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Development, pharmaceutical analysis and invitro evaluation of modified herbal fumigation formulation

Research Article

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Abstract

Ayurveda is science of the life. Maintenance of health of healthy individual and Treatment of diseased are the main principles of *ayurveda* management. *Acharya Sushruta*, The Father of Surgery, explained different types of surgeries and documented procedures in sophisticated manner. He also explained different types of instruments, dressing techniques etc. He clearly emphasised the importance of asepsis in his words *Rakshakarma* and different drugs he enlisted for it. *Rakshakarma* includes various karma (procedures) and *dhoopan* is an important karma among them. In modern era, sterilization and disinfection are the main weapons to deal with the different microbes present in the environment. The incidence of nosocomial infections increasing day by day, thus keeping environment and surfaces microbe free is imperative. Along with operation theatre complex, fumigation of in-patient and out-patient wards is also necessary. In *Ayurveda, Vranitaagar Dhoopan* i.e. herbal fumigation of ward is explained by *Acharyas*. To evaluate the efficacy of *vranitaagar dhoopan*, many researchers are taking efforts and few studies are already completed with promising results. Formaline is most widely used chemical for OT complex fumigation but due to its carcinogenic, irritable properties, it is not preferred for routine fumigation of wards. Many safer chemicals are emerging, still cost effectiveness of these new chemicals is a great hurdle. Hence, an attempt is made to modify herbal fumigation formulation for *Dhoopan* to make ward fumigation effective and safe. This study reveals that 30:70 hydroalcoholic extract of *Dhoopan* medicines has very remarkable antimicrobial effect with minimal dose of 15ml within 3min 48 sec in closed chamber of size 1.5x1.5x1.5 ft^3.

Keywords: *Dhoopan,* Herbal fumigation, Formaldehyde, *Rakshakarma,* Ward sterilisation, Hospital acquired infections.

Introduction

Eco surrounding is ladened by millions of microorganisms. When these microbes invade into host body mechanism led to variety of infections. Such microorganisms may prove dangerous when open wounds are exposed to them and thus these microbes get entry in the host body (1). Hospital acquired infections (HAI) are most common in any healthcare setup, Surgical site infections (SSI) are more dangerous and most common among hospital acquired infections. These infections include catheter associated urinary infections (CAUTI), central line associated bloodstream infections (CLABSI), surgical site infections (SSI), ventilator associated pneumonia (VAP), and Clostridium difficile infections (CDI).(2)

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Risk factors for HAI include immuno-suppression, older age, length of stay in the hospital, multiple underlying comorbidities, frequent visits to healthcare facilities, mechanical ventilatory support, recent invasive procedures, indwelling devices, and stay in an intensive care unit (ICU)(3). In GERMANY, a point prevalence study conducted in 2015-16 included 2.31,459 patients across 947 hospitals concluded that about 19.5% of patients in ICU had at least one HAI.(4) As per another study conducted during March 2014 to August 2014in Pune, Maharashtra, overall prevalence of HAI was 3.76% in a tertiary care unit while in surgical intensive care units it was maximum i.e., 25%.(5) In a study carried out in surgical wards of Guru Gobindsinh Hospital, Jamnagar - surgical site infections were most prevalent with 4.1%(6). According to National Healthcare Safety Network, the common causative organisms for SSI include (in descending order) *staphylococcus aureus*, coagulase-negative *staphylococcus, Enterococcus, E. coli, Pseudomonas aeruginosa, Enterobacter, Klebsiella pneumoniae*.(7)

In *Hindu* mythology '*Raksha*' and '*Bhuta*' are very vital concepts which can be correlated with

prevention from known or unknown causes leading to infections. *Dhoopkalpa* which are composed of several drugs having *krumighna, jantughna* properties producing fumes with aroma. These fumes are helpful to purify air and such aroma makes environment pleasant. *Acharya Sushruta* known as the father of surgery explained variety of surgical and para-surgical procedures and different types of instruments for performing such surgeries. *Acharya* explained in details about *rakshakarma. Dhoopan* is an integral part of *rakshakarm. Dhoopan* is explained as preventive as well as therapeutic measures in *Ayurveda* (10). *Acharya* explained *Dhoopan* for *vranitagar, kumaragar, sutikagar. Dhoopan* is one of the traditional methods mentioned by *Acharyas* for disinfection and purification with the help of drugs of herbal, herbo-mineral and animal origin. In modern science Formaldehyde is mostly used for OT fumigation. As it is carcinogenic (8) and irritable many safer chemicals are emerging but economical value is great hurdle. During covid 19 pandemic, Ministry of AYUSH advised fumigation with medicated herbs as preventive measure (9). There is absolute need to develop organic fumigation formulation which is safe for mankind with potent antibacterial properties. It is proven that many herbal medicines are having potent antibacterial properties and many studies are still ongoing. The method of *Dhoopan* explained in ayurveda classics is tried to be standardized by few scholars (15). The purification from *Dhoopan* considered as *Rakshoghna* method i.e., protection and prevention from unknown microorganisms. There are so many herbs explained in Ayurvedic texts for *Dhoopan* i.e., herbal fumigation.

