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Pharmaceutical Standardization and antimicrobial activity of *Shila Sindoora*

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Abstract

Shila sindoora (SS) is a mercury-based ayurvedic formulation. It is indicated in all types of skin disorders, respiratory disorders, abscess and gonorrhea. Present study aims to standardize its standard manufacturing process (SMP) and analytical tests for *SS batches.* Antimicrobial activity of SS will also be evaluated using microbial strains *Staphylococcus aureus* (*s. aureus*), *Pseudomonas aeruginosa (p. aeruginosa)*. The pharmaceutical procedure of SS was performed as per the ayurvedic pharmaceutical methods. The analytical tests were assessed as per the Ayurvedic pharmacopeia of India (API). The antimicrobial study was conducted using well diffusion method. The average yield obtained is 45.60% and it got deposited in the inner surface of the neck of the bottle (*Galastha Kupipakwa Rasayana*). X-ray diffraction (XRD) study showed presence of HgS and As_2S_2 in the product. Inductively coupled plasma mass spectrometry (ICP-MS) study showed 21.80% of Mercury, 6.23% of Sulphur and 14.34% of Arsenic in the compound. Average size of particles found to be 767.1 nm and distribution of particles are in between 652.9 nm to 941.2 nm. The antimicrobial study results that SS batches is more efficient against *P. aeruginosa* in comparison to *S. aureus*. The present study concludes that standardization of SS along with its antimicrobial effects are established.

Keyword

Shila sindoora, Kupipakwa rasayana, Standardization, *S. aureus, P. aeruginosa*, antimicrobial activity, X-ray diffraction (XRD), Inductively coupled plasma mass spectrometry (ICP-MS).

1. Introduction

The quality check on the manufacturing of ayurvedic formulations is very important. To establish ayurvedic medicines on global level, needs to develop the standard manufacturing procedure (SMP) profile on scientific ground. The uniqueness of ayurvedic formulations is that they are indulged with herbal, mineral and metal-based ingredients which are processed in such a way that they become more compatible, high in therapeutic value and minimal in side effects.

The mercurial formulations which are prepared in glass bottle through specified heating patterns i.e., *kramagni* [*mridu* (mild), *madhyam* (moderate) and *teevra* (higher) i.e., increasing manner of heat] are known as *kupipakwa rasayana* [1]. *Rasa sindoora* [2], *makaradhwaja* [3], *sameerapannaga rasa*[4], *swarana vanga*[5] and *shila sindoora*[6] (SS) are few therapeutically potent and widely used mercurial

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formulation prepared using this method. *SS*, an ayurvedic mercurial based formulation which is indicated for the treatment of several skin disorders, respiratory disorders, abscess and gonorrhea[7].

In present study, SMP of the SS will be evaluated. For this, organoleptic as well as physico-chemical parameters will be determined to detect the chemical and biological configurations. Antimicrobial study using microbial strains *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Till date, no data is available for SMP and antimicrobial profile of SS.

2. Materials and method

2.1 Procurement of raw materials

Ashuddha hingula (unpurified cinnabar), *Ashuddha Gandhaka* (unpurified sulphur), *Ashuddha Manahshila* (unpurified arsenic di sulfide) were procured from Jaipur, Rajasthan, India located herbal raw drugs seller. *Goghrita* (cow butter), *Godugdha* (cow milk), Fresh *ghritakumari* (Aloe *barbadensis miller*) and *nimbu* [*Citrus limon (L.) Burn.* f.] were purchased from local market of Jodhpur, Rajasthan, India.

2.2 Materials

Khalva yantra (mortar and pestle: length:12inch, width:7.9inch, depth:3.6inch, thickness:1inch), *nada yantra* (earthen pot: upper circumference: 88 cm, middle circumference:130 cm, depth: 29 cm)**,** *Kupi* (seven-layer mud smeared glass bottle, capacity:750 ml), Electrical muffle furnace (EMF)-inner hearth (length: 10.2 cm, breadth: 10.2 cm, depth: 25.3 cm, maximum temperature capacity: 850 ^{0}C) were used as per requirement.

