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MICROBIAL SAFETY ASSESSMENT AND STERILIZATION OF SUFOOFE SAILAN: AN UNANI POLYHERBAL FORMULATION

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ABSTRACT
Biggest problem with most of the herbal preparations is that the drugs are not subjected to sterility studies and Unani medicine is also one of them. Instead of curing, they may cause serious problems. It is liable to produce various toxic effects if the drug prepared is not sterile. Unfortunately, no good researches have been carried out to determine appropriateness of sterilization or microbial load reduction method for Unani drugs at the level of both single drugs and compound formulations till date.In the present study microbial quality of pre-sterilized and post sterilized sample of *Sufoofe sailan* had been evaluated and compared for 'Microbial Safety assessment of Sufoofe Sailan (A Unani polyherbal) formulation. Microbiological analysis was carried out for the detection and enumeration of microorganisms viz. total bacterial count, total fungal count and presence of *E.coli* , *Salmonellae*, *Staphylococci* and *Pseudomonas aeruginosa* using standard media. Also, the aflatoxins analysis was performed on *Sufoofe sailan* sample. Representative figures for the microbial status of pre-sterilized and post sterilized SS sample including total bacterial count (T.B.C) and total fungal count (T.F.C) were noted. Before sterilization figures for T.B.C and T.F.C were 30000 and 20CFU/g, respectively. While, sterilized sample showed zero figures i.e., no colonies were found. *E. coli*, *Staphylococcus aureus*, *Salmonella*, *Pseudomonas aeruginosa* and fungi were absent in both before and after sterilized samples of SS. The post sterilized samples were also found free from aflatoxins (B1, B2, G1 & G2). Each preparation must be subjected to chemical and microbiological examination, right from the raw drug to the manufacturing stage and upto finished product. It was concluded that sterility studies should be done for every drug to make the herbal drugs world widely acceptable.

KEY WORDS: Safety assessment, Sterilization, Aflatoxins, Bacteria, Fungi

INTRODUCTION

With the increasing use of herbal medicines worldwide, and rapid expansion of global market for herbal products, the safety and quality of herbal finished products has become a major concern for health authorities, pharmaceutical industries and public as per WHO guidelines.[1] In context of quality assurance, assessing the quality in relation to the safety of herbal medicines with specific reference to contaminants and residues is essential parameter. Quality evaluation of herbal preparation is a fundamental requirement of industry and other organization dealing with herbal products. The growing use by the public is forcing moves to assess the health claims of these agents and to develop.[2] In Unani medicine plant origin drugs are used for treatment of number of ailments. Hundred percent purity of an herbal drug is possible only when the drug is free from adulteration, substitution and contamination. Biggest problem with the most of the traditional preparations is that the drugs are not subjected to sterilization process. Instead of curing, they may cause serious problems. It is liable to produce various toxic effects if the drug prepared is not sterile. Various factors affect the state of drugs like the influence of climate and ageing in relation to medicine, powders are attached by fungi, distilled and aqueous preparations are liable to decomposition, mildews are formed in syrups, and sugary components might settle down, ointments may become malodorous and decompose. It is a major ethical issue as contaminated drug cannot be marketed for human use. Contaminations in herbal medicines may be physico-chemical, biological, agrochemical and organic in nature. Contaminations may be due to contaminated ingredients, preprocess contamination, process contamination, and post process contamination. So, each new preparation must be subjected to microbiological examination, right from the bench to the manufacturing stage, keeping this in view, sterility study of some Unani drugs is undertaken to address this important issue. Also, the use of herbal medicinal plants is generally contributing significantly to the exposure of the population to contaminants.[5]Current practices of harvesting, production, transportation and storage may cause additional contamination with microbial growth.[3]

Sterilization can be defined as any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and prions) from a surface, equipment, foods, medications, or biological culture medium. In practice sterility is achieved by exposure of the object to be sterilized to chemical or physical agent for a specified time. Various agents used as sterilization agents are: elevated temperature, ionizing radiation, chemical liquids or gases etc. The success of the process depends upon the choice of the method adopted for sterilization. Sterilization

is simply the process of microbial destruction. Microbial destruction methods such as heat, chemical, and radiation sterilization are used. Upon exposure of such treatment, microorganisms die according to logarithmic relationship between concentration or population of the living cells and the time exposure or radiation dose. [4]

