Study of microbial quality on different drug formulations widely used in Ayurvedic system of medicine

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Abstract

World Health Assembly (WHA) has given the following limitations for herbal preparations. Aerobic bacteria $1x10^5$/g. for internal use and $1x10^7$/g. for topical dosage forms. Fungi $1x10^4$/g., *E-coli* $1x10^2$/g, and *Salmonellae* – none for internal and topical dosage forms. This study was carried out to study the microbial load, micro-organisms, anti microbial effects of compound preparations. Powders (Choorna), Pastes (Kalka), Pills (Vatika), Fermented dicoctions (Arista and Asawa), Oils (Thaila) and Local applications (Lepa) preparations were tested. Microbial load of some of the Choorna, Lepa and Kalka were exceeded the WHA limitations. Some of the Kalka, Arista and Asawa,Vatika, Thaila and Lepa preparations were within the acceptable limits. Isolated pure cultures were identified using morphological and biochemical tests and slide culture techniques. Most of the bacterial cultures belong to the genus Bacillus. The isolated fungi were identified as Penicillium Sp, Aspergillus Sp, Mucor Sp, Rhizopus Sp, Fusarium Sp. Some of the Thalisadi Choorna and Chandra Kalka preparations were positive for *Salmonellae* and *E-Coli*. It was noted that Hinguastaka Choorna and Manibadra Choorna had anti microbial effect on *Salmonellae typhi*. Thripaladi Choorna had effect on *Pseudomonas aeruginosa*. Sarvavisadee Oil was active against *Pseudomonas aeruginosa*, *Staphylococcus aureas*, *Salmonellae typhi*, and *klebsiella*. Buddharaja Kalka was active against *Pseudomonas aeruginosa*, *Staphylococcus aureas* and *Bacillus cereus*.

Hazard Analysis and Critical Control Paint Analysis was done on the Thalisadi Choorna and found implementation of Good Manufacturing Practice and HACCP principals could contribute to microbiologically safe end products. The steam treatment method was found to be an effective method to reduce the microbial load. Studies on the antimicrobial activity justify the use of these compound preparations in common infective conditions.

Introduction

Herbal medicine has been in practice for several thousand years. In developed countries, the popularity of herbal medicine is continuing to grow. Further medicinal plants are important sources for the biopharmaceutical industry. Hence, medicinal plants and herbal medicines account for a significant percentage of the biopharmaceutical market. All these materials contain a natural inherent microbial flora and may be contaminated during harvesting, processing, preparation and storage. Considering these facts the World Health Assembly (WHA) in its resolutions WHA 31:33, WHA 40:33, WHA 42:43 has emphasized the need to develop and ensure the microbial quality standards of medicinal plant products using modern techniques and applications of suitable standards [1].

Aims and objectives

This study was carried out to assess the microbial load on the final preparations, identify the specific micro-organisms, study the ways and means to control and prevent contamination, establish good manufacturing practice, define an acceptable microbiological quality standards in the final preparation as envisaged by WHA standards and study the anti-microbial activity of the compound preparations.

Materials and Methods

Study on microbial load

The compound preparations of various formulations (Choorna, Kalka, Vatika, Arista, Thaila, and Lepa) of different manufacturers were subjected to the analysis of microbial load of bacteria and fungi. Samples were collected from open market from fifteen different drug manufacturers. Tested Choorna (Powders) are Dathri Choorna, Thripaladi Choorna, Thalisadi Choorna, Hinguastaka Choorna and Manibadra Choorna. Tested Kalka (Pastes) are Buddharaja Kalka, Nawaratna Kalka, Chandra Kalka, Sarkaradi Kalka and Desadun Kalka. Tested Vatika (Pills) are Seetharama Vati, Suranvidura Vati and Jeevananda Vati. Tested Arista (Fermented decoctions) are Dasamoola

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Arista, Saraswatharista, Draksarista Arista, Pipalyadi Asawa and Arawindasawa. Tested Thaila (Oil) are Pinda Thaila, Narayana Thaila, Sarvavisadee Thaila, Visarpahara Thaila and Wathavi-duranga Thaila. Tested Lepa (Topical application) are Dasanga lepa, Sothahara lepa and Rogan e khas [2].

Three different samples in each drug of manufactures were taken for the study. Studies on microbial load was done using a dilution series up to 10^0 to10^{-3} in sterile distilled water. Oil preparations were directly applied on agar plates to study the microbial load. Nutrient agar and potato dextrose agar were used as common culture media for bacteria and fungi respectively. Pour plate technique was used on nutrient agar and spread plate technique was used on potato dextrose agar. Colony forming units were taken after 24 hours and 72 hours for bacteria and fungi respectively. It was assumed that each colony was formed by a single organism.