In the context of immediate post-operative care of the surgical site, Acharya Sushruta explained a set of herbs like *Guggulu, Agaru, Raal, Gaurasarshap*, leaves of the *Nimba* tree, *Vacha, saindhav lavan* along with cow *ghee* to be used for fumigation of the surgical site (10). Previous research works, such as 'Role of *Vranadhoopan* in the Management of Chronic Non-Healing Ulcers Under the Influence of Tab *Gandhak Rasayan*' (11) and 'An Antibacterial Effect of Herbal Fumigation *Kwath* on *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Escherichia coli*,' (16) have shown that there is a good antimicrobial potential in a few of these herbs like *Guggulu, Gaurasarshap*, leaves of the *Nimba* tree, and *Vacha*. Hence, these four herbs are selected for ward fumigation, i.e., *Aagar Dhoopan*, in this study, and instead of using the classical-conventional *Dhoopan* method, an attempt is made to prepare a modified herbal fumigation formulation.

There is asking need for ward sterilization considering increasing hospital aquired infections (HAI) incidences and involvement of resistant microorganisms. In wards mainly 1% sodium hypochlorite solution and phenyl used for clearing of floor but no active fumigation method is used. Fumigation is better tool for sterilization as fumes can reach at any extinct area without bar. Formalin is the most commonly used agent for fumigation of the OT, ICU environment but too frequent use and inhalation of formalin is dangerous to health as it has toxic nature and formalin fumes are very pungent, irritating and cause burning of eyes. Hence formalin is not used in general wards for routine fumigation (20). To overcome this problem with formalin many new safe chemicals are emerging, but the economical limitations are great hurdle. *Ayurveda* has explained *Aagar Dhoopan* i.e., fumigation of wards and lot of work has been done to determine its antimicrobial effects. Still the conventional method of herbal fumigation also encounters certain issues like partial combustion, environmental pollution etc.

In fumigation the required herbo-chemical constituents are mainly available in volatile form, which directly can be extracted with hydro-alcoholic extraction method. So, this study was planned to provide effective, safe, easy to handle, preventive as well as therapeutic, soothing herbal fumigation with medicines like *Guggulu, Gaurasarshap, Vacha, Nimba*(11)

The primary objective of the study was to evaluate effect of modified herbal fumigation formulation (MHFF) for *Aagar dhoopan* (ward fumigation).

The secondary objectives were,

1. To prepare modified herbal fumigation formulation with hydro-alcoholic extract method.
2. To study physico-chemical properties of newly designed herbal fumigation formulation.
3. To assess the antimicrobial action of modified herbal fumigation formulation method.

Materials and Methods

Conceptual study: There are many references given in *Sushrut Samhita* regarding *dhoopan*. One among them is quoted in context of *dhoopan* of *vrana* and the environment around the *vranita* i.e., patient. In *Sutrasthan, Agropaharaniya adhyaya, Acharya* mentioned a yoga for *vrana dhoopan* (9). In the commentary *Acharya Dalhan* quotes, not only the wounds and ulcers but also the beds etc. should be fumigated so that foul smell in the wound/ulcer would be extracted out and flies, mosquitoes would go away.

To enhance the medicinal properties, potency and penetration, hydroalcoholic extraction method was used by using methanol.

Drug review: Previous researchers have proven the antimicrobial and wound healing potential of herbs recommended for *dhoopan karma* such as *Guggulu, Gaurasarshap*, leaves of the *Nimba* tree and *Vacha* (11, 15). Hence these tested herbs were selected for preparation of Modified Herbal Fumigation Formulation in this study and efficacy of this modified form was further evaluated. Selected drugs and their properties explained in table no. 1

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Table 1: Selected drugs for fumigation and their properties

Sr. No.	Name of herb *(Latin name)*	Part used	*Rasa Panchak*					Established antimicrobial activity
			Rasa	*Guna*	*Virya*	*Vipaka*	*Karma*	
1	*Nimba (Azadirachta indica)*	Dried Leaves	*Tikta, Kashaya*	*Laghu, ruksha*	*Shita*	*katu*	*Krimighna*	100% inhibition of *Streptococcus pyogenes (12). S aureus, S. epidermidis, P. aerruginosa*
2	*Gour sarshap (Sinapis alba)*	Seeds	*Katu tikta*	*laghu, snigdha*	*Ushna*	*Katu*	*Rakshoghna, Krimighna*	*P. aeruginosa antibacterial properties against Streptococcus pneumoniae and* antifungal properties *(13)*
3	*Guggulu (Commiphora mukul)*	Resin	*Katu, tikta, kashaya*	*Laghu, ruksha, vishad, sookshma, sara*	*Ushna*	*Katu*	*Krumijayeta*	*Bacillusmegaterium, Enterococcus faecalis, Staphylococcus aureus (10) and fungal strains of Aspergillus niger, A. flavus, Candida albicans, Microsporum Micrococcus luteus, fulvum(13)*
4	Vacha *(Acorus calamus)*	Rhizome	*Katu, tikta*	*Laghu, teekshna*	*Ushna*	*Katu*	*Krimighna, Jantuhar*	*Salmonella typhi, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Staphylococcus aureus(14).*

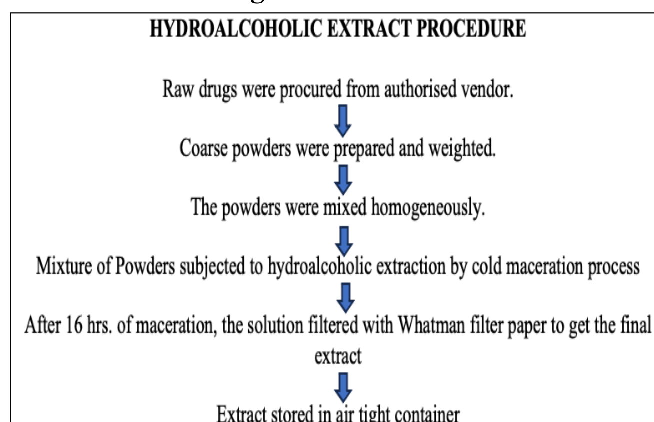
Pharmaceutical and analytical study

All the required herbs procured from standard drug vendor and authenticated from Dravyaguna Department, Parul Institute of Ayurveda, Parul University.

