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Systemic validation of mono-herbal Siddha formulation *Kanduparangi chooranam* through ICP-OES, GC-MS and microbial contamination analysis

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Abstract

Introduction: Standardization of the Siddha drugs is the process that enhances the quality of the drug before entering into industrial purposes in pharmacological and pharmaceutical sciences. The reports from the instrumental analysis give adequate details to claim the therapeutic efficacy of the drugs without any hazardous effects which are already quoted in the Siddha classical textbooks.

Materials and methods: The drug *Kanduparangi chooranam* was analyzed by the following methods, Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), total and specific microbial contamination test and Gas Chromatography-Mass Spectroscopy (GC-MS).

Results and discussion: ICP-OES results of *Kanduparangi chooranam* showed that the toxic heavy metals like As, Cd, Hg and Pb were Below Detection Limit (BDL). The total viable aerobic bacterial count and total viable fungal count of *Kanduparangi chooranam* showed that $9.2x\ 10^2$ and $12.3\ x\ 10^2$ respectively detected level contamination were under the limits value of safety as per WHO guidelines. Specific microbial contamination test of *Kanduparangi chooranam* showed that there was no growth of the following microorganisms, *Salmonella* species, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. GC-MS analysis revealed that there was no trace of chemical compounds of Organo-pesticide residues (Chlorine, Phophorus & Pyrithroids) and AflatoxinsB_1, B_2, G_1 and G_2.

Conclusion: *Kanduparangi chooranam* which was prepared from purified roots of *Pygmaeopremna herbacea* in a proper way as per the Siddha classical method, is useful for treatment purposes devoid of toxic heavy metals, microbes, pesticides and aflatoxins.

Keywords: *Kanduparangi chooranam; Pygmaeopremna herbacea;* ICP –OES; Microbial contamination; GC-MS; Drug standardization

1. Introduction

Standardization of the Siddha drugs is the process that enhances the quality of the drug before entering into industrial purposes in pharmacological and pharmaceutical sciences. The reports from the instrumental analysis give adequate

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details to claim the therapeutic efficacy of the drugs without any hazardous effects which are already quoted in the Siddha classical textbooks [1].

Herbal raw drugs that are collected or purchased from various regions for the preparation of therapeutic drug formulation may be contaminated with pesticide residues like Phosphorus, Chlorine and Pyrithroids, heavy metals like Lead, Mercury, Arsenic and Cadmium, aflatoxins B_1, B_2, G_1 and G_2, and microbes like bacteria and fungi. So, standardization is necessary to avoid these harmful particulars from the drug formulations [2].

2. Materials and methods

2.1. Source of the raw material for the preparation of the drug

The root part of *Pygmaeopremna herbacea* (Roxb.) Moldenke (*Kanduparangi ver)* was purchased from a well-reputed country shop named *M.Gopala Asan Naatu Marunthu Kadai* in Nagercoil, Tamilnadu, India.

2.2. Authentication of the raw drug

The root sample was found and authenticated by Dr. S. Sutha, Ph.D., Head of the Department of Medicinal Botany, Government Siddha Medical College, Palayamkottai.

2.3. Purification of the raw drug

The root of the *Pygmaeopremna herbacea* (Roxb.)Moldenke was purified according to the proper procedure as per classical Siddha literature by removing the adulterants and cutting them into small pieces, drying them in the shade and then collecting them.

2.4. Preparation of the drug

The purified raw drug was dried well in shadow and made into micronized powder based on the Siddha literature, *Gunapadam I -Mooligai vaguppu*[3]. Finally, the powder was sieved using pure white cloth which is mentioned as *Vasthirakayam* in Siddha.

Table 1 Kanduparangi (*Pygmaeopremna Herbacea*) - A review

Sl. No	Comman name Tamil/ English	Botanical name/ Family	Phyto-Chemistry	Pharmacological actions	Usage in Siddha[3]
1	*Kanduparangi* [Blue glory or Beetle killer]	*Pygmaeopremna herbacea (*Roxb.) Moldenke / Verbenaceae	Acid resin Icetexane Sirutekkone (Bharangin) Scutellarein	Antipyretic Antimicrobial Analgesic Anticonvulsant Antiasthmatic Antioxidant Anti carcinogenic Antitumor Antiulcer Hepatoprotective Stimulant Sedative Laxative	Vatha, pitha and kaba disorders, bronchial asthma, fever, mental disorders, sinusitis, burning pain, chronic rheumatism, general body pain, fever with chills and hallucinations