WHO has also developed the technical guidelines for the assessment of microbial quality of herbal medicine.[5] The quality assessment of herbal formulations is very important in order to justify their acceptance in modern system of medicines. It is thus mandatory that the microbiological limit tests of herbal medicinal preparations be done to ensure that the product is free from risk. In India many such formulations are handmade or made by physicians directly. These formulations are not subjected to aseptic conditions during various stages of preparation, packaging, storage, transport etc. as required by regulatory norms. Also, plant materials carry a huge number of bacteria and fungi, mainly originating in soil. Aerobic sporulating bacteria frequently predominate in this to which additional contamination and microbial growth occur during harvesting, handling and production.[6,7] The important precautions to be taken in order to get satisfactory isolated colonies during the performance of all procedures are to avoid air currents by open windows, random movements of hands, heating systems or even talking and contact between the sample and all non-sterile objects and surfaces. Laboratory workers must constantly guard themselves against the potential hazard of acquiring a laboratory infection.

The contaminants that present serious health hazard are pathogenic bacteria such as *Salmonella, Escherichia coli, Staphylococcus aureus, Shigella* spp. and other Gram-positive and Gram-negative strains of bacteria.[3] Unfortunately, no good researches have been carried out to determine the microbiological safety of herbal products. Acceptance criterion for herbal materials for internal use is interpreted in table 1. As the use of herbal preparations by patients is increasing, there is an urgent need for pharmacists and physicians to have knowledge about the safety of these preparations. In the present study microbial quality of three different batches of SS have been evaluated before and after sterilization. *Sufoofe sailan* is a polyherbal powder preparation described in various texts of Unani medicine and is used to treat *Sailanur rahem* (leucorrhea), *uqr* (sterility), *surate inzal* (premature ejaculation) etc. This formulation contains habis (dessicative) and qabiz (constipative) drugs e.g., *samagiyat* (gums), *ral* (resin) with sugar which may be responsible for its early decomposition. Ingredients of *sufoofe sailan* are *gule dhawa* 6g, *gule fofal* 6g, *mochras* 6g, *gond molsri* 6g and *nabat safaid* 24 g (Table 2). All ingredients are powdered, passed through sieve and mixed rigorously to make homogenized form.[5]

MATERIALS AND METHODS

Sources of data: Data was generated from the experimental studies at the laboratory of the Department of Ilmus Saidla, National Institute of Unani Medicine and CPPP Dept., Foundation for Revitalisation of Local Health Tradition (FRLHT) Bangalore.

Procurement of raw drugs

All plant materials used for the formulation of SS were purchased from the raw drug trader during February to July 2013. All the plant materials were confirmed, and a sample of each plant material used was submitted to the drug museum, with voucher specimen no. 19/IS/Res./2014, for future reference. *Gule Dhawa* (*Woodfordia fructosa* L. Kurz.) and *Gule Fofal* (*Areca catechu* L.) were further certified by Herbarium curator, Department of Botany, and specimen was deposited in their museum with accession number 2968 and 2969, respectively.

Preparation of *Sufoofe Sailan*

All ingredients were powdered separately in the electric grinder and sieved through no. 80 mesh. These powdered ingredients were weighed separately in the ratio mentioned in NFUM and mixed rigorously in an electric kitchen mixer to get homogenous powder [Table 1].

TABLE 1: Ingredients of *Sufoofe Sailan*

S.no.	Drug name	Botanical name	Part used	Proportion
1.	*Gule dhawa*	*Woodfordia fructosa* L.Kurz.	Flower	12.5%
2.	*Gule fofal*	*Areca catechu* L.	Flower	12.5%
3.	*Mochras*	*Bombax malabaricum* Dc.	Gum	12.5%
4.	*Gond molsri*	*Mimusops elengi* L.	Gum	12.5%
5.	*Nabat safaid*	Sugar	Crystals	50%

Sterilization technique

Dry steam sterilization was done on SS sample to diminish or reduce microbes in sample. A total of 20-minute process involved treating SS sample with steam at a temperature of approximately 112°C under high pressure.