- Raw drugs: *Vacha (Acorus calamus)*. *Nimba* leaves *(Azadirachta indica)*, *Gour-sarshap (Sinapis alba*), *Guggulu (Commiphora mukul)*, in the ratio 1:1:1:1/2.
- Preparation of medicine: Hydroalcoholic extract: (30-70%)
    - Methanol – 7 litres
    - Distilled water – 3 litres
    - *Vacha churna* – 850gm
    - *Nimba leaves churna*- 850gm
    - *Gaura sarshapa*- 850gm
    - *Guggulu*- 450gm

With 30% raw drug concentration with 30:70 proportion hydroalcoholic extract was prepared. (Figure 1)

Figure 1: Stepwise preparation of Modified Herbal Fumigation Formulation



Final yield – 7.5 litre Hydro-methanolic extract

Analytical study

The findings of Organoleptic characteristics, Physico-chemical parameters and qualitative tests are tabulated in table number 2, 3 and 4, respectively.

Table 2: Organoleptic characteristics

Parameter	Polyherbal hydro-alcoholic extract
Colour	Golden brown
Odour	Aromatic alcoholic
Consistency	Liquid

Table 3: Physico-chemical parameters

Sr. No.	Parameter	Polyherbal Hydroalcoholic Extract
1	Total Ash Value (%w/w)	0.55
2	pH Value	6
3	Specific gravity (cc)	0.8894
4	Refractive index	1.3275
5	Total Solid content (%w/w)	3.17
6	Viscosity (cp)	0.8188
7	Boiling point °C	74

Table 4: Qualitative tests

Sr. No.	Solvent	Result
1	Triterpenoids	Present
2	Essential Oil	Present
3	Tannin	Present
4	Saponin	Present
5	Flavonoid	Present
6	Glycoside	Present

HPTLC fingerprinting report

- Sample: Modified Herbal Fumigation Formulation Sample ID: AD/24/271
- Preparation of test solution: 30:70 hydro-methanolic extract was prepared as elaborated in Figure 1. 20 ml of this solution was taken for HPTLC fingerprinting.
- Preparation of spray reagent [Anisaldehyde-Sulphuric acid reagent]: 0.5 ml Anisaldehyde was mixed with 10ml Glacial acitic acid, followed by 85 ml Methanol and 5ml Sulphuric acid (98%).
- HPTLC of modified formulation was seen at two different wavelengths.

At 254nm total 4 spots were observed and there Rf values were 0.024, 0.778, 0.878, 0.939 (Figure 2)

At 366nm total 9 spots were observed and there Rf values were 0.024, 0.068, 0.117, 0.188,

0.340, 0.601, 0.778, 0.857, 0.942. (Figure 3 and Table 5)

GC-MS Procedure

- Instrument: GC (Perkin-Elmer GCMSs) was equipped with MS detector.
- Sample Preparation: About 100 ml of prepared extract (figure 1) was stirred vigorously using vortex stirrer for 10 s. The clear extract was subjected for GC-MS for analysis.

Figure 2. HPTLC (at 254nm)

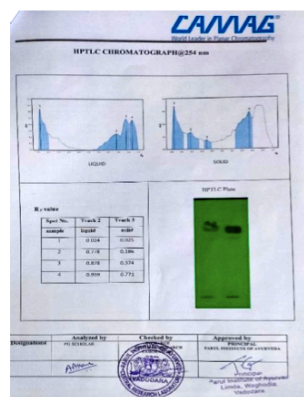


Figure 3. HPTLC (at 366nm)

Table 5: Probable groups of phytochemicals, as per HPTLC of MHFF

SPOT	PHYTOCONSTITUENTS
0.024	SAPONINS
0.068	OLEORESIN
0.117	MANGIFEROLICA
0.188	GLYCOSIDE
0.340	DITERPENOIDS'
0.601	ANTHOCYNIN
0.778	ASARONE
0.857	GUGGULUSTERON
0.878	FIXED OIL
0.939	NIMBOLIDE
0.942	ASARONE

- GC-MS Protocol: Column Elite5 MS (30 mm × 0.25 mm ID ×0.25 µm, composed of 5% phenyl 95% methylpolysiloxane), electron impact mode at 70 eV; Helium (99.999%) was used as carrier gas at a constant flow of 1 ml/min injector temperature 250°C; Maximum temperature: 270°C.
- The oven temperature was programmed from 60°C (Initial temperature- isothermal for 1.0 min), with an increase of 40°C/min, to 170°C C (isothermal for 4.0 min), then 10°C/min to 310°C (isothermal for 10 min) fragments from 45 to 450 Da. Total GC running time was 32.02 min. The compounds were identified by GC-MS Library (NIST and WILEY).