2.3 Preparation of *Shila sindoora* (SS)

Apart from the main procedure, the following sub-procedures also has been carried out in the department of Rasa Shastra and Bhaishajya Kalpana, Post Graduate Institute of Ayurveda, Rajasthan Ayurved University, Jodhpur, India. All the procedures were performed according to the ayurvedic pharmaceutical methods. The *Shodhana* of *hingula* was conducted through the wet grinding process using lemon juice for seven times[8]. The mercury was extracted from the purified cinnabar using the *Nada Yantra* (i.e., *hingulottha parada*)[9]. Sulfur underwent purification through the galana/*dhalana* method (melting and pouring) in *Godugdha*[10]. Additionally, the *Shodhana* process for *Manahshila* involved wet grinding in lemon juice for seven times[11].

The preparation of *SS* was divided into three stages, namely, *Purva Karma* (Pre heating phase), *Pradhan Karma* (Heating phase) and *Pashchat Karma* (Post heating phase) stages.

2.3.1 *Purva Karma* (Pre heating phase)

An equal quantity of *Hingulottha Parada*] (Mercury obtained from Cinnabar) and *Shuddha Gandhaka* (processed Sulphur) were triturated for 32 hours till the mixture (*kajjali*, black sulfide of mercury) become soft, lusterless, black and fine[12]. *Shuddha Manahshila* (processed arsenic di sulfide) *was* added to the *Kajjali*, triturated again. *Ghritkumari Swarasa* (Aloe juice) *was* used for levigating media in *kajjali* and triturated for 8 hours till it gets dry[7]. The dried powder was filled in *kupi*.

Table 1: Ingredients of *Shila Sindoora*

Ingredient	Batch I	Batch II	Batch III
Hingulottha Parada (g)	100	100	100
Shuddha Gandhaka (g)	100	100	100
Shuddha Manahshila (g)	100	100	100
Ghritkumari Swarasa (ml)	100	100	100

2.3.2 *Pradhan Karma* (Heating phase)

Preparation of *SS* was carried out in EMF by providing controlled intermittent and gradually increasing heating pattern that is, *Mridu Agni* for 4 hours (<250 ^{0}C), *Madhyama Agni* for 13 hours (250-550 ^{0}C) and *Teevragni* for 8 hours (550-700^{0}C)[13]. In between the *Madhyama agni*, the hot iron rod was inserted to clean the neck of the bottle, to avoid the blockage due to the deposition of sublimated *Gandhaka* (*shalaka sanchalana*). After observation of the confirmative tests like, complete cessation

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of *Sulphur* and *Arsenic* fumes; copper coin test (as placing a copper coin over the *Kupi's* mouth resulted in a silver-white mercurial coating, indicating the evaporation of mercury particles) and *sheet shalaka* test (After inserting iron rod into the *Kupi*, a thin layer along with shiny light black coating was formed on it, suggesting compound formation) were positive[14], the mouth of the *Kupi* was corked and the temperature was increased to around 40 ^{0}C and was maintained for 4 hours to facilitate complete formation of the compound.