Materials required

HPLC water, 50mL dichloromethane (DCM), anisaldehyde sulphuric acid, ethyl acetate and formic acid, sample or bacterial suspension, 9ml dilution blanks (6 or 7)**,** sterile petri plates (12)**,** sterile 1 ml pipettes (6) **,** colony counter, nutrient agar medium (200ml) **,** chloramphenicol(200ml), sabouraud agar medium (200ml), lactose broth, mac-conkey, tetrathionate bile brilliant green broth, soyabean casein digest medium, deoxycholate citrate agar, and citrimide agar were used for microbial studies. All chemicals and the reagents were of analytical grade.

Safety evaluation and sterility studies

The drug was evaluated for the various safety and toxicological parameter like microbial content and heavy metal determination. In the present study, microbiological analysis on SS samples was carried out according to official microbiological methods of WHO. The samples were prepared as many decimal dilutions as necessary depending on the expected bacterial load of the material being examined. After dilutions prepared, appropriate

media were inoculated and incubated at specific temperature. Sterility studies includes Detection of microbiological contaminations in pre and post sterilized samples of compound drug SS.

Microbial analysis

The determiation of microbiological contamination was established for microbial load viz total bacterial count, total fungal count and specific pathogen (*E.coli, Salmonella spp, Staphylococcus aureus, Pseudomonas aeruginosa*) in the pre and post sterilized SS samples. Maximum permissible limits of pathogens is given in Table 2.

TABLE 2: Maximum permissible limits of pathogens

S.no.	Pathogens	Maximum limit
1.	Aerobic bacteria	10^5 per gram
2.	Yeasts and moulds	10^3 per gram
3.	*Escherichia coli*	10 per gram
4.	*Clostridia,*	absent per 1 gram
5.	*Salmonellae*	absent per 1 gram
6.	*Shigella*	97 absent per 1 gram

The methodology applied for the studies was as follows.

Total microbial load

Enumeration of microorganisms (bacteria/fungi) of the samples was done by serial dilution agar plate method.

Procedure

Fifty ml each of nutrient agar medium and Sabouraud dextrose agar medium was prepared. 0.85% NaCl was prepared and 10ml was suspended to first test tube and 9ml saline was suspended to the remaining tubes labelled as (10^{-1} to 10^{-6}) each. The above media and tubes containing saline were autoclaved at 121^{o}C/15mins. After autoclaving contents were brought to laminar air flow and all the tubes containing saline were cooled. One gram of sample without preservative was weighed and transferred to the first tube containing 0.85% NaCl and mixed gently for uniform suspension. It was allowed to stand for some time. One ml of suspension was transferred from the first tube to the next tube labelled as 10^{-1}. Further dilutions were made till the tube labelled as 10^{-6}. Once the dilutions were done 0.1ml of suspension was taken from tube labelled as 10-^{1} and plated on sterile petri plate. In the same way 0.1ml of suspension was taken from tubes labelled as 10^{-3} and 10^{-5} each and plated on two sterile petri plates. Once the plating was done a thin layer of media cooled to 45^{o}C was poured into the plates containing sample and plates were gently rotated for uniform distribution of cells. [6]

For bacteria

Nutrient agar media was poured and after solidification the plates were incubated at 37^{o}C for 24 hours in inverted position and noted down the observation.

For fungi

Sabouraud dextrose agar media was poured and after solidification the plates were incubated at room temperature for 48-72 hours and noted down the observation. The total number of viable organisms was reported in terms of colony forming units.

Specific Pathogen Tests

The test will be performed using pour plate method to detect any of the micro-organisms if present in the drug. The organism to be deducted will be *Escherichia Coli, Salmoella, Shigella, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans.* The general method used in detailed as follows. The sample will be incubated with broth at specific temperature and sub cultured on plates, again incubated as specified and noted the growth area. Confirmation was done using specific test for each organism. The table attached shows the specific details for tests used for each organism.[7,8] (table 3)

Escherichia coli

A quantity of the homogenized material in lactose broth was transferred, prepared and incubated containing 1 g or 1 ml of SS, to 100 ml of MacConkey broth and incubated at 43-45°C for 18-24 hours. A subculture on a plate with MacConkey agar was prepared and incubated at 43-45°C for 18-24 hours. Growth of red, generally non-mucoid colonies of Gram-negative rods, sometimes surrounded by a reddish zone of precipitation, indicates the possible presence of E. coli. This was confirmed by the formation of indole at 43.5-44.5°C or by other biochemical reactions. No such colonies were detected means that SS sample passes the test.[9]

Salmonella spp.

