Study Protocol and pre-protocol

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

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~~ treatment facilities were severely inadequate.*Roudra Rasa* is a special Ayurvedic preparation for *Arbuda*(Cancer) mentioned in BhaishajyaRatnavali. 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 MCF-7 (Breast), HT-29 (Colon), HOP-62, A549 (Lung), and DU145, PC-3 (Prostate) cancer cell lines have chosen to examine *Roudra Rasa'*santi 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 parameter will be assessed. Anti-carcinogenic activity will be carried out in selected cancer cell lines. AndAcute toxicity testing will be done in accordance with OEC Directive 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)

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. (2) 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. (3)

According to Ayurveda, vitiation of *Vata* and *KaphaDosha* 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 *Kaphadosha*, any *Arbuda*can form in any of the *dushya*, i.e. *Rakta* (blood), *Mamsa* (muscle), and *Medadhatu* (fat). By influencing the *Jatharagni* and *Dhatwagni*, the vitiated *Vata* and *Kaphadosha*s produce *Ama* (autotoxin). By inhibiting the *Srotasa, Ama* together with *apakwadhatu*, creates diverse *dhatugatvikara* in the form of *Arbuda, Shopha, Granthi*, and so on.(4)

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.

Roudra Rasa is one of the *Bhasma* preparation mentioned in BhaishajyaRatnavali for *Arbuda* treatment. *Bhasma*refers to a group of organomineral compounds that have improved stability, bioavailability, bio - compatibility, drug targeting, and therapeutic activity. 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. (5)

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 dramatically. (6)

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 nanoparticle 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 pharmaceutical, analytical and experimental study. The research will take place in M.G.A.C.H.R.C., Salod (H), Central Animal House, DMIMS (DU), Wardha, and other reputable national laboratories as identified or recommended by DMIMS (DU). Institutional Ethics Committee (IEC) and Institutional Animal Ethics Committee (IAC) ethical approval has been obtained (IAEC).

The current project will be carried out under the following headings:

4.1 Pharmaceutical study

In this study *Roudra Rasa* will be prepared in three batches as per classical reference to establish pharmaceutical standardization. This pharmaceutical study will be done according following steps.

1. Selection of raw material
2. Procurement of raw drug – all raw drugs will be collected / procured from the field and authentic reliable sources.
3. Authentication of raw material – all raw materials will be verified and authenticated by department of Dravyaguna of MGACH & RC.
4. All the row materials will be authenticated by FRLHT, Banglore.

4.1.1 Preparation of RoudraRasa : (7)

"शुद्धसूतंसमंगन्धंमर्द्यंयामचतुष्टयम्। नागावल्लीदलयुतंमेघनादपुनर्नवा॥ 59||

गोमुत्रपिप्पलीयुक्तंमर्द्यंरुद्ध्वापुटेल्लघु लिहेत्ख्शोउद्रैरसोरोउद्रोगुञ्जमात्रोअर्बुदंजयेत्। 60|| "

… (B. R.Pg.no.-829/59-60)

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	1 part
2	*Gandhak*(Sulphur)	Sulphur powder	1 part
3	*Nagavalli (Piper bitle)*	Leaf	Q.S.
4	*Meghanad(Amaranthusspinosus Linn)*	Whole plant	Q.S.
5	*Punarnava(Boerhaaviadiffusa)*	Whole plant	Q.S.
6	*Gomutra* (Cow's urine)	Flower	Q.S.
7	*Pippali (Piper longum)*	Fruit	Q.S.

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

Firstly *Paradashodhan* and *Gandhakashodhana* will be done

ShudhaParad + ***ShudhaGandhaka***

Ref.- R.T.-5/29-30 Ref.- R.R.S. – 3/24

↓

With equal quantity *ShuddhaParada* and *shuddhaGandhaka*

Kajjali will be prepared.

Comment [C1]: ?what do you mean?

Comment [C2]: Is this necessary? Only Mercury. For all table items.

Comment [C3]: ?