Table 2: Temperature Pattern and Observations during preparation of SS

Time (hrs.)	Temperature (^{0}C)			Observation
	Batch I	Batch II	Batch III	
0	29	45	48	Furnace switched on
01	72	96	98	No changes were seen
02	124	137	144	Mild smell of SO_2
03	193	202	201	Slight Sulphur aroma was smelt at the *kupi* mouth white fumes started
04	266	264	257	Dense yellow fumes of SO_2were observed
05	294	297	298	Yellowish fumes of Sulphur gradually became dense.
06	327	330	324	Semi-solid state of *kajjali* was felt at the base of bottle
07	372	376	373	Mild yellow flames appeared on the *kupi* mouth
08	398	401	396	*Kajjali* was found in molten state as observed. Bubbling sound was heard during *kajjali* melting.
09	437	433	432	Mild SO_2 fumes were still evolving. orange flames appeared on the neck of *kupi* when red hot iron rod inserted in the *kupi*
10	483	488	481	Light blue flames appeared on the neck of *kupi* when red hot iron rod inserted in the *kupi*
12	502	500	498	Maroon red appearance seen in the bottom of the *kupi*
13	508	503	504	White fumes and blue flames were still coming
14	534	532	534	Light blue flames appeared on the neck of *kupi*
16	586	584	581	Blue-Orange flames appeared on the neck of *kupi* when red hot iron rod inserted in the *kupi*
17	588	590	586	Bright orange flames were produced when red hot iron rod inserted
18	618	618	611	Copper coin test was performed after every 5 mins. and was found with the deposition of black soot over its surface
19	648	647	643	White Yellow fumes coming out when red hot iron rod inserted
20	659	654	657	No flames were produced when hot iron rod was inserted in the mouth of *kupi*. Copper coin test was found negative
21	663	667	662	The examination of bottle's interior with the help of torch revealed maroon red color in the bottom of the *kupi*. Positive copper coin test. Corking of the *kupi* was done with brick cork and mud smeared cloth
22	685	686	681	Intense heat given for 4 hours.
25	720	713	702	EMF Switched off

2.3.3 *Pashchat Karma* (Post heating phase)

After Self-cooling, *Kupi* was taken out from the EMF. The layers of mud smeared cloth were scrapped. A thread soaked in kerosene was tied around the *Kupi*, one inch below from the level of the sublimed product and then ignited. On complete burning, a wet cloth was wrapped around the burning thread. A cracking sound was produced and bottle broke into two parts[15]. Final product was collected from the neck of *Kupi*. It was weighed and packed.

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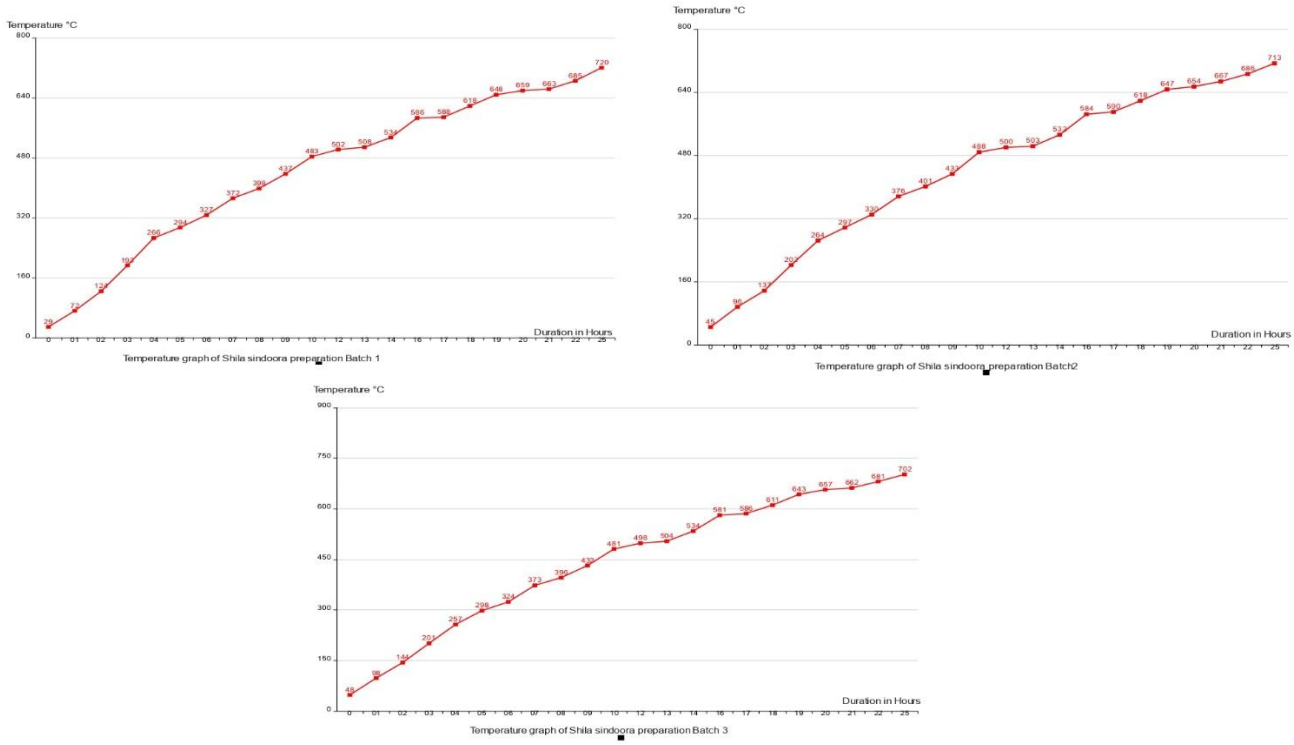