GC-MS report revealed that many active compounds found in the hydroalcoholic extract. Methyl Eugenol, tyrosyl-s-benzylcycteine, oxirane, alpha and beta-asarone etc. which are having antimicrobial and mood elevating properties (Figure 4).

In vitro study

In vitro study was conducted by two different methods with specified purposes-

A. Agar Well diffusion method to determine better effective concentration of solvent for modified fumigation formulation.

B. Inverted petri-plate method inside a closed chamber to determine minimum effective quantity and minimum effective temperature required for conduction of fumigation process.

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Figure 4: Active Phyto-constituents found in MHFF as per GC-MS report

REV	for	Compound Name	M.W.	Formula
938	858	ASARONE	208	C12H16O3
926	813	.BETA.-ASARONE	208	C12H16O3
923	825	ASARONE	208	C12H16O3
914	802	ASARONE	208	C12H16O3
902	782	ASARONE	208	C12H16O3
893	817	.BETA.-ASARONE	208	C12H16O3
892	314	BENZENE, 1-METHOXY-4-(PHENYLETHYNYL)-	208	C15H12O
890	797	BENZENE, 1,2,4-TRIMETHOXY-5-(1-PROPENYL)-	208	C12H16O3
889	735	1,2-DIMETHOXY-4-(2-METHOXY-1-PROPENYL)BENZENE	208	C12H16O3
866	669	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
862	698	BENZENE, 1,2,3-TRIMETHOXY-5-(1-PROPENYL)-, (E)-	208	C12H16O3
858	732	.BETA.-ASARONE	208	C12H16O3
856	752	.GAMMA.-ASARONE	208	C12H16O3
841	649	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
838	711	.BETA.-ASARONE	208	C12H16O3
829	703	ASARONE	208	C12H16O3
789	588	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
777	599	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
777	589	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
769	604	ISOELEMICIN	208	C12H16O3

REV	for	Compound Name	M.W.	Formula
967	926	ASARONE	208	C12H16O3
955	896	ASARONE	208	C12H16O3
953	871	.BETA.-ASARONE	208	C12H16O3
943	927	.BETA.-ASARONE	208	C12H16O3
941	860	ASARONE	208	C12H16O3
938	903	BENZENE, 1,2,4-TRIMETHOXY-5-(1-PROPENYL)-	208	C12H16O3
927	839	ASARONE	208	C12H16O3
908	771	1,2-DIMETHOXY-4-(2-METHOXY-1-PROPENYL)BENZENE	208	C12H16O3
893	335	BENZENE, 1-METHOXY-4-(PHENYLETHYNYL)-	208	C15H12O
886	811	.GAMMA.-ASARONE	208	C12H16O3
876	684	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
872	781	.BETA.-ASARONE	208	C12H16O3
862	773	ASARONE	208	C12H16O3
861	702	BENZENE, 1,2,3-TRIMETHOXY-5-(1-PROPENYL)-, (E)-	208	C12H16O3
846	665	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
841	727	.BETA.-ASARONE	208	C12H16O3
818	636	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
804	645	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
794	624	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	C12H16O3
782	626	ISOELEMICIN	208	C12H16O3

REV	for	Compound Name	M.W.	Formula
929	813	BENZENE, 1,2-DIMETHOXY-4-PROPENYL-, (Z)-	178	C11H14O2
899	807	METHYLEUGENOL	178	C11H14O2
892	827	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	C11H14O2
881	809	METHYLEUGENOL	178	C11H14O2
879	810	METHYLEUGENOL	178	C11H14O2
856	768	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	C11H14O2
846	785	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	C11H14O2
830	755	METHYLEUGENOL	178	C11H14O2
794	701	2-ALLYL-1,4-DIMETHOXYBENZENE	178	C11H14O2
793	743	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	C11H14O2
791	712	METHYLEUGENOL	178	C11H14O2
730	575	BENZOIC ACID, 4-(1-METHYLETHYL)-, METHYL ESTER	178	C11H14O2
728	538	BENZENEPROPANOIC ACID, ETHYL ESTER	178	C11H14O2
727	452	3H-2-BENZOPYRAN-3-IMINE, 1,4-DIHYDRO-	147	C9H9ON
722	621	OXIRANE, [(4-ETHYLPHENOXY)METHYL]-	178	C11H14O2
721	540	BENZENEPROPANOIC ACID, ETHYL ESTER	178	C11H14O2
711	505	BICYCLO[4.3.0]NONAN-2-ONE, 8-ISOPROPYLIDENE-	178	C12H18O
708	398	TYROSYL-S-BENZYLCYSTEINE	374	C19H22O4N2S
703	359	BENZENEMETHANOL, 2-HYDROXY-5-METHOXY-.ALPHA.,.ALPHA.,4-TRIMETHYL-	196	C11H16O3
701	541	BENZENEPROPANOIC ACID, ETHYL ESTER	178	C11H14O2

In vitro study conducted at VASU Pharmaceuticals and research Centre, Vadodara with the objective of defining better effective concentration of solvent for modified fumigation formulation. Two hydroalcoholic (solvent) concentrations of same solute quantity for extraction viz., trial A and trial B with 30:70 and 50:50 hydroalcoholic concentrations respectively and two blank solutions (Blank A1- plain 30:70 and Blank B1- plain 50:50 hydroalcoholic solutions) were selected for the study. The study was performed using Agar Well diffusion method. The results were encouraging and suggestive that sample A showed very good antimicrobial activity against *E. coli, P. aureus, P. aeruginosa* and *C. albicans* with 30-70 concentration (Figure 5).