2.5. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) Analysis[4,5]

ICP-OES is an analytical technique used for the detection of trace metals. The intensity of this emission is indicative of the concentration of the elements within the drug. Specification of Siddha, Ayurveda and Unani products intended for exports:

BDL- Below Detecting Limit, 1% = 10000 ppm, (1 ppm = 1/1000000 (or) 1 ppm = 0.0001%)

Table 2 Toxic heavy metals and the permissible limits as per WHO & FDA[5]

Sl. No	Heavy metals	WHO & FDA permissible limits
1.	Arsenic (As)	10ppm
2.	Mercury (Hg)	1ppm
3.	Lead (Pb)	10ppm
4.	Cadmium (Cd)	0.3ppm

2.6. Total Microbial Contamination Analysis[5]

2.6.1. Evaluation of Total Aerobic Bacterial Count

Preparation of Sample for Experimental Work

Weighed 10 gm of the drug sample aseptically and dissolved in 10 ml of sterile water and made up to 100 ml with the sterile water. The insoluble drug product was suspended in 100 ml of buffered sodium chloride-peptone solution (pH 7.0).

Serial dilution of Sample

A serial dilution is the dilution of a sample, in 10-fold dilutions. From the sample, 1 ml of the sample was added to 9 ml of sterile distilled water and mixed well. This dilution was denoted as 10^{-1} dilution. From this dilution, one ml was taken from that mixture added to 9 ml, and designated as 10^{-2} dilution. The same procedure was repeated up to 10^{-4}.

2.6.2. Isolation of Total Viable Aerobic Microbial Count

Isolation of Bacteria by Plate Count Method

In this test, the bacteria in the sample were made to grow as colonies, by inoculating a known volume of the sample into a solidifiable nutrient medium (Casein Soybean Digest agar or Nutrient agar medium) in a Petri dish. The agar plate was prepared by mixing growth medium with agar and then sterilized by autoclaving. Once the agar was cooled to 45 ^{o}C, approximately 15 to 20 ml of medium was poured into a sterile Petri dish under aseptic condition and left to solidify for 15 minutes.

After solidification, each plate was smeared with 0.1 ml of sample from the dilution of 10^{-1} and 10^{-2}. After inoculations, all the plates were incubated at 37 ^{o}C for 24 hours. After incubation, the bacterial colonies were developed as visible to the naked eye and the number of colonies on a plate was counted using the Quebec Colony Counter. Plates with an average of 30 to 300 colonies of the target bacterium were selected for colony count. Because of the statistical problems, plates with lower than 30 colonies greater than 300 colonies were rejected.

Composition of Nutrient Agar Media

- Peptone : 5 gms
- Sodium chloride :5 gms
- Beef extract: 1.5 gms
- Yeast extract: 1.5 gms
- Agar: 5 gms
- Distilled water: 1000 ml
- pH: 7.4 $\pm$ 0.2

Isolation of Fungi

From each of the above-prepared samples, 0.1 ml of sample was transferred to Sabouraud Dextrose agar (SDA) prepared with Chloramphenicol. The plates were then incubated for 5 days at room temperature (20 to 25 ^{o}C). After incubation, the fungal colonies were observed and calculated.

Composition of Sabouraud Dextrose agar (SDA):

- Dextrose: 40 gms
- Peptone: 10 gms
- Distilled water: 1000 ml
- Agar: 15 gms

2.7. Specific Microbial Contamination Analysis

Preparation of drug extract solutions for the experiment

The drug was weighed and dissolved in sterile distilled water to prepare appropriate dilution to get the required concentrations of about 10, 20 and 30µg/ml. They were kept under refrigerated conditions unless they were used for the experiment.

Bacterial Inoculums Preparation

Inoculums of *Salmonella* species, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were prepared in nutrient broth medium and kept for incubation at 37 ^{o}C for 8 hrs.

2.7.1. Isolation & Identification of Escherichia coli

One ml of the prepared sample was added to a sterile screw-capped container containing 50 ml of nutrient broth and mixed well. Then, it was allowed to stand for 1 hour and mixed well again. After one hour, the screw caps of the bottle were loosened and incubated at 37 ^{o}C for 18 to 24 hours.