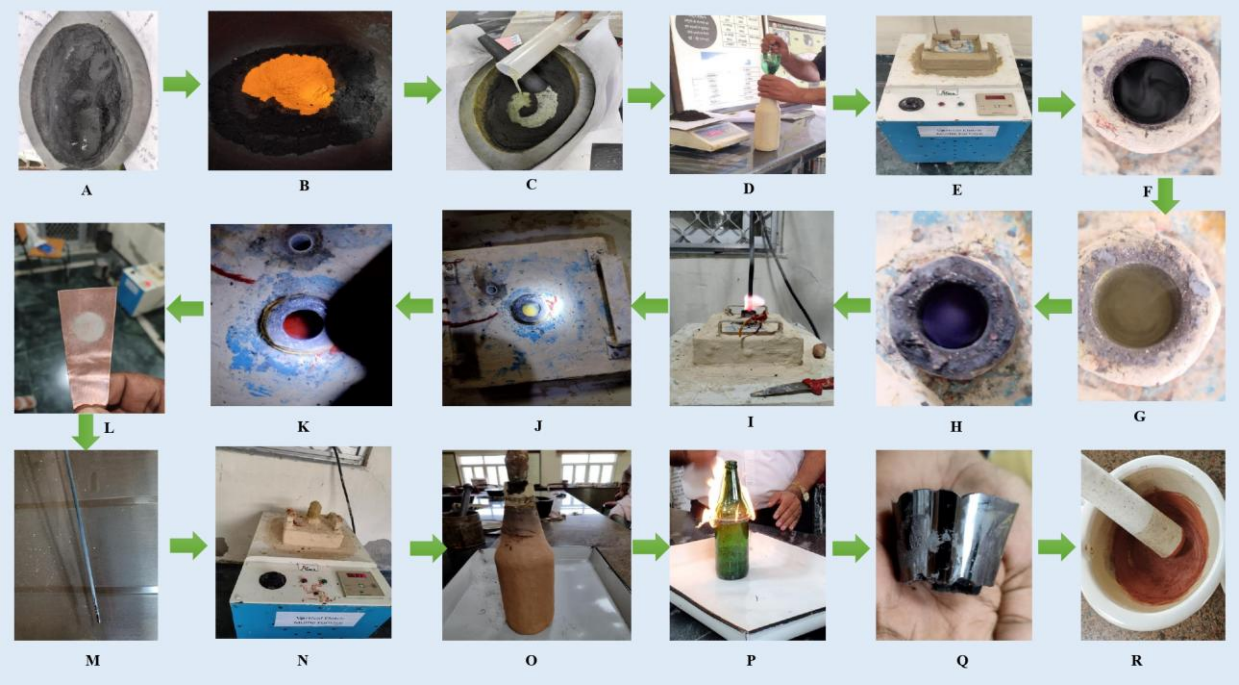

Figure1: The pharmaceutical procedure of SS. A) *Kajjali*; B) *Kajjali* mix with *Manahshila*; C) Adding *Ghrita Kumari Swarasa;* D) Filling of *Kajjali* in the *Kupi;* E) Placement of filled *Kupi* in EMF; F) White fumes; G) Yellow fumes; H) Blue flames; I) Flaming stage of *Kupi*; J) Yellow appearance of bottom; K) Maroon red appearance of bottom; L) Copper coin test positive; M) *Sheet Shalaka* test positive; N) Corking of the *kupi;* O) Removal of *kupi* from EMF; P) Breaking of *kupi; Q) Shila sindoora; R)Shila sindoora* after trituration