Table 6: Details of samples, cultures and media used during Agar Well diffusion method

<table>
<tr><th rowspan="2">Sample Used:</th><th colspan="2">Cultures Used</th><th colspan="2">Media Used</th></tr>
<tr><th>Name of Culture</th><th>ATCC number</th><th>Media</th><th>Lot No. (Make)</th></tr>
<tr><td rowspan="4">1.Sample -A (30:70 conc.) 2. Blank A1 (30:70 conc.) 3. Sample-B (50:50 conc.) 4.Blank B1 (50:50 conc.)</td><td>S. aureus</td><td>6538</td><td rowspan="3">Mueller Hinton Agar (MHA)</td><td rowspan="3">0000509408 (Hi-media)</td></tr>
<tr><td>E. coli</td><td>8739</td></tr>
<tr><td>P. aeruginosa</td><td>9027</td></tr>
<tr><td>C. albicans</td><td>10231</td><td>Sabouraud Dextrose Agar with Chloramphenicol (SDA)</td><td>0000508784 (Hi-media)</td></tr>
</table>

Culture Preparation

To generate stocks of microorganisms in test tubes, slants were prepared (as the nutrient agar solidifies when the test tubes are kept in slanted i.e., tilted position) (16). Freshly prepared agar slants were used to generate stocks of microorganisms under study i.e., *S. aureus, E. coli, P. aeruginosa* and *C. albicans*. These slants were washed by using 10 ml of sterile Normal saline solution.

Method: Cylinder Plate Method

Method for

Media Preparation: Mueller Hinton Agar (MHA) was used for determining the activity of *S. aureus, E. coli, P. aeruginosa.* Sabouraud Dextrose Agar (SDA) was used for determining the activity of *Candida albicans*. Media were prepared as per Manufacturer's Instruction (17,18). The media were then autoclaved at 121°C temp. & 15lbs pressure for 15 minutes.

Sample Preparation: Approximately 6 gm (3+3 gm) of herbal powder was weighted and added into two different 20 ml test tubes and labelled them Sample-A & Sample-B. 10 ml of 70:30 (Methyl alcohol: Water) was added into sample -A & 10ml of 50:50 (Methyl alcohol: Water) was added into Sample-B. The samples were sonicated for 10 mins. Samples were kept for 48 hrs, at room temperature. After 48 hrs. the samples were centrifuged & the supernatant liquid was collected. It was used as a test sample for activity. Additional 10 ml of plain 70:30 and 50:50 Methyl alcohol: Water solutions were kept ready in other 2 test tubes, labelled as Blank A1 and Blank B1 respectively.

Testing Procedure for Anti-microbial activity: Sterile media MHA & SDA were cooled down up to 55°C and 10 microlitre of bacterial cultures was added into different MHA flasks, 10 microlitre of yeast culture was added into SDA flask. It was mixed slowly; the plates were labelled and then 25 ml of media was poured by sterile measuring cylinder. The plate was solidified and required wells were made on plates at proper distance by sterile borer. Test and blank samples were poured in respected labelled well. As soon as samples were diffused completely in well, MHA plate was incubated into Bacteriological incubator at 35°C for 24 hours & SDA plate into biological oxygen demand incubator at 25°C for 48 hours. The zone of inhibition was observed.

Table 7: Effect of Modified herbal fumigation formulation in Agar Well diffusion method

Sr. No.	Name of Sample	Zone of Inhibition (mm)			
		S.	*E. coli*	*P.*	*C.*
1	Sample A	22	13	23	15
2	Blank A1	NZI	NZI	NZI	NZI
3	Sample B	14	5	8	6
4	Blank B1	NZI	NZI	NZI	NZI

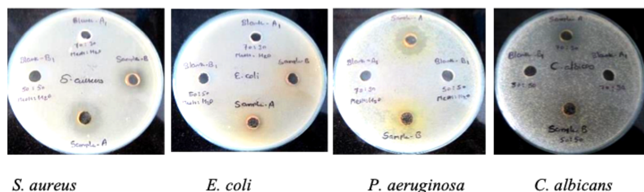
NZI: No (significant) Zone of Inhibition

Results of agar well diffusion method

From the available data, Sample-A showed very good anti-microbial activity on *S. aureus, E coll. P. aeruginosa & C. albicans* in comparison with Sample-B (Table 7).

Hence, it was confirmed that 70:30 (Methyl alcohol: Water) was better effective concentration of solvent for modified fumigation formulation.

Figure 5: Observations: In-vitro anti-microbial activity of Herbal Fumigation Liquid



S. aureus *E. coli* *P. aeruginosa* *C. albicans*

In vitro study of modified herbal fumigation formulation inside a chamber using inverted petri plate method:

After knowing the better effective concentration of solvent for modified fumigation formulation, further in vitro study of *dhoopan* inside a chamber using inverted petri plate method was planned with objective to know the minimum effective quantity of this formulation and minimum effective temperature to be provided to the formulation during fumigation process. This study was conducted at Department of Microbiology, Parul institute of applied sciences, Vadodara, Gujarat.

