Study Protocol

Evaluation of Repeated Dose (Sub Chronic) Toxicity Study of Shilasindur Prepared by Two Different Methods Along with Its Characterization and Antimicrobial Activity

Abstract

Introduction: Rasaushadhies (metal and mineral) preparations are unique preparations that include Bhasma, herbo-mineral preparations and Kupipakwa rasayana hold a significant place in Ayurvedic pharmaceutics. Nearly 70 % of formulations include a combination of one or more metallic/mineral with several herbs which have a supporting role in improving efficacy, relieving symptoms of the disease and to achieve long and healthy life. Shilasindur is a one of the Kupipakwa rasayana containing mercury, sulphur and arsenic disulphide and indicated in skin disorders, respiratory tract disorders and other infectious diseases.

Aim and Objectives: Evaluation of repeated dose (Sub chronic) toxicity study of Shilasindur along with its characterization and antimicrobial activity.

Material & Methods: Shilasindur will be prepared in three batches as per classical reference and by using Electric Muffle furnace. The prepared formulation will be assessed with Organoleptic characters, physicochemical parameters and ICP – AES (Inductively coupled plasma atomic emission spectroscopy), FTIR (Fourier-transform infrared spectroscopy), XRD (X-Ray Diffraction). Sub chronic toxicity study will be carried out according to the OECD guidelines 408 and 452 as well as CCRAS guidelines of safety and toxicity. Epsilometer test (E-test) will be used to check antimicrobial activity of Shilasindur.

Results: The analytical parameters will be assessed to establish the pharmaceutical standardization. Toxicity study will be done in three doses to ensure its safety. Statistical analysis will be done by applying unpaired t-test and ANOVA test.

Conclusion: If significant positive results obtained in this work then it will be a valuable contribution and evidence that the drug Shilasindur is safe for consumption at treatment doses.

Key words: Kupipakwa , standardization Sub chronic toxicity study, antimicrobial study.

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Introduction

Herbo-mineral formulations hold a significant place in Ayurvedic pharmaceutics. Nearly 70 % of formulations include a combination of one or more metallic/mineral with several herbs which have a supporting role in improving efficacy, relieving symptoms of the disease and to achieve long and healthy life (1-2). Rasaushadhies (metal and mineral) preparations are unique preparations that include Bhasma, herbo-mineral preparations and Kupipakwa rasayana.

Heavy metal toxicity as a major threat and become a risk factor to the community. Mercury and its compounds, which are one of the most toxic metals itself widely attracted the attention of modern present scenario (3) Ancient texts quoted by Acharya Nagarjuna, Mercury and its compounds have tremendous potency. One of its miraculous, highly significant and unique preparations is Kupipakwa rasayana. It has very specific and highly extensive rapid pharmaceutical action with very smaller dose and faster palatability (4). Due to these properties it is one of the superior preparation which is prepared in Kupi (Glass bottle) with parad (Mercury), Manashila (Arsenic di-sulphide), Somal (Arsenic oxide),Swarn (Gold), Rajat (Silver) etc. Apart from that these drugs are highly potent metal and Mineral which is included in the list of poisonous drug by Drug and Cosmetic act 1940 under schedule E because of its highly toxic nature in crude form (5). Though its toxicity varies with reducing its toxic effect and convert it into medicinal form after processing and preparing medicine or various Kalpana. That is the magical knowledge, which is quoted by Rasashastra and by Rasasiddha seers.

Shilasindur is a Kupipakwa rasayana (6) and it is sagandha (processed with Sulphur), Sagni (processed with heat i.e. temperature in increasing order),Kanthastha (found in neck of the glass bottle) and Murchhita Paradjarit (Processed with mercury). Its contents Parad (Hg), Gandhak (S) and Manashila (Arsenic di-sulphide- AS_2S_3) as ingredients. It is indicated in all type of skin disorders, respiratory tract disorders and other infectious diseases (7-8)

Kupipakwa formulations are widely used by Ayurveda practitioners. Shilasindur, which is available in market, is frequently used in the treatment. However, there is a lack of scientific evidence related to acute toxicity, sub-acute toxicity, chronic toxicity of Shilasindur. Moreover, the efficiency and utility of Shilasindur is needed to understand in the terms of its cost effectiveness and superiority as a standard formulation (9). A few researches conducted on the Shilasindur but still there is a lack of its sub chronic toxicity studies. Hence, this study is

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undertaken to evaluate sub chronic toxicity profile of Shilasindur along with its characterization and antimicrobial activity.