2.4 Analytical tests

SS *sample* was subjected to various organoleptic and physicochemical analysis for confirmation of physical, chemical and biological configuration of the compound. They include texture,

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color, taste, odor, pH, Loss on drying, Total ash, Acid insoluble ash, Alcohol-soluble extractive value, Water-soluble Extractive value[16], determination of crystallinity of a compound [by X-ray diffraction (XRD)], Particle size analysis and percentages of Mercury, Sulphur and Arsenic [by Inductively coupled plasma mass spectrometry (ICP-MS)][17].

2.5 Antimicrobial study

The effectivity of the SS formulation was determined through antimicrobial study using microbes *S. aureus* and *P. aeruginosa*. The Muller Hinton agar well diffusion was selected as study procedure. The testing was conducted at Drug Testing Laboratory Cultivator Phyto Lab, Jodhpur, An AYUSH approved laboratory. Luria Bertani Agar (LBA) bacterial medium was prepared as per SOP of Media Preparation (CPL/SOP/B/01). 60 ml of LBA was inoculated with 1ml of bacterial culture *S. aureus* and *P. aeruginosa* from dilution of cell suspension having 10^8 cfu/ml. The LBA medium with the culture inoculum was poured into petri plate & allowed to solidify at room temperature. Solidified LBA agar plate was used to determine antibacterial property of different sample through well diffusion assay. Equidistant wells of 6mm diameter were cut on each plate. Mark the wells A, B &.C for positive control, negative control, and sample under test respectively. Pipette transfer 100µl of antibiotic, water & sample to the wells respectively. Antibiotic Cefixime was used as positive control and DMSO was used as negative control. Antibiotic cefixime of concentration 1:2 (10mg/ml) used for Staphylococcus aureus & Pseudomonas aeruginosa. The well diffusion assay depends on diffusion of the sample in the well through a solidified agar layer in a petri plate. Plates for antibacterial assay was incubated for 24 hours at 37±1°C. After incubation, the agar plate was analyzed for the formation of zone of inhibition (ZOI) around the well to determine the antibacterial property of the sample under test.

3. Results

3.1 Pharmaceutical study

Table 3: Comparative results of SS preparation all batches

Batch	Initial weight of the material (g)	Time (hrs.)	Max Temp. (^{0}C)	Status of *Kupi*	Weight of product (g)	%Yield
I	288	25	736	Blast	98.80	34.30
II	288	25	713	Blast	152.90	53.09
III	288	25	703	Intact	142.30	49.40

g: gram; %: percentage; hrs: hours

3.2 Analytical study

Table 4: Organoleptic and Physico-chemical tests

Organoleptic tests								
Color	**Color (After trituration)**		**Taste**		**Odor**		**Texture**	
Maroon Red	Brick Red		Tasteless		Odorless		Compact	
Physico-Chemical Tests								
pH (10% Aq. Solution)	**Loss on Drying** (%w/w)	**Total Ash** (%w/w)	**Acid-insoluble Ash** (%w/w)	**Alcohol-soluble Extractive** (%w/w)	**Water-soluble Extractive** (%w/w)	**Mercury** (%)	**Sulphur** (%)	**Arsenic** (%)
6.84	0.03	0.09	BLQ	0.25	0.45	21.802	6.23	14.345

BLQ: Below limit of Quantification; **LOQ**: Limit of Quantification (LOQ:0.1)

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Table 5: XRD of SS

Sample Name	Obs. Max	d (Obs. Max)	Net Height	Net Area	Intensity
	2-Theta °	Angstrom	Cps	Cps x 2-Theta °	%
HgS	26.6814	3.34114	439.58	0.2362	100.00
As_2S_2	28.3645	3.14659	104.85	0.2362	23.85
As_2S_2	31.3696	2.85167	420.18	0.2066	95.59