Preparation of suitable growth media

Plan: To evaluate sensitivity of herbal fumigation formulation, colonies of pure strain of *E. Coli, Pseudomonas, Klebsiella, Staphylococcus aureus, Candida albicans* were developed in petri dishes. Two identical glass chambers of size 1.5ft x 1.5ft x 1.5 ft were prepared. Petri dishes containing bacteria i.e., *S. aureus* and other were placed inverted in glass chamber A and C. Fumigation was done with modified herbal formulation in chamber A and the petri dishes in chamber C were placed without fumigation. After that petri dishes were closed and kept in incubator at 37°C for 48hrs time period. After 48 hours petri dishes were observed to evaluate antibacterial and antifungal activity.

- Group A –Modified herbal fumigation formulation (HAE),
- Group C – CONTROL (without any intervention).

Results mentioned in specially prepared table.

Growth media: Nutrient agar prepared according to SOP of preparation i.e., Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.

Preparation of Nutrient Agar

1. 28 g of nutrient agar powder was suspended in 1 litre of distilled water in a flask labelled as 'Agar flask'.
2. This mixture was heated while stirring to fully dissolve all components.
3. The dissolved mixture was autoclaved at 121°C for 15 minutes to sterilize the media.
4. Once the nutrient agar has been autoclaved, it was allowed to cool but not to solidify.
5. Nutrient agar was poured into each petri dish which were left on the sterile surface until the agar has solidified.
6. Total 10 petri dishes of nutrient agar were prepared, the lid of each Petri dish was replaced, sealed with paraffin strips and stored the plates in a refrigerator.

Preparation of nutrient broths

1. 13gm of broth powder was diluted in 1 litre of distilled water in a flask labelled as 'broth flask'.
2. This mixture was heated while stirring to fully dissolve all components.
3. The dissolved mixture was autoclaved at 121°C for 15 minutes to sterilize the media.
4. The flask was allowed to cool to atmospheric temperature.
5. Then the broth was transferred in test tubes which were previously autoclaved.
6. With a wire loop single colony from plates will be inoculated in the broth in each tube respectively.
7. Broths incubated at 37°C for 24 hrs.

Nutrient broth was prepared with distilled water as per SOP of microbiological studies (19)

1. 'Broth solution " was inoculated with a single colony from 50 µL of a frozen stock of *Staphylococcus aureus* in flask. This flask with broth solution for *S. aureus* was placed into an incubator at 37°C overnight with shaking to allow time to replicate the target microorganism (here *S. aureus*).
2. Now Nutrient broth for *S. aureus* was ready and the flask was labelled as 'Nutrient broth for *S. aureus*'.
3. 0.2 ml of nutrient broth was inoculated on one petri dish of nutrient agar and with the help of sterilised spreader, even spreading was done. The petri dishes were incubated at 37°C for 24 hours. It was observed that, after incubation, the petri dish developed huge number of colonies (almost uncountable), hence to reduce the number of colonies of *S. aureus* systematically, serial dilution of Nutrient broth of *S. aureus* was done.
4. Method of serial dilution (19):

- The flask labeled "Nutrient broth for *S. aureus* " was removed from the incubator and shaken vigorously.
- Ten test tubes capable of storing 20 mL or more were prepared in a rack with label $T1^{SA}$-$T10^{SA}$. Each tube number is consistent with the dilution factor it corresponds to (i.e., T5 = 1×10^{-5} or 0.00001 or 1/1,00,000th of stock concentration).
- 9 mL of sterile water was added with the help of pipet into each of the 10 test tubes.
- Each of these test tubes was sterilised in the autoclave for a minimum of 15 minutes at 121°C, 15 psi.
- Serial dilution was done up to 10^{-5} by adding 1 ml of the nutrient broth to test tube $T1^{SA}$ containing 9 ml of distilled water and taking 1ml of it to be added to next test tube $T2^{SA}$. This method was repeated till tenth test tube ($T10^{SA}$) was added with 1ml from $T9^{SA}$.
- With the help of micro pipette 0.2 ml broth from test tube $T3^{SA}$, $T5^{SA}$ and $T9^{SA}$ were inoculated on

nutrient agar petri dishes (described in step A above) and with the help of sterilised spreader, even spreading was done. The petri dishes were incubated at 37°C for 24 hours. It was observed that, the petri dish incubated from $T3^{SA}$ developed 503 countable colonies, while $T5^{SA}$ petri dish developed 80 countable colonies and $T9^{SA}$ petri dish developed 12 countable colonies. As the colonies developed were very high with dilution $T3^{SA}$ while for $T9^{SA}$, the count was too less, we considered solution from test tube $T5^{SA}$ for further study.

