Pharmaceutico Analytical Study of Roudra Rasa and Evaluation of its Anti-Carcinogenic Effect in Selected Cell Lines and Acute Toxicity Studies in Albino Wistar Rats:A Study Protocol

ABSTRACT

Introduction: - There are expected to have become 1.9 million new cancer diagnoses and 609,360 cancer - related deaths in 2022, or about 1,670 mortality each day. Regardless of human development level, throughout the world, the disease is a leading source of morbidity and mortality. Cancer like disorders have been documented also in Indian subcontinent since antiquity. In the 19th century, the very first cancer cases were diagnosed, and the burden of cancer began to climb in the 20th century, when current treatment facilities were severely inadequate. *Roudra Rasa* is a special Ayurvedic preparation for *Arbuda* (Cancer) mentioned in Bhaishajya Ratnavali. It has already been tested clinically in Oropharengeal cancer, with Ayurvedic treatment incorporating *Roudra Rasa* showing a greater benefit. Cancer cell lines of human are useful models for exploring cancer biology and testing the treatment effectiveness of anti - cancer drugs in the lab. . That's why Michigan Cancer Foundation-7 (MCF-7 - Breast), Human Colorectal Adenocarcinoma Cell Line (HT-29 - Colon), Human Lung Adenocarcinoma (HOP-62), Lung cancer epthelial cell (A549 - Lung), and cell line from prostate carcinoma (DU145), prostate cancer cell line (PC-3 - Prostate), these cancer cell lines have chosen to examine *Roudra Rasa's* anti cancer activity

Aim and Objectives: - Pharmaceutico analytical study of *Roudra Rasa* and evaluation of its Anti-carcinogenic and Acute toxicity studies in selective cell lines and albino Wistar rats respectively

Materials and Methods: - *Roudra Rasa* will be prepared by traditional method and quality control parameters will be assessed. Anti-carcinogenic activity will be carried out in selected cancer cell lines. and acute toxicity testing will be done in accordance with OECD guidelines 423.

Observations and Results: - Observations of pharmaceutical, analytical and experimental studies will be noted and presented in the forms of table, charts, photographs etc.

Conclusion: - If *Roudra Rasa* shows any significant Anti-Carcenogenic activity then it would be safe and efficient medicine for Cancer.

Keywords: - Cancer, Roudra Rasa, Anti-carcinogenic cell line study, Acute toxicity study

1 INTRODUCTION

One of the most horrible diseases of the twenty-first century is cancer, which is defined as uncontrolled cell proliferation. Many efforts have been made, but success remains elusive, which is why illness anxiety is greater than disease. However, as a result of the dedicated efforts of all areas of medical science, some conceptions have emerged and some light has been shed on the cancer therapy process[1-2].

After heart disease, cancer remains the second leading cause of mortality. There are expected to be 1.9 million new cancer diagnosis and 609,360 cancer deaths in 2022, or about 1,670 mortality each day [3]. With an anticipated 2.3 million new cancer cases (11.7 %), female breast cancer has passed lung cancer as the most often recognised malignancy, followed by lung (11.4 %), colorectal (10.0 %), prostate (7.3 %), and stomach (5.6 %) cancers. Lung cancer remained the leading cause of mortality, with 1.8 million deaths predicted (18%), following by colorectal cancer (9.4%), liver cancer (8.3%), stomach cancer (7.7%), and female breast cancer (6.9 %). Although mortality differed by two times for males and the little for females, total incidence as 2- to 3 times larger in developed countries than that in developing countries for both sexes.

In contrast, female breast cancer death rates in transitional regions were significantly higher than in advanced countries. Despite growing risk factors related to global capitalism and a growing economy, in 2040 the worldwide cancer burden is anticipated to climb to 28.4 million cases, a 47 % rise from 2020, with transitioning (64 % - 95 %) countries experiencing a bigger increase than transitioned (32 %- 56 %) countries. The concept of sustainable framework for the distribution of cancer preventive actions and preventative medicine in developing nations is critical for global cancer control [4].