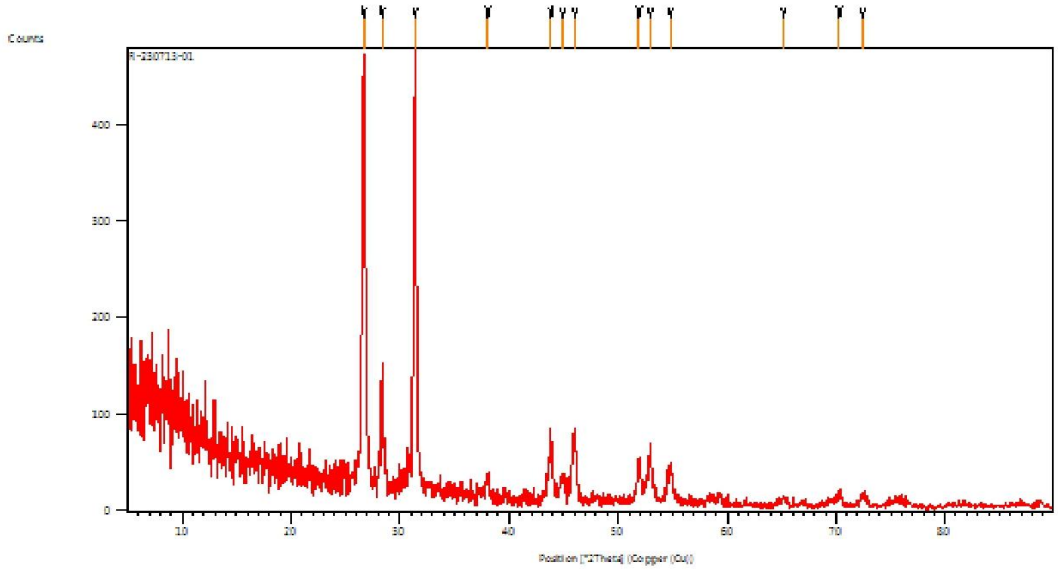

Figure 2: XRD of SS

Table 6: Particle Size Analysis of SS

Sample name	Average Particle Size (nm)	Poly Dispersity index %
SS I	707.2	9.1
SS II	652.9	16.5
SS III	941.2	26.3
Average	767.1	

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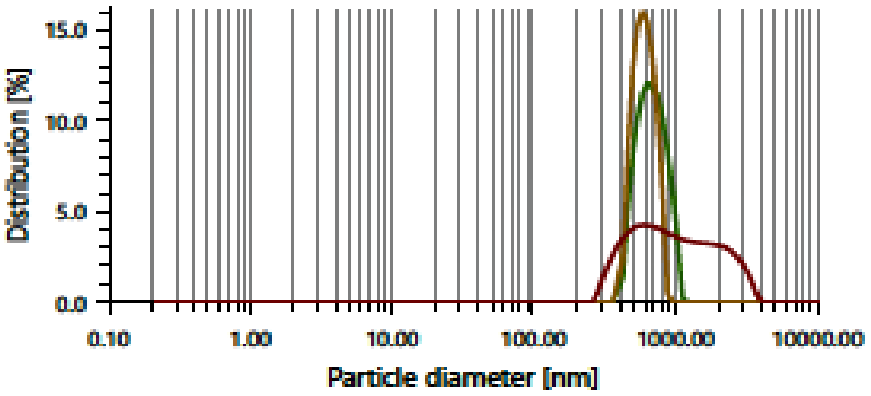

Figure 3: Particle size distribution (intensity)

Table 7: Elemental analysis (by ICP-MS) of SS

Test Parameter	Unit	Results	Test Method
Mercury (Hg)	%	21.802	CPL/STP/C/67 (ICP-MS)
Arsenic (As)	%	14.345	CPL/STP/C/67 (ICP-MS)
Sulphur (S)	%	6.23	CPL/STP/C/67 (ICP-MS)