Standardization is a measure for ensuring the quality, which is used to describe all measures, which are taken during the manufacturing process and quality control leading to reproducible quality. It is also need to create scientific evidence for Herbo-mineral preparations for ensuring uniformity and consistency at larger scale production. In view of high demand of Shilasindur there is an urgent need to bring about standardization of its preparation process and the end product by using various physicochemical, microbiological and analytical methods (10-11)

Present study will be revealed Pharmaceutical preparation of Shilasindoor prepared with two different methods, its analytical standardization and evaluation of sub chronic toxicity profile of Shilasindur along with its antimicrobial activity on gram positive and gram negative organisms.

Aims and objective:

1. Evaluation of repeated dose (Sub chronic) toxicity study of Shilasindur along with its characterization and antimicrobial activity.

Objectives:

1. To establish SOP of Shodhana (Purification) of all ingredients of Shilasindur i.e. Parad, Manshila and Gandhak according to the references.
2. To establish SMP of Shilasindur by two different methods i.e. Traditional (by Valuka yantra) and Modern (Electric Muffle furnace)
3. To study quality control parameters of Shilasindur.
4. To evaluate repeated dose (Sub chronic) toxicity study of Shilasindur in wistar strain albino rats.
5. To assess antimicrobial potential of Shilasindur.

Materials and Methods:

Present work will be conducted under following headings

A. Pharmaceutical study

In this study, Shilasindur will be prepared in three batches as per classical reference and by using Electric Muffle furnace to establish the pharmaceutical standardization. This pharmaceutical study will be done according following steps.

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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. Raw drugs will be standardized as per API.

Preparation of Shilasindur:

Preparation of Shilasindur includes the following steps

Table 1: Shodhana(Purification) of Ingredients of Shilasindur:

S.N.	Ingredients	Shodhana Drug	Process	Reference
1.	Parad (Hg)	Lime stone powder, Rason (Garlic)	Mardan (Trituration)	Rastarangini (12)
2.	Gandhaka (S)	Cow's Milk, Cow's ghee	Swedan (Steaming)	Ayurveda Prakasha (13)
3.	Manashila (Realgar)	Ginger Juice	Mardan (Trituration)	Rastarangini (14)

i. Preparation of Shilasindur [Rasyog sagar] (15)

The study material Shilasindoor will be prepared according to the Rasayogsagar

The Shilasindur shall be prepared using following steps:

All procured and authenticated drugs will be cleaned properly

⇩

All ingredients will be subjected to Shodhana (Purification) process

⇩

Shodhana (Purification) of Parad will be performed with lime stone and Garlic through trituration process as per the reference of Rastarangini text.

⇩

Shodhana of Gandhak will be performed with Godugdha (cows milk) through Swedan procedure as per the references of Ayurved prakash text.

⇩

Shodhana of Manashila will be performed by triturating with Ardrak Swaras (Juice of *Ziziber officinale*) for seven times as per the reference of Rastarangini text.

⇩

Kajjali will be prepared by triturating equal quality by weight of Shuddha parad (purified mercury) and Shudhha Gandhak (Purified Sulphur).

⇩

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Trituration of Shuddha Manshila with this Kajjali

⇩

Kanchakupi (Glass bottle) will be filled by with Kajjali and will be placed in ValukaYantra (Iron vessel filled with sand)

⇩

Setting of entire apparatus on fire wood on Koshti/ EMF

⇩

Heat will be given in gradual increasing order viz; Mruduagni (Mild heat:150^0C – 250^0C) Madhyam agni (Moderate heat: 350^0C – 600^0C) and Tivragani (Intensive heat above 600^0C) for 24 hours

⇩

After performing with copper coin test bottle will be sealed.

⇩

Kanchkupi will be allowed to be self-cooled

⇩

Breaking of Kanchkupi using kerosin and thread. Final product Shilasindur will be collected from the neck of Kupi(glass bottle)

⇩

This obtained Shilasindur will be powdered in Khalvayantra and stored in air tight glass bottles.

⇩

This procedure of preparation of Shilasindur will be repeated 3 times (3 batches) to establish the pharmaceutical standardization.

ii. Preparation of Shilasindur by Electric muffle furnace:

The Shilasindur shall be prepared using Electric Muffle Furnace (EMF) instead of Valuka Yantra following the same process.