5. Procedure of preparation of nutrient broth for *Escheria coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* was same as described for preparation of nutrient broth for *S. aureus*. All the micro-organisms were taken 50 µL from their respective frozen stocks.
6. Nutrient agar petri dishes were inoculated with 0.2ml nutrient broth of each of *Escheria coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* by the method as described for inoculation of nutrient broth of *S. aureus*. After incubation, it was seen that, all the petri dishes developed countable colonies (within range of 30 to 300 colonies) of each species and hence there was no need of further serial dilution of nutrient broths of these species.
7. Sabouraud Dextrose Agar (SDA) was prepared as per Manufacturer's Instruction (18) and used for preparation of SDA petri dishes and broth of *Candida albicans*. SDA petri dishes were inoculated with 50 µL of *Candida albicans* and after incubation 36 countable colonies were observed, hence no serial dilution was needed.
8. Petri dishes were prepared by inoculating SDA broth of *Candida albicans,* nutrient broths of *Escheria coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and dilution $T5^{SA}$ of nutrient broth of *S. aureus*. Six separate petri dishes were prepared for each micro-organism.

- These six sets of petri dishes were further distributed in two groups namely, Group C-control and Group A-fumigation with modified herbal fumigation formulation.
- Plates in group A were adhered to lid of a specially designed glass chamber of 1.5 x 1.5 x 1.5 ft^3. and the effects of fumigation were tested by inverted petri dish method.
- Plates from group C were placed in another glass chamber of same size, without any intervention.
- Three observations were taken by using 15, 20 & 25ml of modified herbal fumigation formulation.
- After fumigation, plates from both the groups were closed and placed in incubator for 48 hrs.
- For every observation with different quantity of modified herbal fumigation formulation, new set of petri dishes were taken in both Group C and Group A.
- All plates from group C and group A were observed after 48 hrs. for results.
- Results were entered in specially designed observation tables to compare the results of fumigation in terms of number of countable colonies.

Observations

30:70 Hydroalcoholic extract exhibited better antimicrobial potential than 50:50 extract. HPTLC and GC-MS studies revealed abundance of phytoconstituents having antimicrobial properties. 3 min time duration was required to fill chamber with vapours of modified fumigation formulation (figure 6B). Inverted petri dish method is useful but little bit difficult to carry out experiment.

Temperature should be around 80° C to get proper results. With higher temperature, the modified herbal fumigation solution (MHFS), which was in liquid form, evaporated fast. This resulted in high vapour concentration in the chamber leading to washout of microbial flora in petri dishes. Hence fast evaporation of test drug due to higher temperature is not beneficial.

Figure 6 A and B: Chamber set up (Group A) in vitro antimicrobial study- Chamber size 1.5 x 1.5 x 1.5 ft^3. Petri dishes are adhered to inner side of lid, after placement of lid, the petri dishes are in inverted position. The induction stove is kept at the centre of floor of chamber and fumigation formulation is poured in a bowl kept on the induction stove. 6A: Before commencement of fumigation. 6B: 3 minutes after fumigation.

As the temperature was kept constant, the time duration was increased with increasing volume of modified herbal fumigation solution to allow complete evaporation. The time required for complete evaporation of 15ml, 20ml and 25ml of modified herbal fumigation solution was 3 minutes 48 seconds, 5 minutes and 7 minutes 50 seconds respectively.

External heating source setup was not suitable and convenient hence heating source apparatus i.e., induction stove was placed inside the chamber (Figure 6A).

Strict aseptic and safety precautions were followed while doing antimicrobial studies as little contamination can vary the results.

With higher quantity of fumigation solution, more time was required to evaporate the solution and better the antimicrobial effect was observed. This clearly shows that more the contact time of the fumigation solution's vapours with the microorganisms, better the results.

Results

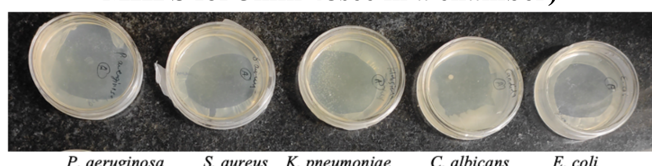
All the quantities *viz.,* 15, 20 and 25ml of MHFF showed remarkable antimicrobial effect on *Staphylococcus aureus, Escheria coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Candida albicans* with 70:30 (Methyl alcohol: Water) concentration (Table 8).

Hence, it's clear that, for the given volume (1.5 x 1.5 x 1.5 ft^3) of chamber, minimum effective quantity of 15 ml at temperature 80° C and minimal exposure time of 3 minutes and 48 seconds, the modified herbal fumigation formulation under study remains effective.

Table 8: (Observations of antimicrobial study in terms of countable colonies)

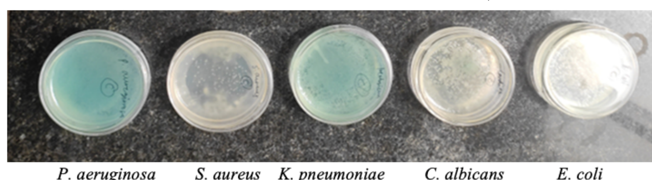
Sr. No.	Microbial specimen	No. of colonies post fumigation					
		Group A (Fumigation)			Group C (No intervention)		
		15 ml	20 ml	**25 ml**	15 ml	20 ml	**25 ml**
1	*Staphylococcus aureus*	25	16	9	138	155	150
2	*Escheria coli*	24	14	7	468	480	>500
3	*Pseudomonas aeruginosa*	42	30	22	65	58	50
4	*Candida albicans*	18	9	5	60	72	75
5	*Klebsiella pneumoniae*	52	32	24	160	172	180

Figure 7: Group A (After fumigation with 15 ml of MHFS for 3min 48sec in a chamber)



P. aeruginosa *S. aureus* *K. pneumoniae* *C. albicans* *E. coli*

Figure 8: Group C (After 3min 48sec, without any intervention in a chamber)



P. aeruginosa *S. aureus* *K. pneumoniae* *C. albicans* *E. coli*

Discussion

Herbs belonging to *krimighna, kandughna* and *vranashodhan vranaropan-gana* were used for their antimicrobial properties in herbal fumigation (Table 1). Different studies shows that these individual drugs having virucidal, bactericidal, fungicidal, mycobactericidal (23,24) properties. *Dhoopana* is an example of drug delivery through the fumes which having very high penetrability in various things exposed to fumigation. These fumes can easily reach up to any corner of the room and hence prove very potent against various disease-causing vectors and microorganisms (15).