The solution, suspension or emulsion of the pretreated material prepared was incubated at 35-37°C for 5-24 hours. 10 ml of the enrichment culture was transfered to 100 ml of tetrathionate bile brilliant green broth and incubated at 42-43°C for 18-24 hours. Subcultures on two agar media: deoxycholate citrate agar and brilliant green agar. It was incubated at 35-37°C for 24-48 hours. No colonies were produced so no secondary was carried out.

Pseudomonas aeruginosa

The SS sample was pre-treated using buffered sodium chloride-peptone solution, pH 7.0. 100 ml of soybean-casein digest medium with a quantity of the solution, suspension or emulsion thus obtained was inoculated containing 1g of SS. It was then mixed and incubated at 35-37°C for 24-48 hours. A subculture was prepared on a plate of cetrimide agar and incubated at 35-37°C for 24-48 hours. Growth of colonies of Gram-negative rods occurs with a greenish fluorescence, an oxidase test was applied and the growth in soybean-casein digest medium at 42°C was tested. 2 or 3 drops of a freshly prepared 0.01 g/ml solution of N'-tetramethyl-p-phenylenediamine dihydrochloride R was placed on filter-paper and applied a smear of the suspected colony; purple color was produced within 10 seconds means test was positive. Cultures do not appear, means test was positive.

Staphylococcus aureus

An enrichment culture was prepared for *Pseudomonas aeruginosa*. A subculture was prepared on a suitable

medium such as Baird-Parker agar and incubated at 35-37°C for 24-48 hours. No growth of microorganisms is detected means SS passes the test. Black colonies of Gram-positive cocci often surrounded by clear zones may indicate the presence of *Staphylococcus aureus*. Cultures do not appear means the material passes the test.[8]

Aflatoxins analysis

25 gm from extracted SS sample were tested for aflatoxins (B1, B2, G1 and G2). Tests were performed accordingly.[9]

TABLE 3: Test for specific micro-organism; calculation per 1gm or 1ml of the sample

Name of micro organism	Inculation (Broth)	Incubation	Transfer media	Incubation	Sub-culture on plates	Incubation	Growth appearance	Conformation test
Escherichia Coli	100ml casein soyabin digest	35°-37°C 18-48 hrs	Mac conkey agar 100ml	43°-45°C 18-24 hrs	Mac conkey agar	35°-37°C 18-72 hrs	Red non-mucoid gram –ve rods	Indole test
Salmoella	100ml casein soyabin digest	35°-37°C 18-24 hrs	Rappaport vassiliadia broth 10ml	30°-35°C 24-48 hrs	Xylose-lysine deoxy-cholate agar	30°-35°C 24-48 hrs	Red colonies with/ without black centers	Identification test
Shigella	100ml casein soyabin digest	30°-35°C 18-24 hrs	100ml G.N broth	30°-35°C 24-48 hrs	Xylose-lysine deoxy-cholate agar	30°-35°C 24-48 hrs	Red coloured translucent colony without black center	Identification test
Pseudomonas aeruginosa	100ml casein soyabin digest	35°-37°C 18-48 hrs	-	-	Cetrimide agar	30°-35°C 18-72 hrs	Greenish colour colony	Identification test
Staphylococcus aureus	100ml casein soyabin digest	35°-37°C 18-48 hrs	-	-	Barid parker agar	35°-37°C 18-72 hrs	Black colour colonies surrounded by clear zone	Coagulase test or DNAse test
Candida albicans	Suitable amount of sabouard dextrose broth	30°-35°C 3-5 days	-	-	sabouard dextrose agar	30°-35°C 24-48 hrs	Cream colour colonies	Identification test

RESULTS:

TABLE 4: Total bacterial and fungal count in pre and post Sterilized samples of Sufoofe Sailan