According to Ayurveda, vitiation of *Vata* and *Kapha Dosha* causes the emergence of numerous non-inflammatory swellings, which are categorised as *Arbuda* (tumour), *Granthi* (swelling), and others (glandular swelling). *Galaganda* (cervical lymphadenopathy) and other conditions. On the basis of its indications and symptoms, the *Arbuda* can be linked to cancer. Although there are no definitive aetiological reasons for *Arbuda* in Ayurvedic texts, the causative variables for *Granthi* and *Vranasopha* have been studied for *Arbuda* therapy. Due to vitiation of *Vata* and *Kapha dosha*, any *Arbuda* can form in any of the *dushya*, i.e. *Rakta* (blood), *Mamsa* (muscle), and *Meda dhatu* (fat). By influencing the *Jatharagni* and *Dhatwagni*, the vitiated *Vata* and *Kapha doshas* produce *Ama* (autotoxin). By inhibiting the *Srotasa, Ama* together with *apakwa dhatu*, creates diverse *dhatugat vikara* in the form of *Arbuda, Shopha, Granthi*, and so on[5-6].

The underlying idea of Ayurveda is that "no disease manifests without the involvement of *Tridosha*," and that the vitiation of *Tridosha* can be controlled with the use of herbal / herbomineral formulations and rasayan drugs [7-8]. *Roudra Rasa* is one of the *Bhasma* preparation mentioned in Bhaishajya Ratnavali for *Arbuda* treatment. *Bhasma* refers to a group of organomineral compounds that have improved stability, bioavailability, bio - compatibility, drug targeting, and therapeutic activity [9]. To be sure, *Bhasmas* are bioactive nanoparticles. Nanotechnology-based efforts to improve cancer treatment are still in the early stages of development, and it would be ideal if the anticancer medicine was also a nanoparticle[10].

Cell lines provide an endless source of widely available, easy-to-produce material, and hence serve as the foundation for reasonably greater tests. This allows large-scale medication combination investigations with cell lines possible. The proficiency to control environment of the in vitro tissue growth has increased the value of cell cultures in pharmacological research dramatically11]. The goal of this study is to test *Roudra Rasa's* anticancer potential on a selective cancer cell lines.

2 AIMS

Pharmaceutico analytical study of Roudra Rasa and evaluation of its Anti-carcinogenic and Acute toxicity studies in selective cell lines and albino wistar rats respectively

3 OBJECTIVES

3.1 Phase I

1. To prepare *Roudra Rasa* described traditionally.
2. To assess the quality control parameters of the *Roudra Rasa*
3. To assess the nano particle size of *Raudra Rasa*

3.2 Phase II

3.2.1 Part I

4. To assess the Anti carcinogenic activity of *Roudra Rasa* on selective human cancer cell lines.
5. To compare relative potency of *Roudra Rasa* in selective cell lines.

3.2.2 Part II

6. To evaluate acute toxicity of *Roudra Rasa* in albino wistar rats.

4 MATERIALS AND METHODS

Study design: This is an experimental, analytical, and pharmacological investigation. The study will be conducted in recognized national laboratories such as M.G.A.C.H.R.C., Salod (H), Central Animal House, DMIHER (DU), Wardha, and others as approved by or suggested by DMIHER (DU). Study will be approved by the Institutional Ethics Committee (IEC) and the Institutional Animal Ethics Committee (IAEC).

The following categories will be used to carry out the current project:

4.1 Pharmaceutical study

To create pharmaceutical standardisation, Roudra Rasa will be manufactured in this study in three batches in accordance with traditional references. The conduct listed below will be followed to complete this pharmaceutical study.

1. Raw material selection
2. Raw drug procurement: All raw ingredients will be obtained from reputable, trustworthy sources in the field.
3. Authentication of raw materials: The Dravyaguna department of MGACH & RC shall verify and authenticate all raw materials..
4. FRLHT, Banglore, shall certify the authenticity of all the row materials.