B. Analytical study (16)

a) Descriptive –Subjective parameters of finished product – (Organoleptic Characters)

The prepared Shilasindur will be evaluated by these organoleptic characters

1. Shabda (Sound)
2. Sparsh(Sensation)
3. Rupa (Color)
4. Rasa (Taste)
5. Gandha (odour)

1. Objective parameters of finished product (Physiochemical parameters)
    a. Specific gravity

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b. Loss on drying at 110c
c. Acid insoluble ash
d. Ph
e. HPTLC
f. Total Ash Value
g. AAS (Atomic Absorption Spectrophotometry) for
    i. Mercury as Hg w/w%
    ii. Arsenic as As w/w%
    iii. Sulphur as S w/w%
h. XRD (x-ray diffraction)
i. ICP – AES (Inductively coupled plasma atomic emission spectroscopy)
j. SEM - EDX (Scanning electron microscope-Energy dispersive x-ray spectroscopy)
k. FTIR (Fourier transform infrared spectroscopy)

C. Experimental study: (17-19)

Animal study will be conducted according to OECD guidelines.

i. Repeated dose oral toxicity (sub Chronic) study:

This study will be carried out according to the OECD guidelines. TG 408 and 452 as well as CCRAS guidelines of safety and toxicity

Experimental design

a. **Selection of animals** – Male and female healthy albino rats will be selected from animal house, DMIMS (DU). Total 40 wistar strain albino rats of either sex weighing between 140-200 gm will be taken randomly for study.
b. **Treatment of experimental rats** – the rats will be acclimatized for 14 days' prior starting the study. Animals will be kept regular maintained room temperature and will be fed with palate diet supplied from animal house and drinking water ad libidum.
c. **Experimental design** – Total 40 wistar albino rats of either sex will be randomly divided into 5 experimental groups with 8 rats each as shown in table 2.

Group1 – Control group (4M,4F)

Group2 – Vehicle control (4M,4F)

Group 3 - Experimental group (4M,4F)

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Group 1 – Control group will be given no medicine for 90 days

Group2 – Vehicle control will be orally administered with honey for 90 days

Group 3 - Experimental group will be administered orally with compound containing Shilasindur with Therapeutic equivalent dose as 1 TED, 5TED and 10 TED with honey for 90 days.

d. **Dose calculation**- The dose for experimental study will be calculated by exploring standard dose calculation procedure from recommended clinical human dose.

e. **Conversion of doses** – The dose of Shilasindur clinically and therapeutic is 250 mg / day. this is converted to animal dose bared on body surface area of wistar albino rats orally for 90 days (FDA) (20-21)

Standard Formula=Human clinical dose X conversion factor (6.2)

Human dose=250mg / 60 kg = 4.16 mg/kg

Conversion of dose = 4.16 X 6.2=25.79 mg /kg of rat

Dose for rat = 5.16mg / 200 gm rat

Table 2: Grouping of animals (Sub chronic toxicity study)

Group	Drug	Dose	No. of Animals	Duration	Route
1.Group I Control group	No Medicine	-	8 (4 M + 4 F)	90 days	-
2.Group II Vehicle control	Honey	1 mL	8(4 M + 4 F)	90 days	Oral
3.Group III Experimental group					
a. Study group-1TED	Shilasindur +Honey	5.16 mg(SS) + 1ml Honey	8 (4 M + 4 F)	90 days	Oral
b. Study group-5TED	Shilasindur +Honey	25.80 mg(SS)+ 1ml Honey	8 (4 M + 4 F)	90 days	Oral
c. Study group-10TED	Shilasindur +Honey	51.60 mg(SS) + 1ml Honey	8 (4 M + 4 F)	90 days	Oral

Recovery phase: Recovery phase will be 30 days (after completion of repeated dose 90 days oral toxicity study). In order to investigate the recovery phase from toxic changes, 50%

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of animals in each group will be allowed to live for, at least for 30 days after cessation period of administration of the test substance.

The experimental methodology

The experimental group 1 TED, 5TED and 10 TED Wistar rats will be administered the study drug containing suitable vehicle i.e. honey with mentioned dose. The animals will be deprived of food 4 hrs prior of dosing and 2 hrs after dosing. Drinking water will be allowed ad libidum. All the animals will be observed for 90 days after dosing. Individual rat will be observed carefully at least first 30 min after dose administration. Keen attention will be given during first 4 hrs. Animals will be observed at least 3-4 times a day on day 1 of administration of study drug. Then after for 90 days drug will be administered and animals shall be observed. Same way dosage will be given to the group 2 and observed.