Morphological and biochemical tests

Isolated microbial colonies from above compound preparations were purified by re-streaking in the same agar medium and stock cultures were prepared and stored at 4°C until biochemical tests were done for identification. Periodically cultures were renewed to maintain the viability of the microorganism. Morphological study and identification of bacteria were done through biochemical tests and fungi were identified through slide culture techniques [3]. Stock cultures were sub cultured on Nutrient agar plates by using streak plate technique. Culture plates were used for morphological and biochemical tests within 24 hours.

The following bio chemical tests were done on these cultures: Grams stain, Spore stain, Motility test, Catalase activity, Oxidase activity, Acid production from Glucose, Oxidative and fermentative activity tests for Carbohydrate utilization, Methyl red test, Voges proskauer reaction, Indole test, Starch hydrolysis, Growth in 7% Sodium chloride, Growth in 65°C, Growth in 45°C, Gelatin hydrolysis and Casein hydrolysis.

Tests for *Salmonellae*

Buffer peptone was used as non-selective enrichment media. One gram of the solid preparation or one ml of the liquid preparations was added to 20 ml of the buffer peptone and kept on gentle shaking position for 24 hours (at the ratio of 25 grams in 475 ml).

Selanite broth and tetra thionate broth were used as selective media (these media were prepared according to the specifications of the Oxoid-manual. Within 48 hours of incubation period in the selective media one loop of these test solutions were streaked on Bismuth Sulphite agar (B/SAgar) and Brilliant Green agar (B/G Agar) plates separately (Bismuth Sulphite agar and Brilliant Green agar were prepared according to the specifications of Oxoid manual). Black colonies on B/ S agar and pink colonies on B/G agar was considered as positive for *Salmonellae* organisms. These specific colonies were picked and streaked on already prepared nutrient agar plates and incubated for 24 hours to obtain pure cultures. Within 24 hours these pure cultures were biochemically tested for *Salmonellae*. These stock cultures also identified during the tests done for identification of the other micro-organisms. Urease test, Indole test, and Hydrogen sulfide production tests were done as biochemical confirmation tests for *Salmonellae* [4].

Test for coliforms

One gram or one ml of each of the samples was dissolved in 10 ml of sterile distilled water. Using this stock solution series of dilutions up to 10^{-3} was made. One ml of this solution was transferred into 09 ml of single strength sterile MacConky broth tubes which contain a Durham tube. Mix the tubes by gentle rotation. These tubes were incubated at 37°C for 48 hours. The tubes which gave yellow colour with gas production were assumed as positive for Coliforms. These positive tubes were sub cultured into sterile Brilliant Green Bile broth tubes which also contained a Durham tube in two sets. One set was kept at 37°C and another was kept at 44°C for 48 hours. The tubes which showed gas production at 37°C were considered as positive for Coliforms and the tubes which showed gas production at 44°C were considered as positive for faecal Coliforms. *E.coli* was confirmed by Indole test [4].

Test for anti microbial activity

Compound preparations below two years of manufacturing date were selected. Minimum adult dose of Choorna and Kalka were dissolved in 15 ml of sterile distilled water and kept in a shaker at 100 r.p.m. continuously for four hours in order to get the maximum soluble water extract of these preparations. 0.1 ml of these extracts was used to the antimicrobial assay. In case of oils, 0.1 ml of oil was used directly. 50 ml of the Nutrient broth was prepared, taken into five metal capped tests tubes and sterilized. These tubes were inoculated separately using sterile inoculating needle with pure American Type Culture Collection (ATCC test cultures).

The test organisms were ATCC cultures of *Pseudomonas aeruginosa, Escherichia coli, Salmonellae typhi, Klebsiella, Bacillus cereus* and *Staphylococcus aureus*. Nutrient agar plates were prepared and kept in an incubator at 37°C for 24 hours in order to exclude contamination and to reduce the moisture content of these plates. These agar plates were seeded with above ATCC test cultures separately using spread plate technique. Sterilized metal cylinders (assay cup) were placed in each sector using sterile forceps. The cylinders were pressed gently until the edge of the cylinders sink into the agar to keep it firmly in place. 0.1 ml of the drug extract was placed into these cylinders and plates were sealed. In case of oil 0.1ml of oil was placed into these cylinders and plates

were sealed. Within 24 hours the growth of the seeded organisms were noted. The clear zones around the assay cups were measured and recorded. Width of the zones was measured and considered as the efficacy of the drugs against the test organism [4].