4.1.1 Preparation of Roudra Rasa : [12]

शुद्धसूतंसमं गन्धं मर्द्यं यामचतुष्टयम् | **नागावल्लीदलयुतं मेघनादपुनर्नवा** || 59||

गोमुत्रपिप्पलीयुक्तंमर्द्यं रुद्ध्वा पुटेल्लघु | **लिहेत् ख्शोउद्रैरसो रोउद्रो गुञ्जमात्रोअर्बुदंजयेत्** || 60||

… (B. R. Pg.no.- 829/59-60)

Shuddha Parad and *shuddha Gandhaka* should be taken in equal quantity and prepare *nishchandra* (lusterless) *Kajjali. Kajjali* then subjected to *bhavana* (Wet grinding) of *Nagavalli Swarasa* (Juice), *Meghanad panchang* (whole plant) *kwath* (Decoction), *Punarnava mula* (Root) *Kwath* (Decoction) or *Patra* (Leaf) *swarasa* (Juice), *Gomutra* (Cow's urine)and *Pippali kwath* respectively. Each *bhavana* should be of four hours. After *bhavana sharava samputa* (Enclosed earthen plates) and *sandhibandhana* (Binding of joints) should be done, and dry it. Dried *sharava samputa* then subjected to *Laghuputa* (Low heat/temperature). After *swangasheeta* (self cooling) obtained *Roudra Rasa* triturate it and collect in a glass jar. This *Roudra Rasa* in 1 *Ratti* (125 mg) doses along with Honey, twice a day cures *Arbuda Roga*.

Standard operative procedure is used after the assurance of quality by using classical and modern scientific quality parameters

Table No. 1: Ingredients and quantity of *Roudra Rasa*

Roudra Rasa			
S.N.	**Ingredients**	**Part used**	**Quantity**
1	*Parad* (Mercury)	Mineral	300 gm
2	*Gandhak* (Sulphur)	Sulphur powder	300 gm
3	*Nagavalli (Piper bitle)*	Leaf	500 gm
4	*Meghanad (Amaranthus spinosus Linn)*	Whole plant	500 gm
5	*Punarnava (Boerhaavia diffusa)*	Whole plant	250 gm
6	*Gomutra* (Cow's urine)	Urine	500 ml
7	*Pippali (Piper longum)*	Fruit	250 gm

Chart 1: Flow chart showing the method of preparation of *Roudra Rasa*

Firstly *Parada shodhan* and *Gandhaka shodhana* will be done

Shuddha Parad + ***Shuddha Gandhaka***

Ref.- Rasatarangini-5/29-30 Ref.- Rasaratna Samucchaya. – 3/24

↓

With equal quantity *Shuddha Parada* and *shuddha Gandhaka*

Kajjali will be prepared.

↓

Kajjali will be subjected to **four hours *Bhavana*** of each Drug.

↓

First ***Nagavalli patra Swarasa*** *Bhavana* will be given for 4 hrs

↓

Then ***Maghanad Kwath*** *Bhavana* will be given for 4 hrs

↓

Then ***Punarnava Kwath*** *Bhavana* for 4 hrs

↓

Then ***Gomutra*** *Bhavana* for 4 hrs

↓

Lastly ***Pippali Kwath*** *Bhavana* for 4 hrs will be given

↓

Final obtained mixture of *Kajjali*, from which ***Chakrika*** will be prepared and dried

↓

sharav samputa will be sealed by keeping *Chakrikas* in that and dried

↓

Then will give *Laghu puta*

↓

After ***swangasheeta***, obtained final product will be collected and stored.

4.2 Analytical Study:

The created formulation will be examined in a significant laboratory. We shall evaluate the organoleptic properties of Roudra Rasa (in accord of API). Some physiochemical measures include pH value, loss on drying, ash value, acid insoluble ash, specific gravity[13]. AAS (Atomic Absorption Spectrophotometry) for mercury as Hg w/w%, arsenic as As w/w%, and sulphur as S w/w%. Additionally, XRD (X-ray defraction), FTIR (fourier transform infrared spectroscopy), and ICP-A ES (Inductively Coupled Plasma Atomic Emission Spectroscopy) will be done. Using SEM-EDX (Scanning Electron Microscop- Energy Dispersive Spectroscopy) the particle size of Roudra Rasa will be calculated[14].