3.3 Antimicrobial activity

Table 8: Antimicrobial activity of SS

Cultures used	ZOI at inoculum density of 10^8 cfu/ml (mm)			ZOI at inoculum density of 10^7 cfu/ml (mm)			ZOI at inoculum density of 10^6 cfu/ml (mm)		
	Positive control	Dilution 1:2	Dilution 1:10	Positive control	Dilution 1:2	Dilution 1:10	Positive control	Dilution 1:2	Dilution 1:10
P. aeruginosa (NCIM-2200)	28	22	16	30	24	18	33	27	21
S. aureus (NCIM-5345)	32	15	14	34	17	16	37	20	19

NCIM: National collection of industrial microorganisms; ZOI: Zone of inhibition; mm: Milimetre; cfu; Colony forming unit; 1:10: 10% concentration of SS; 1:2: 50% concentration of SS

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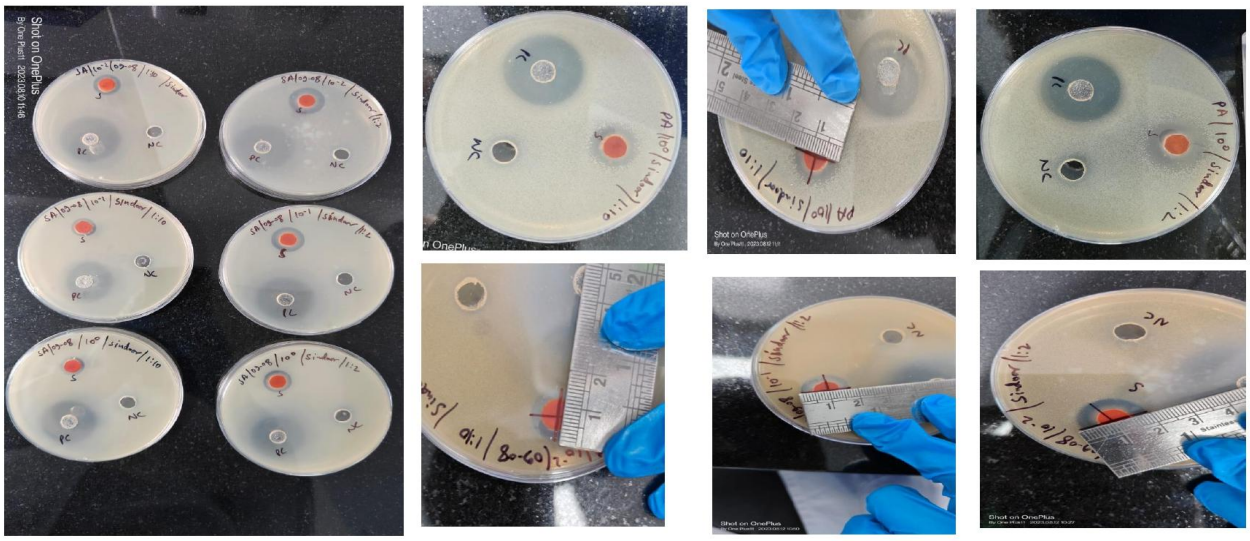

ZOI of SS against *S. aureus* **ZOI of SS against *P. aeruginosa***

Figure 4: ZOI of SS

Discussion

This present research work aims to develop the standard manufacturing process (SMP) and antimicrobial study. The time duration taken for the preparation of SS is 48 hours. The slight modification in time duration that is, 25 hours was referred from previous studies. The trituration of mercury and Sulphur for 32 hours leads the formation of black sulfide of mercury- α HgS. This state of mercury is far less toxicity and more stable form. Mercuric sulfides pharmacological action is not yet clear. This compound is insoluble in vivo but further, addition of purified arsenic di sulfide, leads to the formation of arsenic bonded mercuric sulfide compound[18]. During this pre pharmaceutical process, around 2.9% loss was found. The possible rationalities were the mixture spillage during trituration; adherence of minute particles of mercuric sulfide to the mortar and pestle surface which is difficult to collect and some amount was used while performing the confirmatory tests. This homogenous mixture was wet-grinded with *aloe juice* for 8 hrs. Total 100 ml *aloe juice* was required for wet grinding and 2.38% of weight gain was observed due to the incorporation of solid components from the liquid media.

