solution. The molecular weight of study drug was not known and hence, the solution was prepared in a proportion of 1:100(stock solution: star solution). The same concentration was used for the vehicle control and also for positive control. Ethanol was used as a star solution of the drug. Solution is made using ratio 1:100 in a test tube and it was mixed gently with the help of magnetic stirrer. The experimental drugs were solubilized in solvent at 400-fold the desired final maximum test concentration and stored frozen prior to use. At the time of drug addiction an aliquot of frozen concentrate was thawed and diluted to 10 times the desired final maximum test concentration with complete medium containing test article at a concentration of 100, 200, 400 and 800µg/ml.

2.2.3 Plate preparation and drug addition[23, 26]: For in vitro anticancer screening, 100 µl cells/ well were seeded into 96-well microtiter plates so that every well receives $5X10^3$cells. After cell inoculation, the plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 24h. The incubated plates were then placed in drug dispensing machine inside the laminar flow hood to avoid bacterial contamination. The study drug was tested in 96 well plates with its 4 dilutions in triplicates at 4 dose levels at 10, 20, 40, 80 µg/ml. Adriamycin (Doxorubicin) was used as a positive control drug for comparative screening and ethanol was used as a vehicle control.

2.2.4 SRB assay[26, 27]: On addition of the drug, the plates were incubated further for 48 hours at 37^oC in humidified CO_2 (5%) incubator. After incubation, 50µL of 30% TCA was added to fix the cells to the bottom of the wells. After 60 minutes incubation at 4°C, plates was washed gently under tap water and air dried at room temperature. Then 100 µL SRB (sulforhodamine B) reagents was added into each well and left for 15minutes and the SRB dye was removed by washing the plates with tap water. 1% acetic acid was used to remove unbound SRB dye. After air drying, 0.1ml of 10mM UN buffered TRIS base was added and the absorbance was read on the Elisa plate reader at the wavelength of 540nm with reference to 690nm. Optical density of drug treated cells was compared with that of control cells and growth inhibition as calculated as percent values.

2.2.5 Endpoint measurement[23]: Using the six absorbance measurements [time zero (Tz), control growth(C) and the test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels. Percentage growth was calculated on a plate by plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of control wells. Percentage growth inhibition was calculated as [(Ti-Tz)/ C-Tz)] x 100 for concentration for which Ti>/=Tz (Ti-Tz) positive or zero and [(Ti-Tz)/ Tz)] x 100 for concentration for which Ti<Tz. (Ti-Tz) negative. The dose response parameters were calculated for each test article. Growth inhibition of 50%

(GI50) was calculated from [(Ti-Tz)/ C-Tz)] x 100= 50. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti=Tz. The LC50 was calculated from [(Ti-Tz)/ Tz)] x 100= -50. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameters were expressed as greater or less than the maximum or minimum concentration tested. From the values graph were plotted and results were given in terms of LC50, TGI and GI50 values.

3. Observation and results

Table 1: % Control growth of human breast cell line MCF-7

	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
KG	83.7	82.3	80.4	73.7	92.1	91.6	77.3	71.2	91.2	79.5	75.2	67.4	89.0	84.5	77.6	70.8
ADR	-28.9	-34.6	-55.2	-56.1	-22.6	-30.9	-52.7	-53.9	-26.1	-27.5	-43.5	-44.6	-25.9	-31.0	-50.5	-51.5

Table 2: % Control growth of human breast cell line MDA-MB-435

	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
KG	100.0	100.0	100.0	84.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0	86.7	100.0	100.0	100.0	90.4
ADR	-71.7	-78.6	-80.2	-81.3	-78.8	-81.2	-81.5	-83.6	-74.0	-83.6	-84.0	-84.1	-74.8	-81.1	-81.9	-83.0

Table 3:% Control growth of human breast cell line MDA-MB-468:

	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	40	80	120	160	40	80	120	160	40	80	120	160	40	80	120	160
KG	100.0	100.0	100.0	100.0	100.0	100.0	96.0	85.4	100.0	100.0	97.6	95.6	100.0	100.0	97.9	93.6
ADR	-36.9	-41.7	-44.0	-46.2	-41.0	-44.1	-45.0	-47.6	-45.2	-46.1	-47.8	-57.7	-41.0	-44.0	-45.6	-50.5