4.3 In-Vitro Study - Anticancer Study:

4.3.1 Cancer cell line:

MCF-7 (Breast), HT-29 (Colon), HOP-62, A549 (Lung), DU145, PC-3 (Prostate) are cancer cell lines have chosen from Tata memorial centre advanced centre for treatment, research and education in cancer, Kharghar, Navi Mumbai.

4.3.2 Procedure for cell treatment:

To achieve the desired density of 1105 cells/ml, the monolayer cells will be detached using trypsin-EDTA to generate a single cell suspension. The number of viable cells will then be counted using a hemocytometer before being diluting with media supplemented with Fetal Bovine Serum (FBS 5%). 100 microliters of inoculum each well, implanted into 96-well plates with an accumulate of 10,000 cells per well, will be cultured to permit cell adhesion at 37°C temparature, 5% CO2, air 95% , and 100% humidity.Following 24 hrs, the cells are being exposed with varied concentrations of the test substances. Then, a fraction of the test solution will be dilute in serum-free media to double the final intended maximum test concentration. They would first dissolve in ordinary dimethylsulfoxide (DMSO).

By carrying out four sequential dilutions, a total of five different concentrations will be acquired. By adding aliquots of 100 µl of each of these different sample diluted sample to the appropriate wells that were initially containing 100 µl of medium, the necessary final concentrations of sample can be attained. The plates should be incubated for a further 24 hours at 37 °C, 5% CO2, 95% air, and 100% humidity once the sample has been introduced. All concentrations will be performed in triplicate, and a medium without samples will be used as a control.

4.3.3 MTT assay

The 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), a yellow, water-soluble tetrazolium compound, is used in the MTT test, a commonly used screening method. Other names for it include the tetrazolium salt assay and the microculture tetrazolium test. Succinate-dehydrogenase, a mitochondrial enzyme present in living cells, disassembles the tetrazolium ring, converting MTT into an insoluble purple formazan. As the result, the quantity of formazan produced depends on the number of live cells.

The MTT test will be used to evaluate a substance's cytotoxicity in cancer cells (Mosmann *et al.,*1983). Cells (1 105/well) will be plated on 24-well plates with 5 ml of media per well (Costar Corning, Rochester, NY). Confluence occurs following 48 hrs of incubation for the cell. Following that, the cells will be cultivated over 24 to 48 hrs at 37°C in the presence of samples. Each well will receive 1 ml of % MTT cells, phosphate-buffered saline solution (5 mg/ml) after being rinsed using phosphate-buffered saline (pH 7.4) and disposing the sample solution. 4. After 4 hours of incubation, 0.04M HCl/isopropanol was used.

To determine which cells were viable, the absorbance at 570 nm will be employed. Based on the collected results, the concentration required to inhibit viability by 50% (IC50) would be visually calculated. The absorbance at 570 nm will be measured using a UV Spectrophotometer with blank wells that don't contain any sample-carrying cells. The effectiveness of the samples against the aggressive growth of the cancer cells of choice will next be evaluated [15].

4.3.4 Sample preparation

Following its dissolution in 100 mg/ml dimethyl sulfoxide, *Roudra Rasa* will be dilute at 100 g/ml, 200 g/ml, 400 g/ml, and 800 g/ml. Then, it will be tested using four different concentrations in a few distinct cell lines: 10 g/ml, 20 g/ml, 40 g/ml, and 80 g/ml. For comparison screening, Adriamycin (doxorubicin) will be employed as a positive control medication [4].

4.4 Acute Toxicity Study:

The current study will be conducted in accordance with the Organization for Economic Cooperation and Development's (OECD) guideline 423 for chemical testing.

4.4.1 Procurement and selection of Animals: - Required healthy Albino Wistar Rats, 180-200g in weight, both Female & Male will be selected from animal house for study.