HACCP analysis

Analysis was done at the Nawinna Ayurvedic Drugs Corporation. The sites which were subjected to identify any critical points are main drug stores, pre preparation site, drug manufacturing site, packaging site, labeling site and stores of the finish product. Product Thalisadi Choorna was selected as a test product on the basis of the test results of the microbial load. A flow chart of the product was prepared. Possible critical points were identified. A test preparation was prepared according to identified critical points in order to minimize the contamination and to reduce the microbial load. Microbial load was repeated in the test product.

Methods tested to control microbial load on compound preparation "Thalisadi Choorna"

1. Heat treatment. 100g of the above sample was subjected to heat treatment in a hot air oven at 80°C for 10 minutes for three consecutive days.
2. Ultra violet radiation at 256 wave length continuously for 24 hours (100 g).
3. 100 g of the sample was steamed under atmospheric pressure in a closed container up to 10 minutes for three consecutive days.

Then the study of microbial load was thereafter repeated in every sample. Then Thin Layer Chromatographic (TLC) patterns and the volatile oil content of the steamed and original samples were done on sample which showed relatively low microbial load and the original sample which was not subjected to any pretreatment methods.

Study on thin layer chromatographic patterns

Steamed in an open container, steamed in a closed container and original sample of Thalisadi Choorna was subjected to this study.

Moisture content of the 3 gram above samples was measured. 3 gram of the sample and water content of the samples was taken for extraction. Extraction with 50 ml of Ethanol and 50 ml of water were taken separately. Extracts were concentrated and dissolved in 3ml of same extracted solvent and using a sterilized micropipette. 1-2 micro liter of this solution was used to spot the TLC plates. Different solvent systems were used to study the TLC. Using Vanilline + Conc-Sulphuric acid spray reagent the bands were fixed and noted under the UV lamp (254 and 365 nm). Number of bands, R.f values, distance of the solvent front from the spot and the distance of the bands from the spot also recorded.

Study on the volatile oil content

Steamed in an open container, steamed in a closed container and original sample were taken for the study. 80 gram of each drug samples was taken with 250 ml of distilled water. "Dead and Stark Distilled Arm" apparatus was used to measure the volatile oil content. Each sample was refluxed in hot water bath for 2-3 hours. Volatile oil collected at the upper end of the distillery arm was measured and recorded.

Results and Discussion

Results of the microbial load on different formulations were summarized as below (Table 1).

Table 1

	Range for bacterial counts /gr	*Range for fungi counts /gr*
Choorna (Powders)		
Dathri Choorna	2.0×10^4 to 1.1×10^6	4.7×10^4 to 1.7×10^6
Hinguastaka Choorna	9.8×10^4 to 4.0×10^6	2.0×10^4 to 4.5×10^4
Manibadra Choorna	8.4×10^4 to 4.2×10^7	2.0×10^3 to 1.5×10^5
Thalisadi Choorna	3.0×10^3 to 6.2×10^7	00 to 1.9×10^4
Thripaladi Choorna	2.0×10^5 to 1.2×10^7	00 to 1.5×10^4
Kalka (Pastes)		
Buddaraja Kalka	00 to 7.5×10^3	00 to 5.0×10^2
Chandra Kalka	1×10^2 to 6.1×10^4	00 to 1.3×10^3
Desadun Kalka	00 to 3×10^2	00 to 1.8×10^4
Nawaratna Kalka	6.0×10^3 to 1.2×10^7	4.8×10^5 to 2.0×10^6
Sarkaradi Kalka	1.0×10^3 to 1.7×10^6	00 to 4.0×10^4

(Contd)

	Range for bacterial counts /gr	*Range for fungi counts /gr*
Vatika (Pills)		
Seetharama Vati	00 to 7×10^3	00 to 1.5×10^4
Jeevananda Vati	00	00
Suranvidura Vati	00	00
Lepa (Local applications)		
Dasanga Lepa	9.0×10^2 to 2.1×10^5	80 to 1.3×10^4
Sothahara Lepa	00	00
Rogan E khas	00	00
Thaila (Oil)	*Range for Bacterial counts /ml*	*Range for Fungi counts /ml*
Pinda oil	00 to 59	00 to 10^5
Narayana oil	00 to 23	800 to 54
Sarvavisadee oil	00 to 26	00 to 26
Visarpahara oil	00 to 41	00 to 71
Vathaviduranga oil	00 to 17	00 to 47
Arista (Fermented decoctions)		
Dasamoola Arista	00 to 3.4×10^3	00 to 4.8×10^2
Pippalyadi Asawa	4×10^1 to 1.3×10^4	00 to 1×9
Aravindasava	00 to 1.6×10^4	00 to 4.8×10^2
Sarasvatharista	00 to 2.7×10^5	00 to 3.5×10^1
Draksa Arista	00 to 1.1×10^5	00 to 4.5×10^1