Table 4: % Control growth of human breast cell line Zr-75-1:

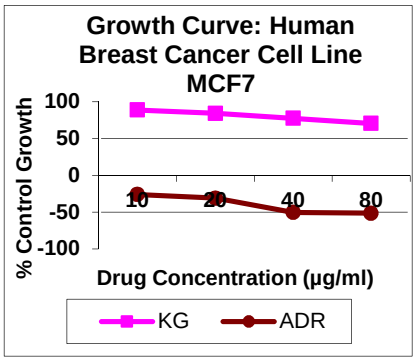
	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	40	80	120	160	40	80	120	160	40	80	120	160	40	80	120	160
KG	100.0	100.0	97.8	91.4	100.0	100.0	100.0	93.8	100.0	100.0	100.0	100.0	100.0	100.0	99.3	95.1
ADR	-2.0	-2.1	-4.4	-4.9	-2.2	-2.8	-3.8	-7.4	-7.2	-8.8	-10.7	-13.7	-3.8	-4.6	-6.3	-8.7

Table 5: % Control growth of human breast cell line BT-474:

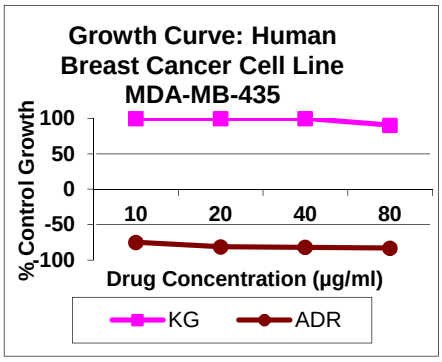
	% Control Growth															
	Drug Concentrations (µg/ml)															
	Experiment 1				Experiment 2				Experiment 3				Average Values			
	40	80	120	160	40	80	120	160	40	80	120	160	40	80	120	160
KG	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
ADR	-41.0	-49.6	-64.0	-70.1	-38.5	-50.1	-68.7	-69.1	-40.3	-50.0	-66.0	-66.6	-39.9	-49.9	-66.3	-68.6

Growth curves of Human breast cancer cell lines:

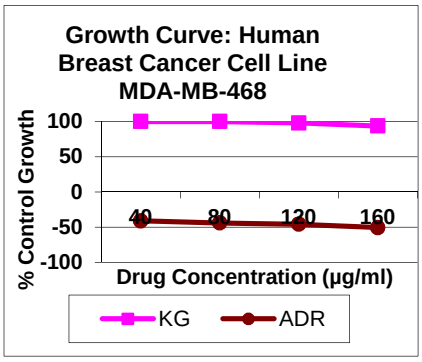
Graph no. 1



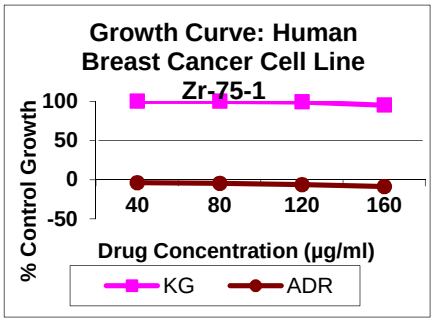
Graph no. 2



Graph no. 3



Graph no. 4



Graph no. 5

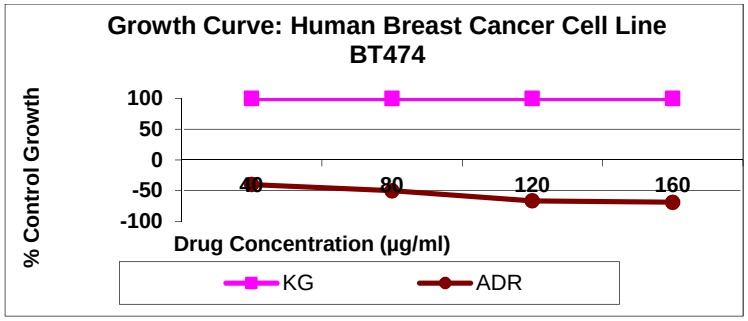


Table no 6: Drug concentrations (µg/ml) calculated from graph for:

	1. MCF7			2. MDA-MB-435			3. MDA-MB-468			4. Zr-75-1			5. BT474		
	LC 50	**TGI**	**GI 50**	**LC 50**	**TGI**	**GI 50**	**LC 50**	**TGI**	**GI 50**	**LC 50**	**TGI**	**GI 50**	**LC 50**	**TGI**	**GI 50**
KG	>80	>80	>80	>80	>80	>80	>160	>160	>160	>160	>160	>160	>160	>160	>160
ADR	60.1	22.0	<10	35.5	<10	<10	59.6	17.3	<10	>80	49.2	<10	48.3	13.8	<10

***LC50**: Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning indicating a net loss of 50% cells following treatment. **GI50**: Growth inhibition of 50 % (GI50) resulting in a 50% reduction in the net protein increase.**TGI:** Drug concentration resulting in total growth inhibition (TGI) **KG:** Kukkutnakhi guggul, ADR: Adriamycin*

DISCUSSION

In the ayurvedic formulary, there are thousands of herbal and herbo-mineral formulations which hold very sound position in the treatment of chronic human ailments. But, as per grant permission of *charkacharya*, researchers can formulate the new formulations as per their clinical experiences.[39]The plant *Kukkutnakhi* is being used as a folklore remedy for the treatment of *shotha* and *shula* etc successfully. Botanically the plant is identified as *Aspidium cicutarium* of the family Drypteridaceae.[3]*Guggul kalpana* a by-product of *vati* has evolved as the most potent and preferable drug delivery mode amongst ayurvedic practitioners. The proprietary drug i.e. *kukkutnakhi guggul* was formulated by using authenticated *kukkutnakhi* and purified *guggul* (*Commiphora mukul* Hook. Ex Stocks*)* in the same proportion for oral administration as per a text "*Guggul kalpana*"[15] *Triphala kwath* was used for purification of crude *guggul*. *Kukkutnakhi guggul* consists of *katu* and *tikta rasa* predominating *vayu* and *akash mahabhuta*, so it acts on disorders of *mamsa* and *medodhatu* like *arbuda* by its *lekhana* property due to dominance of *prithvi and aap mahabhuta.*[15]

Preclinical and clinical research are essential to evaluate integrate new herbal drugs into clinical routine. In-vitro mechanism based on screening of herbal medicine is mandatory in the initial phases of plant drug research before taking them to in-vivo study to evaluate their efficacy.[28]In the current study, *kukkutnakhi guggul* was prepared in the teaching pharmacy and was analysed in the research laboratory of the institute. Standardized *kukkutnakhi guggul* was having total ash% 6.49 ±0.553, acid insoluble ash% 1.323± 0.547, water soluble extract% 35.64±0.590, alcohol soluble extract% 20.45±0.822, pH 3.81±0.041, moisture content% 9.63±0.851, hardness(Kg/cm^2) 1.42±0.080, uniformity of weight %13.267±0.163, friability% 1.566±0.232, disintegration time (min) 9.35±0.229.[29] Its in-vitro anticancer study was conducted at ACTREC, Kharghar, Navi Mumbai. Cryopreservation of 5 selected breast cancer cell lines at -20^{0}C was done by using DMSO and liquid nitrogen and washing of cell line was done by normal saline. DMEM or RPMI medium can be used for cell

growth in tissue culture flask and then cell lines were incubated to maintain pH of the solution. Solvents like Ethanol, water, DMSO were used for preparation drug solution. To avoid the poisonous effect of solvents like ethanol, DMSO prepared solution gets diluted 10 times before addition to cell lines. Solvents are needed to extract the herbo-mineral components for in vitro screening. Water is a universal solvent. Alcohol is the most commonly used solvent for extraction, because of its good polarity & easy penetrating power in cell membranes of plant. Acetone, DMSO, Ethers etc. are the other solvents used for extraction depending upon the solubility of herbo-mineral components.[22, 23]