4.4.2 Procedure: - The first dose is determined by selecting one of four preset oral doses: 5, 50, 300, or 2000 mg/kg body weight. For some of the drug administered animals, the initial dose would be the most probably to induce death.

Table No- 2 - Experimental design

Sr. No	Group	Drug	Dose	No. of animals	Route	Duration
1.	Group I: Normal control	Vehicle(Honey)	2ml/kg	6	P.O	14 days
2.	Group II: Treatment group1	*Roudra Rasa* +Honey	2000mg/kg	6	P.O	14 days
3.	Group III: Treatment group2	*Roudra Rasa* +Honey	300mg/kg	6	P.O	14 days
4.	Group IV: Treatment group3	*Roudra Rasa* +Honey	50mg/kg	6	P.O	14 days

4.4.3 Limit Test at 2000 mg/kg body weight

The mortality rate will be determined at the maximum beginning dose of 2000 mg/kg body weight, followed by a main test.

4.4.4 Main Test

The 2 ml/kg dose volume will be used for study. Female Wistar rats will be fasted for minimum 16-18 hours for acute oral toxicity study. The food will be withheld prior to dosing and 3-4 hours post dosing but drinking water will be provided ad libitum. The development, duration, and severity of toxic symptoms will decide the time interval between dosages. The first set of rats (Group-II) will be dosed with 300 mg/kg body weight. If mortality would not be observed at this dose levels, then Group-III female rats will be dosed with 50 mg/kg body weight. Due to no mortality/morbidity observed further dosing stopped as per the test procedure.

4.4.5 Observations

1. **Clinical Observation :** After test dose administration on day 0 (day of dosing), individual animals will be observed at 30 minutes, 1, 2, 3 and 4 hours of post dosing. Subsequently, all survived animals will be observed once a day during the 14 day observation period.
2. **Mortality:** Animals will be observed for mortality on the day of dosing and subsequently twice daily during the experimental period.
3. **Body weight:** During the observation period, all rats will be weighted on days 0 (before to treatment), 7^{th}, and 14^{th}.
4. **Pathology:** All survived rats after 14 day observation period, will be euthanized by overdose of CO2 and found dead animals also subjected to gross pathology examination, for external and internal observations. The World Health Organization's guidelines for acute toxicity testing will be followed.

4.4.6 Statistical analysis

Statistical analysis will be done by applying –Paired and unpaired T test and one way ANOVA test.

5 OBSERVATION AND RESULTS:

Obdervations and results will be noted and presented in the form of tables, chart, photographs etc.

6 DISCUSSIONS:

Arbuda is characterized by an aggregation of *vatadi dosha* vitiated *mamsa* and *rakta dhatu,* according to Acharya Sushruta. It is round, stiff, and painful, with a wide and deep spread in the base, slow progress, and no chance of becoming *pakaavastha*. *Arbuda* is the name for this sort of *mamsa* gland. Acharya Charak confirmed this. In current science, we can find similar descriptions. As a result, it is apparent that ancient Acharyas were aware of Cancer[1].

As leading cause of cancer prevalence in 2020 female breast cancer may have superseded lung cancer , accounting for 11.7 % of all cancer cases, with just 2.3 million new cancer cases expected. With 685,000 fatalities globally, that is the seventh leading cause of cancer death. One out of every four women has affected by breast cancer and accounts for one out of every six cancer

deaths, ranking first in terms of incidence in the vast majority of nations and fatality in 110. In many nations, breast cancer incidence rates climbed substantially during the 1980s and 1990s.

With 2.2 million new cancer cases and 1.8 million fatalities Lung cancer is expected to be the second most commonly diagnosed cancer and the leading cause of cancer mortality in 2020. It is accountable for one out of every ten diagnoses and one out of every five cancer deaths. Thus Lung cancer is the most prevalent cause of cancer morbidity and death in males, and the 3rd highest common form of cancer prevalence and death in women, after breast and colorectal cancer. Almost two-thirds of lung cancer deaths occur as a result of smoking. Survival rates for lung cancer patients five years from diagnosis are only 10% to 20% in most countries for those diagnosed between 2010 and 2014.