Nimba, Vacha, Gaura Sarshapa and *Guggulu* are having strong antimicrobial properties specially against *Staphylococcus aureus, E-coli* and other bacteria (11,12,13), during previous works using same herbs, combined antibacterial and antifungal properties of these herbs against *Staphylococcus aureus, E-coli, pseudomonas aeruginosa, candida albicans, klebsiella pneumoniae* were studied and shown very promising results (11, 16). In classical method of *dhoopan*, fumes can cause irritation and burning of eyes and there may be chance of liberation of harmful gases like CO2 and CO which can cause air pollution. When fumigation done with modified form, this irritation and burning of eyes can be prevented (15). Recent study conducted at Parul Institute of Ayurveda 'A comparative clinical study of Vranadhupan by burning and heating of Vranadhupan dravyas in the management of dushta Vrana' has proved that conventional method of burning of herbs for fumigation purpose actually produces smoke (not fumes!) and affects AQI (Air quality index) badly due to liberation of high amounts of toxic products of partial combustion i.e. toxic gases (CO2 and CO), high quantity of particulate materials (PM 2.5 and PM 10) and high temperature in comparison with *Sushrutokta* method of fumigation by heating the herbs (21, 22).

In classical method of herbal fumigation, chances of burn with contact of hot plate or hot *sharav* can lead to *pramad dagdha* or accidental burn, that can be prevented when used in modified form (15). Use of modern equipment of fumigation i.e., fumigator or fogger machine is only possible if drug is in liquid form. Previous researchers have studied antimicrobial properties of kwath (decoction) prepared from herbal fumigation medicines to address this issue (16). Still, considering purely aqueous base of decoction, possibility of water vapour deposits on wooden and metallic surfaces in the ward with repeated use of kwath as fumigation agent may lead damage to them. Hence Hydroalcoholic extract was considered as better option for fumigation while planning this study.

Physicochemical analysis of MHFF revealed that pH of modified herbal fumigation formulation is 6 which is mild acidic in nature (Table 3). Mild acidic nature of fumigation solutions helps in inhibiting bacterial growth (20). More the drug concentration, higher alcohol percentage, more the exposure duration shown better results (Table 8).

GC-MS report revealed that many active compounds found in the hydroalcoholic extract. Methyl Eugenol, tyrosyl-s-benzyl cysteine, oxirane, alpha and beta asarone etc. which are having antimicrobial and mood elevating properties.

The modified formulation of *dhoopan* i.e., hydroalcoholic extract show good antimicrobial effect and hence can be used for fumigation of wards.

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Merits

The use of Hydroalcoholic extract was done first time for fumigation purpose and no any other similar studies underwent before. Modified herbal fumigation formulation is having potent action against commonly responsible bacteria for hospital acquired and surgical site infections. It is in liquid form and hence fumigation apparatus or fogging machine can be used for fumigation. Post fumigation residue is minimum which is better sign of good fumigation material. The fumes are having strong aroma with mild fragrance and not irritable to eyes which usually happened with conventional classical method of dhoopan and even with formalin.

Limitations

As it is a very novel approach to modify fumigation formulation in the form of hydroalcoholic extract very first time, few points might be missing in this study. Also, the availability of Alcohol at a large quantity was difficult in a state like Gujarat. Due to the higher tax policy and increased raw drugs value at the market, preparation cost of hydroalcoholic extract in smaller quantity was higher than formalin.

Further scope of research

Drug quantity, concentration and time of exposure can be increased to get more better results. Other bacterial species can be checked for antimicrobial properties even if possible antiviral study can be done. Comparison with formalin or even with the classical method of *dhoopan* can be done. Inside a small room or large chamber of 10 x 10 x 10ft same study can be repeated to know minimum effective dose and duration of the exposure. In the classical formulation along with these 4 drugs *Agaru, Sarjarasa, Saindhav* and *Ghrita* were mentioned in this combination and advised to do *dhoopan* twice a day. So, that method if followed may give better results.

Conclusion

The results of phytochemical analysis and antimicrobial studies during this research work indicate that the medicine has promising antimicrobial properties. Physiochemical and in vitro parameters show it can be used for fumigation. It's having potent antimicrobial activity against the most commonly occurring microbes responsible for hospital acquired infections like *E coli, p. aureus, p. aeruginosa, k pneumoniae* and *c. albicans*. More drug concentration, more alcohol concentration and more the duration of exposure will give better results.

This study has certain limitations and many positive outcomes. Further studies required to prove its potency at large area and with other microorganisms' species. Also required to compare these results with standard method of *dhoopan* explained in classics to verify its potency, advantages and demerits.

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