Every signs of illness, behavioral changes and reactions to the experimental group will be observed and recorded for every single albino rats. The body weight will also be recorded before and after administration of drug on day 7, day 14, day 28 and 90 days or mortality. Hematological (CBC) and Biochemical values (LFT, KFT, CRP etc.) will be calculated before and after the treatment.

Histopathological study

Animals will be sacrificed by administrating mild anesthesia. Liver, kidney, heart, brain, intestine, lungs, stomach will be processed for histopathological studies as per prescribed procedure. Organization of Economic Cooperation and Development guidelines will be followed for the sub-acute toxicity tests. OECD TG 408, 452 and CCRS guidelines.

Statistical analysis

The results of analytical tests for samples of Shilasindur prepared by two different methods will be presented statistically by unpaired t-test. The mean and standard deviation of the treated groups will be done by applying unpaired t-test and ANOVA analysis.

D. Antimicrobial study

Different types of methods are used to assess the antimicrobial activities In the present study Epsilometer test (E-test) means Antibiotic strip test method will be used to check the specificity of drug Shilasindur as it has an effective antimicrobial activity

Antimicrobial agent – Sample of Shilasindur will be prepared as fine powder.

Epsilometer test (E-test): (22-24)

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1. The E-test is a method for measuring MICs of antimicrobial agents against bacteria and is based on diffusion of a preformed antibiotic gradient from a plastic strip. The performance of E-test is evaluated by comparison with a conventional agar dilution MIC method. The E-test is technically straight forward as test are set-up in the same way as the disc diffusion method. The versatility of E-test and easy to use make E-test method an attractive alternative to conventional dilution tests.

2. The antimicrobial gradient method combines the principle of dilution method with that of diffusion method in order to determine the MIC value. It is based on the possibility of creating concentration gradient of the antimicrobial agent tested in the agar medium. The E-test is the commercial version of this technique. In the procedure a strip impregnated with an increasing concentration gradient of antimicrobial agent from one end to other is deposited on agar surface, previously in occulated with the microorganism tested. This method is used for the MIC determination of antibiotics, antifungals and antibacterial. MIC value is determining at the inter section of the strip and the growth inhibition ellipse. It is simple to implement thus it is routinely used.

This technique can also be performed to investigate the antimicrobial interactions between two drugs. To study the combine effect of two antibiotics and E-test strip, impregnated with first antibiotic is placed on the pre in-occulated agar plate surface. After one hour the strip is removed and replaced by another on impregnated with a second antibiotics. The synergy is detected by a decreased of MIC of the combination by at-least two dilutions compared to that of the most active antibiotic tested alone. Also for the same purpose E-test strips can be deposited on the agar medium in cross formation with 90degree angle at the intersection between the scales at the respective MICs for the micro-organism tested.

3. The E-test is the method of determining the antibiotics susceptibility of an organism used in conjunction with the agar plate lawn method. The strips themselves are thin strips impregnated with a predefined gradient of antibiotics. Using an antibiotic strip to test the sensitivity of an organism it provides the concentration of antibiotic required to inhibit the bacteria being tested. The lowest concentration of antibiotics needed to inhibit an organism is known as the minimum inhibitory concentration (MIC) of the organism.

Detailed methodology -

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- Agar plates should be prepared in the same way as described in the disc diffusion method.
- Ensure the antibiotics strips allowed to settled down at room temperature.
- Once the bacteria have been in-occulated onto the agar plate to form a lawn the antibiotic strip should be carefully removed from the packet using sterile forceps. Ensuring that it holds the strip only at the top edge of the strip away from where antibiotic is impregnated into the strip. Once the strip has been removed from its packaging it can be placed in the center of the plate if only one antibiotic is being tested in pairs 9 cm agar plate. Up to six strips can be tested by placing them radiating out in a star shape from the center of the large 15 cm agar plate. The highest concentration should be placed towards the edge of the plate. If multiple antibiotic strips are being tested. The strips should be placed so that the scale is facing up so it can be read. As with the disc ensure that the whole strip is in contact with the agar. Sometime an air bubble can become trapped under the strip if any large bubbles are observed. Try to move the bubble up and out from under the strip by gently pushing the bubble up from the low concentration end of the strip to the high concentration end. Small bubble should not affect the zone of inhibition generated so they can be left under the strip.
- The plate is then incubated in an inverted position at 35 degree centigrade for 18 to 24 hours. But this can be changed depending on which organism you are using and which manufactures products are being used. After the incubation period remove the plate from the incubator and check that there is a confluent lawn of growth on areas of the plate where the bacteria have grown. If the growth looks wrong (too heavier or too light) disregard the plate and repeat the experiment. If bacteria are susceptible to the any of the antibiotic strips used a zone of clearing will be seen on the agar. To read the MIC look for the point at which the zone of inhibition intersects the antibiotic strips. The MIC is the concentration of the antibiotic written on the strip at the point. If the bacterial growth intersects the strip between two values. The higher value will give the more conservative MIC, which means the bacteria is less likely to be accidently recorded as susceptible when resistance. If results for between two concentration other options are re run the susceptibility test or confirm the MIC by another method.