In 2020 More than 1.9 million new colorectal cancer cases are projected, included anus cancer cases with 935,000 deaths, accounting for about one out of every ten cancer cases and deaths. In the United States, it is the third most frequent cancer, but also the second most deadly. Transitioned nations have a 4-fold greater incidence rate than transitioning countries, although there is less fluctuation in death rates due to the higher fatality rate in transitioning countries.

Prostate cancer will become the second most frequent disease in males and the fifth leading cause of cancer death with an estimated 1.4 million new cases as well as 375,000 deaths globally in 2020. In transitioned countries, the incidence rate is three times higher than in transitioning countries. Prostate cancer has a surprisingly unknown aetiology for a disease as frequent as it is. Older age, a family medical history of such a cancer, and even certain genetic abnormalities and diseases (Lynch syndrome) are the only known risk factors. [6]

In Ayurveda, *Bhasma* is a metallic-mineral mixture that has been homogenized with herbal juices or decoctions and then heated (*Puta*) to purportedly eliminate heavy metals. Rasa-Shastra is a branch of Ayurveda that deals with herbal, mineral, and metal preparations (*Bhasmas). Bhasma* is a very well herbo-mineral/metals/non-metals preparation with *Rasayana* (immunomodulatory and anti-aging capabilities) and *yogavahi* (capacity to deliver drugs to specific tissues) properties, it's also non - toxic, gentle to absorb, adaptive, and digestible in the body.The *Bhasma* of Rasashastra are indeed the tune of modern nanoparticle technology[16-18].

Roudra rasa is an Ayurvedic herbomineral concoction used to cure *Arbuda*. *Roudra rasa* is made up of two primary steps: *kajjali* preparation utilizing *Parada* and *Gandhaka*, and *Roudra rasa* preparation. As described in the literature, these important constituents are thus made to give the *bhavana* of different herbal juices as well as decoctions, and step two additionally include subjecting *bhavana* given *Kajjali* to a specific degree of heat is called as *puta*. *Roudra rasa* is said to be prepared with *Laghu puta*. The *Roudra rasa* formulation has been recommended for the treatment of *arbuda*. *Parada* in Ayurveda explained as it removes the *Jara* (Geriatric diseases), *Ruk* (Disease), and *Mrutyu* (Death). And is the centre of the Rasashastra. *Parada* itself or its preparations can cure the dreadful diseases[19]. Also Roudra Rasa contains ingredients *Nagavalli* [20], *Meghanad* [21], *Punarnava* [22], *Pippali* [23] and *Gomutra* [24] has analysed for its anti carcinogenic activity, it is likely that in combination it may enhance its affectivity likely to be increased to show its anti carcinogenic activity..

Arbuda is a term that can be used in a variety of ways. A bulge, a tumour as a lengthy spherical mass, a mass of tissue with a rough surface, consistent bloodletting, and so on are all summarized in this way. The terminology is a collection of terms. The terms used as *Arbuda* is a term that can be explained as a reflection of uncontrollable cell growth. According to Acharya Sushruta, exacerbated *doshas* create *mamsa* vitiation, it causes a spherical, static, mildly painful muscle swelling that is large in size, broad in base, expands slowly, and does not ripen. *Arbuda* is the name given to it [25].

7 CONCLUSIONS:-

In this study Cancer cell line study to analyse Anti-carcinogenic activity and Acute toxicity study of *Roudra Rasa* has planned. If this experiment yields significant results, it will be a valuable addition to ongoing clinical research in anti-carcinogenic studies and provide evidence that Roudra Rasa is safe to take at Ayurvedic therapeutic levels.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

ETHICAL APPROVAL

All the experiments will be conducted on the animals in accordance with the standards set for the use of the laboratory animal use and the experimental protocols have duly approved by the IAEC (Institutional Animal Ethical Committee) of D.M.I. M.S. (D.U.), Sawangi, Wardha
(IAEC Ref. No: DMIMS/CPCSEA-IAEC PA/20-21/18., 05-10-21).

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