Choorna (Powders)

WHA limitations for microbial contamination – Total aerobic bacteria = 10^5, yeast and moulds maximum 10^3 per gram. All the powder preparations exceeds the WHA limitations for the microbial load. Colony forming units (CFU) per gram for bacteria varies from 3×10^3 to 6.2×10^7. Colony forming units for fungi varies from 1×10^0 to 1.7×10^6.

Kalka (Paste)

According to Table 1, only Buddharaja Kalka is below the WHA limitations in both bacterial and fungal counts. Chandra Kalka and Desadun Kalka were under the limits in bacterial counts but exceeds in fungal counts. Nawaratna Kalka and Sarkaradi Kalka preparations exceeds the WHA limitation for both bacterial and fungal counts. Nawaratna Kalka shows the highest range of microbial load. The microbial load in confections for bacteria per gram varies from 1×10^0 to 7.5×10^3. The microbial load for fungi in confections varies from 1×100 to 1.2×10^7.

Thaila (Oil)

All these Oil preparations were below the WHA limitations. In oil samples the colony forming units for bacteria per ml was 1×10^0 to 5×10^1 and fungi was 1×10^0 to 10^2.

Vatika (Pills)

According to the above range Jeevananda Vati and Suranvidura Vati were totally free from microbial contaminations. Only Seetharama vati exceeds the microbial load in fungal count.

Lepa (Topical applications)

Lepa preparations are topical dosage forms. The WHA limitations for bacteria and fungi differ from the internal dosage forms. Aerobic bacteria maximum – 10^7 per gram and yeast and moulds, maximum 10^4 per gram. According to this range all three Lepa preparations were below the WHA limitations for bacterial counts. But Dasanga Lepa preparation was exceeds the fungal counts. The microbial load for Bacteria on Dasanga lepa varies from 1×10 to 2.1×10^5 per gram. Fungi varies from 8×10^1 to 1.3×10^4 per gram Sothahara Lepa and Rogan e ghas preparations does not show any growth.

Arista (Fermented decoction)

According to the above table Dasamoola Arista, Pipalyadi Asawa and Aravinda Asawa were below the WHA limitations in the microbial load. Saraswatha Arista and Draksa Arista are exceeding the WHA limitations in bacterial counts but both were below the WHA limitations in fungal count. The microbial load varies from 1×10^0/ml to 2.7×10^5 /ml in Nutrient agar and 1×10^0/ml to 5×10^2/ml in potato dextrose agar.

Results of the biochemical tests on the different formulations

Most of the bacterial stock cultures belonged to the following Bacillus species (Table 2).

Table 2: Results of the biochemical tests on the different formulations

Thalisadee Choorna	*Bacillus brevis, Bacillus megaterium, Bacillus pumilus*
Dathree Choorna	*Bacillus brevis, Bacillus laterosporus, Bacillus ceres, Bacillus pumilus, Bacillus licheniformis, Kurthia, Bacillus firmus*
Hinguastaka Choorna	*Bacillus cereus, Bacillus brevis, Bacillus firmus, Entrobacter spp, Bacillus licheniformis*
Manibadra Choorna	*Bacillus megaterium, Bacillus brevis, Bacillus pantothenticus, Bacillus pumislus*
Thriphaladi Choorna	*Bacillus firmus, Bacillus brevis*
Buddharaja Kalka	*Bacillus brevis, Entrobacter spp*
Chandra Kalka	*Bacillus pantothenticus, Bacillus brevis, Bacillus firmus*
Desadun Kalka	*Bacillus pantothenticus, Flavobacterium*
Nawaratna Kalka	*Bacillus Megaterium, Entrobacteria, Pseudomonas, Entrobacter spp*
Sarkarade Kalka	*Bacillus firmus, Bacillus brevis, Bacillus Megaterium, Flavobacterium*
Narayana Oil	*Bacillus pantothenticus*
Pinda Oil	*Bacillus firmus, Bacillus brevis, Bacillus pumilus*
Sarvavisadee Oil	*Bacillus brevis*
Vathaviduranga Oil	*Bacillus pantothencius, Bacillus firmus, Streptococcus*
Visarpahara Oil	*Bacillus pantothenticus*
Dasamoola Arista	*Bacillus firmus*
Pippalyadi Asawa	*Bacillus firmus*
Draksa Arista	*Bacillus brevis, Bacillus firmus*
Aravinda Asawa	*Bacillus firmus, Bacillus brevis*
Saraswatha Arista	*Bacillus firmus*
Dasanga Lepa &Sothahara Lepa	*Staphylococcus, Bacillus brevis*