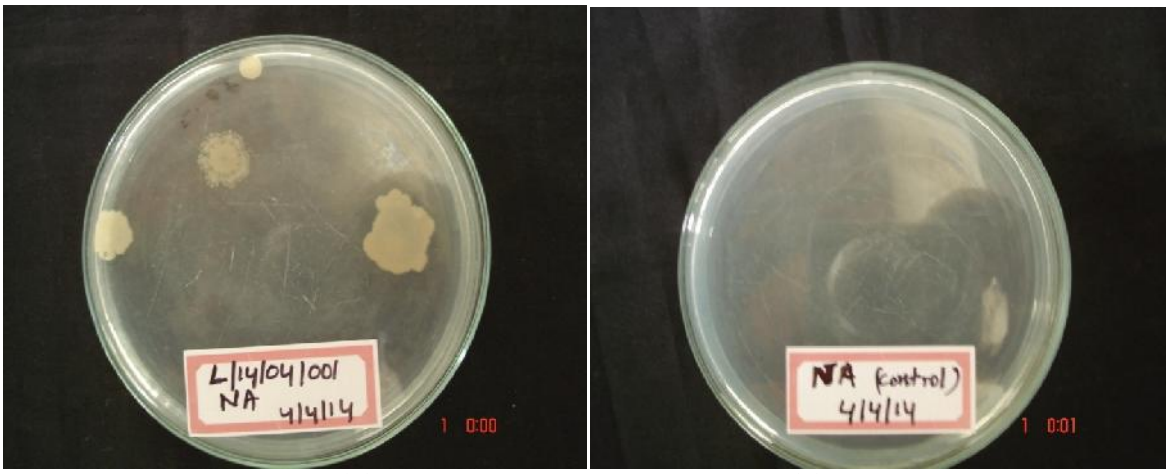
Samples	Total bacterial count (Cfu/gm/ml)	WHO limit	Total fungal count (Cfu/gm/ml)	WHO limit	Inference
Pre sterilized sample	30000	10^5/gm	20	10^3/gm	Within Limit
Post Sterilized sample	0	10^5/gm	0	10^3/gm	Within Limit

TABLE 5: Presence of pathogenic bacteria in pre and post Sterilized samples of Sufoofe Sailan

Sample	*E.coli*	*Salmonella*	*Staph. aureus*	*P. aeruginosa*
Pre-Sterilized sample	Absent	Absent	Absent	Absent
Post-Sterilized sample	Absent	Absent	Absent	Absent

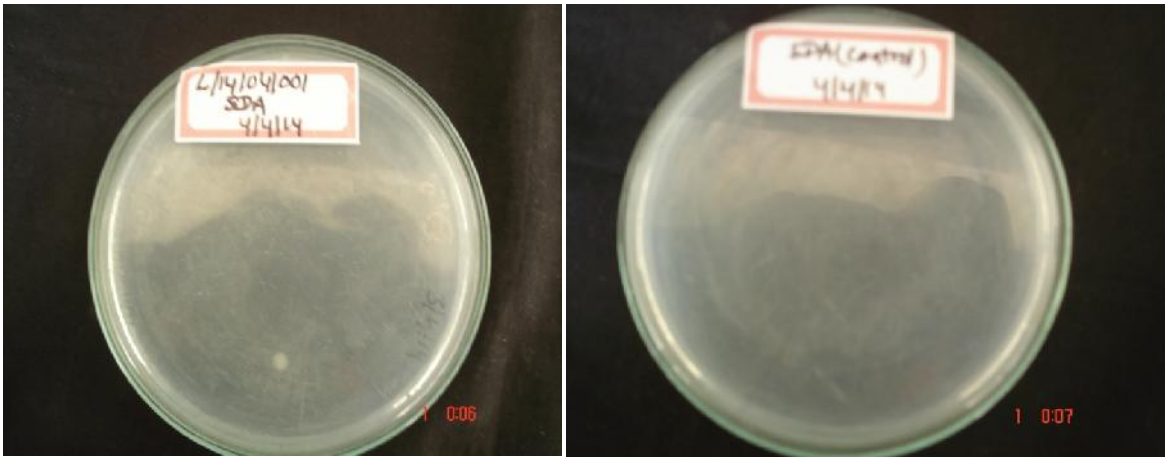
TABLE 6: Aflatoxin count in pre and post Sterilized samples of Sufoofe Sailan

Sample	Test	Limit (maximum)	Inference
Pre-Sterilized sample	Aflatoxin B1	5 µg/kgb	Not detectable
	Sum of aflatoxins B1, B2, G1 and G2	10 µg/kgb	Not detectable
Post-Sterilized sample	Aflatoxin B1	0 µg/kgb	Not detectable
	Sum of aflatoxins B1, B2, G1 and G2	0 µg/kgb	Not detectable



(A) **(B)**

FIGURE1: Total bacterial count in Pre-sterilized samples of *Sufoofe Sailan* using *nutrient agar* as medium
FIGURE2: Total bacterial count in post sterilized samples of *Sufoofe Sailan* using *nutrient agar* as medium.