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

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First ***NagavallipatraSwarasa****Bhavana* will be given for 4 hrs

↓

Then ***MaghanadKwath****Bhavana* will be given for 4 hrs

↓

Then ***PunarnavaKwath****Bhavana*for 4 hrs

↓

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

↓

Lastly***PippaliKwath****Bhavana* for 4 hrs will be given

↓

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

↓

sharavsamputa will be sealed by keeping *Chakrikas* in that and dried

↓

Then will give *Laghuputa*

↓

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

4.2Analytical Study:

Analysis of prepared formulations will be carried out at authentic laboratories. (As per API) Organoleptic Characters of Roudra Rasa will be evaluated. Physiochemical parameters such as pH value, loss on drying, ash value, acid insoluble ash, specific gravity, AAS (Atomic absorption spectrophotometry) (for Mercury as Hg w/w%, Arsenic as As w/w%, Sulphur as S w/w%), XRD (x-ray defraction), ICP-AES (Inductively coupled plasma atomic emission spectroscopy), FTIR (Fourier transform infrared spectroscopy) will be done. **Particle size** estimation of *Roudra Rasa* will be done by SEM-EDX (Scanning electron microscop- Energy dispersive spectroscopy)

4.3In-Vitro Study - Anticancer Study:

4.3.1Cancer 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.

4.3.2Procedure for cell treatment:

The monolayer cells will be separated using trypsin-EDTA to create a single cell suspension, and also amount of viable cells will be counted with a hemocytometer prior to getting diluted with media supplemented with 5% FBS to obtain the goal density of 1105 cells/ml.

At 37°C, 5% CO2, 95% air, and 100% humidity, 100 micro litres of inoculum per well will be implant into 96-wellplates at a plate accumulation of 10,000 cells per well and grown to allow for cell adhesion.

After 24 hours, the cells would be treated with different doses of the test chemicals. They'll dissolve first in plain dimethylsulfoxide (DMSO), and then a portion of the test solution would be diluted in serum-free media to twice the final desired maximum test concentration.

A total of five sample concentrations will be obtained by performing four successive dilutions. The requisite final sample concentrations can be achieved by adding aliquots of 100 µl of all of these various sample diluted sample to a appropriate wells initially holding 100 µl of medium. After the sample is being added, the plates will be incubated for another 24 hrs at 37°C, 5% CO2, 95 % air, and 100 percent humidity. As a control, a medium with no samples will be utilised, and all concentrations will be done in triplicate.

4.3.3MTT assay –

Tetrazolium salt assay or Microculturetetrazolium test are additional names for it.

The MTT test is a widely used screening procedure3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT), a yellow tetrazolium salt that is water soluble. The tetrazolium ring is broken down by succinate-dehydrogenase, a mitochondrial enzyme found in live cells, turning MTT to an insoluble purple formazan. As a result, the number of viable cells determines the amount of formazan generated.

The MTT test will be used to determine the cytotoxicity of substances on cancer cells (Mosmann et al.,1983). In 24-well plates, cells (1×10^5 / well) will be plated in 5ml of medium per well (Costar Corning, Rochester, NY). The cell reaches confluence after 48 hours of incubation. The cells will then cultured at 37°C for 24 to 48 hours in the presence samples. After rinsing with phosphate-buffered saline (pH 7.4) and discarding the sample solution, each well will receive 1ml of 0.5 percent MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells) phosphate-buffered saline solution (5 mg/ml).Following a 4-hour incubation period, 0.04M HCl/isopropanol was applied.

The absorbance at 570nm will be used to assess which cells were viable. The concentration need for this 50% inhibition of viability (IC50) will be visually determined based on the data acquired. A UV Spectrophotometer will be used to measure the absorbance at 570 nm, using wells no sample carrying cells serve as blanks. The samples' efficacy against the rapid proliferation of chosen cancer cells will then be determined. (8)

4.3.4Sample preparation

Roudra Rasa will be diluted to 100 g/ml, 200 g/ml, 400 g/ml, and 800 g/ml after being dissolved in 100 mg/ml dimethyl sulfoxide. It will then evaluate in selected different cell lines at four different concentrations: 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 guideline 423 for chemical testing.

4.4.1Procurement 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 ays

4.4.3Limit 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. Ifmortality would not beobserved 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.

4.4.6Statistical 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.

6DISCUSSIONS:

Arbuda is characterized by an aggregation of *vatadidosha* vitiated *mamsa* and *raktadhatu,* 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.

Comment [C4]: This description is not related to this part of the article.

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. (9) *Roudra rasa* is an Ayurvedicherbomineral 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 *Laghuputa*. 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.(10) Also Roudra Rasa contains ingredients *Nagavalli* (11), *Meghanad*(12), *Punarnava*(13), *Pippali*(14) and *Gomutra*(15) 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. (10)

7CONCLUSIONS:-

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.

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 dulyapproved 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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