According to the macroscopic and microscopic appearance the isolated fungi were identified as Penicillium Sp, Aspergillus Sp, Mucor Sp, Rhizopus Sp, Fusarium Sp.

Results of the *Salmonellae* tests

None of the drug samples was positive for *Salmonellae*. Except three samples of Thalisadi Choorna and two samples of Chandra kalka preparations.

Results of the Coloiform tests

None of the Choorna, Kalka, Arista, Vatika and Thaila gave positive results for Coliforms in presumptive test. However, some of the Dasanga lepa preparations gave positive results in presumptive tests and test for faecal coliform confirmation tests.

Results of the antimicrobial activity

Choorna (Powders), Kalka (Pastes), and Thaila (Oil) preparations were subjected to this study. Powder preparations of Hinguastaka, and Manibadra showed anti bacterial effect on *Salmonellae typhi*. Thripaladi Choorna showed anti bacterial effect on *Pseudomonas aeruginosa* None of the tested powder preparations showed anti-bacterial effect on *E-Coli*. The Oil preparation of Sarvavisadee is active against *Pseudomonas aeruginosa*, *Staphylococcus aureas*, *Salmonellae typhi*, and *Klebsiella*. The preparation of Buddaraja kalka was active against *Pseudomonas aeruginosa*, *Staphyloccous aureas*, and *Bacillus cereus*.

Results of the HACCP analysis

According to the microbiological studies carried out on products, the product of Thalisadi Choorna of every manufactures relatively contained more microbial load and other contaminations. A flow chart of the product was prepared and the following critical points, i.e. C.P. 01 – Out dated or wrong raw materials, C.P. 02 – Impurities (Soil particles etc.), C.P. 03 – Adulterants and C.P. 04 – Improper Washing of raw materials and packaging materials were identified. A test sample was prepared according to identified critical points in order to minimize the contamination and to reduce the microbial load. Again the microbial load was studied in the test product.

It was revealed that the microbial load was very low in the test product. This study clearly indicates that the implementation of good manufacturing practice regulations will lead to microbiologically safe end products.

Results of the quality assurance testing

Steam treatment for powder preparations for a short time period consecutively for three days showed a very low microbial load. Same time it does not bring a major effect on quality of the product.

Conclusions

The above study reveals that most of the powder preparations of every manufacturer were above the acceptable limits of microbial load. Adopting good manufacturing practice can reduce the microbial load and exclude the contaminations. Most of the Oil preparations and Vatika preparations are within the acceptable limits. Most of the Kalka preparations (except the Buddharaja Kalka) were also above the acceptable limits of microbial load. The individual data analysis shows the Dasamoola Arista, Pippalyadi Asawa and Arawinda Asawa preparations were below the acceptable limits. The microbial load on Lepa preparations were below the acceptable level. The positive results for specific micro organisms may be due to poor GMP. HACCP analysis confirmed improving the GMP will ensure the microbiologically safe end products. Steam treatment may be an effective method to reduce the microbial load. Studies on the antimicrobial activity highlight the effectiveness of these preparations in common infective conditions.

Recommendations

1. Educational programmes on G.M.P for the factory workers,
2. Educational programme should be repeated periodically,
3. Separate building sites for pre preparation stores and drug manufacturing sites,
4. These sites should be restricted for other workers in the factory,
5. Quality check on raw materials should be performed before sending for production,
6. Quality checkup on factory water resources should be performed periodically,
7. Preparation of raw materials for drug manufacture should be monitored by a medically qualified trained person,
8. Quality control laboratory with basic infra structure facility should be established in every commercial drug manufacturing establishment,
9. Quality check-up should be performed in between the production line,
10. Packaging materials should be subjected to quality check-up prior to packing,
11. Batch re-call facility is necessary between the production line and from the market [5, 6].

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