Prior to 400°C, yellow and white fumes were observed which indicates Sulphur and arsenic vapors in form of oxygen compounds (SO_2, As_2O_2). When the temperature reached to around 410-430°C, flames started to appear at the mouth of the *Kupi*, likely to indicate the ignition temperature of Sulphur. This caused Sulphur to catch fire and produces flame. Around 660-670°C temperature, the bottom of the *Kupi* turned maroon red in color. If a black coating was observed on the copper coin, it meant that the compound still contained Sulphur, which needed to be burned off before corking. When Sulphur is completely burnt and copper coin test becomes positive then proper corking of *Kupi* should be done. Corking is very crucial to maximize the final product yield.

After corking, the temperature was gradually raised till 700°C in duration of 4 hours and maintained for an additional hour. This stage allowed the compound to evaporate and sublimate on the neck of the *Kupi (bottle)*. Increasing the temperature after corking is necessary because it facilitates the evaporation process and ensures the highest possible yield. The batch 1st and 2nd *Kupi* were blasted, due to excessive accumulation of Arsenic, Sulphur fumes and high temperature (around 720^{0}C temp.) inside the *Kupi*.

No sufficient changes were found in SS sample. pH of 6.84 leads to indicate the slight alkaline nature of the formulation. The high temperature heating leads to the compactness or sublimation of the compound. Thus, loss on drying, and total ash data were in limit. In XRD findings, Sharp peaks observed that major compounds as Mercuric sulfide (β-HgS) of majorly at 100% intensity on 26.6814,

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2-theta value with amorphous shape and structure. In the particle size analysis, Means Average size of particles found 767.1 nm and distribution of particles are in between 652.9 to 941.2 nm. The findings of ICP-MS showed 21.80% of Mercury, 6.23% of Sulphur and 14.34% of Arsenic in the sample compound.

The antimicrobial study findings reports that *S. aureus* inhibition zone at 10% of concentration was 14mm, 16mm, and 19mm and at 50% concentration was 15mm, 17mm, and 20mm. *P. aeruginosa* inhibition zone at 10% of concentration was 16mm, 18mm, and 21mm and at 50% concentration was 22mm, 24mm, and 27mm. Thus, *P. aeruginosa* had shown significant effect compared to *S. aureus.* Therefore, this herbo-mineral formulation might prove as potent antimicrobial agent.

This research work establishes the pharmaceutical and antimicrobial activity of SS which could be utilized for large scale manufacturing.

Conclusion and future perspective

In this present study, SMP and antimicrobial validation of SS was established its potent effectivity.

Due to the lack of funding, comparative testing of all three batches were not carried out. Therefore, comparative analytical tests of all the batches should be performed. Some tests such as SEM (Scanning electron microscope), FTIR (Fourier transform infrared spectroscopy) etc. should be done. It is very important to perform the toxicity studies of ayurvedic mercurial formulations. Thus, the toxicity studies of SS should be carried out to ensure the safety profile of the drug. To further determine the potential of SS, Clinical study should be carried out.

Data availability statement

The data, reports, and supplementary material presented in this article is original in nature and for further queries can be directed to the corresponding author.

Ethical statement

This research work protocol was reviewed and approved by Institutional Ethical Committee (IEC), Postgraduate Institute of Ayurved, Rajasthan Ayurveda University, Jodhpur, India. (IEC Approval number: DSRRAU/UPGIAS&R/IEC/ 20-21/434)

Authors contributions

SK, GS, MG, RA, KS, DY and RS had designed the whole research protocol. SK performed the pharmaceutical study. SK and DY observed and documented the data obtained from analytical and antimicrobial testing. SK and DY wrote the manuscript. SK, KS and MG contributed the critical review of the manuscript. All the authors gave their significant contribution to the present research work and approved the submitted version of the manuscript.

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