(a) **(b)**

FIGURE 3: Total fungal count in Pre-Sterilized samples of *Sufoofe Sailan* using *Sabouraud dextrose agar* as medium.
FIGURE 4: Total fungal count in post Sterilized samples of *Sufoofe Sailan* using *Sabouraud dextrose agar* as medium.

DISCUSSION

Representative figures for the microbial status of pre sterilized sample of *Sufoofe Sailan* including total bacterial count and total fungal count were 30000 and 20CFU/g, respectively. While post sterilized sample showed zero figures i.e no colonies were found. *E. coli, Staphylococcus aureus, Salmonella, Pseudomonas* were absent in both before and after samples. The sterilized samples were also found to be free of aflatoxins (B1, B2, G1 and G2).

The WHO limit for total bacterial count is 10^5/gm. WHO limit for total fungal count is 10^3/gm. The total viable count of microbes was within limit as per WHO guidelines. Further, pathogenic microbes were absent in the sterility study samples. Thus, sterility study of SS sample confirmed to the standard quality prescribed by WHO,[10] API[11] and other guidelines.

Generally, testing for pathogens in supplements which have been appropriately processed and are homogeneous need not be repeated throughout a product's shelf-life, provided the batch was tested and found to be free of relevant pathogens at the time of manufacture. The contamination of herbal drugs by microorganism not only cause bio deterioration but also reduces the efficacy of herbal drugs.[12] The toxins produced by microbes makes herbal drugs unfit for human consumption because the contaminated drug may develop unwanted diseases instead of disease being cured. Considerable interest therefore lies in investigation pertaining to the microbial contamination associated with drug samples. The plant material used in herbal drugs preparations are organic in nature, it provides nutrition to microorganisms as well and facilitates the multiplication of microorganism which lead to contamination, deterioration and variation in composition. This give rises to inferior quality of herbal product with little or no therapeutic efficacy. The antimicrobial activity of drug plants has been studied in India and abroad but there is very less literature regarding microbial contamination of herbal drugs. However, some workers have reported fungi from plants part used in drug preparation.[13] Herbals tend to show much higher levels of microbial contamination than synthetic products according to European Pharmacopoeia. The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflatoxins. Aflatoxins in herbal drugs can be dangerous to health even

if they are absorbed in minute amounts (WHO, 2000). Aflatoxin-producing fungi sometimes build up during storage. Procedures for the determination of aflatoxin contamination in herbal drugs are published by the WHO (2000).[14]

Microbiological test results if exceed the specifications established for the product, may also cause undesirable organoleptic and chemical changes. Microorganisms require readily accessible water in appreciable quantities for growth.[18] Scott (1953) established that only moisture does not have significant effect, but the water activity is the key to determine if microorganisms will grow or not. Water activity of aqueous formulations can be reduced by the addition of high concentration of sugars or PEG or by reducing moisture content. Ingredients with low water activity (*i.e*. those which are very dry, or which contain high levels of salt or sugar), highly acidic or alkaline pH (i.e. having a low pH or high pH), or high levels of alcohol are also generally resistant to microbial growth.[19] Drying is the most common and fundamental method for preservation of medicinal plants because it allows for the quick conservation of the medicinal qualities of the plant material in an uncomplicated manner. Kulshrestha et al., mentioned that drying at a specific temperature decreases the total microbial count in plant material as it lowers the water activity.[20]But drying process cannot compete with the sterilization as sterilization process contributes to physical, chemical and microbiological stability of the medicinal herbs. In present date, drying is not sufficient and appropriate method for full destruction of microorganisms to zero figure. So, sterilization technique is most appropriate technique in today's world to make the herbal drugs world widely acceptable.

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