Microorganism selection:

1. Gram positive bacteria – *Staphylococcus aureus, Streptococcus mutans*

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2. Gram negative bacteria – *Escherichia coli, klebsiella pneumonia, Pseudomonas aeruginosa*.
3. Fungal group – *Candida albicans*

Table 3: Activity of microbial agent Shilasindur

Sr. no.	Microorganism	Activity by E-test method	Zone of inhibition Shilasindur in mm	Dose 100mg	Control group with Gentamycin or Cephalosporin zone of inhibition in cm
1	*Staphalococcus aureus*				
2	Streptococcus *mutants*				
3	*Escherichia coli*				
4	*Klebsiella pneumoniae*				
5	*Candida albicans*				
6	*pseudomonas aeruginosa*				

Study design

1. Pharmaceutical, Analytical, Experimental. (Sub chronic toxicity study and Antimicrobial activity)
2. Study period – 24 months
3. Study centers – present work will be conducted at following study centers
    a. Mahatma Gandhi Ayurvedic college, hospital and research Institute Salod (H), Wardha
    b. Animal house of DMIMS, Wardha, Sawangi
    c. Histopathological lab of JNMC, Sawangi, Wardha
    d. Other institutes recommended by DMIMS, Wardha as per need of study.

Statistical analysis: After antimicrobial activity

1. Results will be expressed in mean value and standard error of mean
2. Statistical analysis will be assessed by unpaired t-test and ANOVA test

Observations and results-

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Stepwise observations will be recorded and presented in the form of tables, charts and pictures etc.

Discussion:

In *Ayurveda* herbs and minerals are the major source of drugs for the preparation of various forms of medicines. According to the requirement, these drugs are flourished by undergoing varied modulations. This aids the physician for minimum dose, palatability, easy administration, increased shelf life and bioavailability (25-26). Shilasindur is one such herbomineral preparation prepared by *Kupipakwa* method in Ayurveda indicated in skin disorders, respiratory tract disorders and other infectious diseases. Difficulty finds in preparing Shilasindur by traditional method to provide and maintaining gradual heat in mild to intense form. Electric Muffle Furnace (EMF) is better option and can be replaced with traditional method in preparation of Shilasindur to save fuels, efforts and pollution as well[27].

Reference of safety profile of Shilasindur is found for acute and subacute toxicity while searching through search engines but repeated dose toxicity study of Shilasindur for longer duration is not conducted yet to prove its efficacy with high dose and longer duration. In view of the above fact, present study is planned. Different types of methods are used to assess the antimicrobial activities In the present study Epsilometer test (E-test) means Antibiotic strip test method will be used to check the specificity of drug Shilasindur as it has an effective antimicrobial activity. E –test can be used for testing anti-microbial interactions between two drugs. It is a cost effective method [28]. Studies on efficacy of various Ayurvedic formulations were reported. Few of the related studies were reviewed (29-30).

Conclusion:

Present study is planned for repeated dose toxicity study (sub chronic) of Shilasindur along with its antimicrobial activity. This a genuine attempt to fulfill the gap of few scientific evidences available and if significant positive results obtained in this work then it will be a valuable contribution and evidence that the drug Shilasindur is safe for consumption at treatment doses prescribed in classics for a longer duration.

Ethical clearance: This will be taken from institutional ethical committee of Datta Meghe Institute of Medical Sciences, Sawangi, (DMIMS) Wardha and from Animal ethics committee. Study will be followed as per instructions of IAEC of DMIMS.

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Scope of study:

1. Pre-clinical studies of Shilasindur will help to serve as a supportive evidence for clinical trials of Shilasindur.

2. Data of antimicrobial study will be helpful to plan modified dosage forms such as ointment, cream etc.

3. Quantitative elemental analysis will create standard for assessing quality of this product as well as helpful in judging pharmacokinetic and pharmacodynamic actions.

NOTE:

The study highlights the efficacy of “Ayurvedic" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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