4.1 SRB assay:

Sulforhodamine B assay is a well-established in vitro method for cytotoxicity against cancer cell lines and here it was utilized to determine the selective activity of the potential anticancer drug. Amongst current available methods for advanced in vitro screening techniques are SRB, MTT, clogenic, flourescent assays & dye exclusion test. SRB assay is particularly useful for qualitative analysis. It provides a better linearity with cell number than MTT assay and is also highly sensitive. Hence, it is more appropriate assay for screening. Due to its accuracy and feasibility, is valued by the researchers. The antiproliferative SRB assay was performed to assess growth inhibition.[27, 30-35]

As per result obtained from 5 breast cancer cell lines, the concentration of study drug was increased up to 160 µg/ml the therapeutic doses of ayurvedic formulations are more when compared to modern drugs like adriamycin which was used as control group. But the concentration was exceeded more than 160 µg/ml, due to the limited capacity of micro titre well plate Values obtained from optical density were estimated and average of observed values of each plate was mentioned in table and labelled as experiment 1, 2, 3 respectively. Graphs were plotted on the basis of average value obtained from each experiment which denotes the effect of study drug and control drug on selected cancer cell lines. The results have been discussed of anticancer study on breast cancer cell lines were as follows:

a. MCF-7 breast:

As per table no. 1 and graph no.1, study drug had shown results in a range of 70-89 and inclination of line in graph, as the drug concentration increases which indicates moderate activity at maximum drug concentration i.e. 80µg/ml. According to the SRB assay protocol, compound is said to be significantly active if the value gets estimated within the limit of 80.

b. MDA-MB-435:

As per table no. 2 and graph no. 2, study drug had shown results in range of 90-100 and very slight inclination in a graph at maximum concentration level i.e. 80 µg/ml This indicates the negligible activity, even if the concentration of drug was increased. Hence, it can be said that *Kukkutnakhi guggul* has negligible effect on MDA-MB-435 cell line.

c. MDA-MB-468:

As per table no. 3 and graph no. 3 study drug had shown result in a range of 93-100 and very slight inclination in a graph at maximum concentration level i.e.160µg/ml which indicates the study drug shows negligible activity even if the concentration of drug was increased. Hence it can be said that study drug has negligible effect on MDA-MB-468 cell line.

d. Zr-75-1:

As per table no. 4 and graph no. 4 study drug had shown result in range of 95-100 and

very slight inclination in a graph at maximum concentration level i.e.160µg/ml which indicates the study drug is not effective even if the concentration of drug was increased. Hence, it can be said that study drug has negligible effect on Zr-75-1 cell line.

e. BT-474:

As per table no. 5 and graph no. 5 study drug had shown result in terms of 100 and no inclination in a graph at maximum concentration level i.e.160µg/ml which indicates the study drug shows no activity even if the concentration of drug was increased. Hence, it can be said that *Kukkutnakhi guggul* does not show any anticancer activity on BT-474 cell line.

4.2 Limitations of current research:

There are certain limitations of srb assay like numerous wash steps involved, but fixation required, less sensitive with non-adherent cells, if drug is insoluble in solvent then it's difficult to perform assay. Compounds may show negative results which gets activated after body metabolism or vice a versa.[36-38]

4.3 Innovation of current research:

On the basis of retrospective literary review, the proprietary herbal formulation like *kukkutnakhi guggul* is evaluated for its anticancer effect in 5 selected cancer lines. The study revealed the positive efficacy of the study drug in breast cancer originated by MCF- 7 cancer cell line. The current study will be pioneer research source for the further anticancer research on plant originated products.

CONCLUSION

In the present in-vitro anticancer study, a sincere attempt has been made to draw the conclusions. According to SRB assay protocol, the study drug had shown moderate activity on MCF-7 breast cancer cell line and had shown negligible activity on MDA-MB-435, MDA-MB-468, Zr-75-1 and no activity had been noted on BT-474 breast cancer cell lines. *Kukkutnakhi guggul* definitely proved to be a safe for oral administration and non- toxic at cellular level as its LC50 values were > 160. To study its effect on targeted cancers, specific in vivo scientific studies and human clinical trials should be carried out by further researchers.

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