Primary Test

From the above-prepared enrichment culture, 1.0 ml was taken and transferred aseptically into a tube containing 5 ml of Mac-Conkey broth. Inoculated tubes were incubated in a water bath at 36 ^{o}C to 38 ^{o}C for 48 hours.

Secondary Test

From the primary test, 1.0 ml of the enrichment culture was taken and transferred aseptically into 5 ml of peptone water. It was then incubated in a water bath at 43.5 ^{o}C to 44.5 ^{o}C for 24 hours and the tubes were observed for acid and gas. Then, the culture was subjected to biochemical tests of IMViC and the results were observed and correlated.

Alternative test

It was done by a loop full of enriched culture in the primary test streaked on a sterile Mac-Conkey agar medium. Then, the plates were inverted and incubated at 37 ^{o}C for 24 hours. After incubation, the pink or brick red color colonies were examined and transferred individually onto the surface of Eosin Methylene Blue agar medium (EMB), on Petri dishes. Inoculated plates were inverted and incubated at 37 ^{o}C for 24 hours. After incubation, the colonies on the medium were checked for their color appearance like a green metallic sheen under reflected light. The colonies were subjected to confirmation by further suitable cultural and biochemical tests.

Components of Eosin Methylene Blue Agar Media

- Pancreatic digest of gelatine: 10.0 gms
- Dibasic potassium phosphate : 2.0 gms
- Lactose : 10.0 gms
- Eosin Y : 400 mgs
- Methylene blue: 65 mgs
- Agar: 15.0 gms
- Distilled water: 1000 ml

2.7.2. Isolation and Identification of Salmonella sp

One ml of the prepared sample was added to a sterile screw-capped container containing 100 ml of nutrient broth and mixed well. Then, it was allowed to stand for 1 hour and mixed well again. After one hour, the screw caps of the bottle were loosened and incubated at 37 ^{o}C for 18 to 24 hours.

Primary Test

From the above-prepared enrichment culture, 1.0 ml was taken and transferred aseptically into a tube containing 10 ml of Selenite F broth. Inoculated tubes were incubated in a water bath at 36 °C to 38 °C for 48 hours. After incubation, the culture was subcultured on two of the agar media namely Bismuth sulphite agar and Deoxy cholate citrate agar and incubated the plates at 36 °C to 38 °C for 18 to 24 hours. After incubation, colonies were observed on the medium and confirmed the genus *Salmonella* based on guidelines.

Secondary test:

The suspected colonies of the primary test were subcultured on the slant of triple sugar-iron agar in a test tube and urea broth. Both media were incubated at 37 °C for 24 hours. After incubation, the results were observed according to the development of color change and acid/gas in media. The presence of *Salmonella* was confirmed by agglutination tests.

Composition of Salmonella Shigella Agar Media

- Beef Extract : 5.0 gms
- Enzymatic Digest of Casein: 2.5 gms
- Enzymatic Digest of Animal Tissue: 2.5 gms
- Lactose : 10 gms
- Bile salts: 8.5 gms
- Sodium Citrate: 8.5 gms
- Ferric Citrate: 1.0 gms
- Brilliant Green: 0.00033 gms
- Neutral Red: 0.025
- Agar: 13.5 gms
- Distilled water: 1000 ml

2.7.3. Isolation and Identification of Pseudomonas aeruginosa

From the above-prepared enrichment culture, 1.0 ml was taken and transferred aseptically into 100 ml of fluid soybean-casein digest medium and mixed well. The inoculated tubes were incubated at 37 °C for 24 hours. After incubation, the growth of bacteria was checked. From this, a loop full of culture was streaked on the surface of the Cetrimide agar medium and *Pseudomonas* Isolation Agar medium and incubated at 37 °C for 24 hours. After incubation, the colonies from the agar surface of these two media were checked for detection of fluorescein and pyocyanin.

Composition of Cetrimide Agar Media:

- Pancreatic digest of gelatin : 20.0 gms
- Magnesium chloride: 1.4 gms
- Potassium sulphate: 10.0 gms
- Cetrimide: 0.3 gms
- Agar : 13.6 gms
- Glycerin : 10.0 gms
- Distilled Water : 1000 ml

2.7.4. Isolation and Identification of Staphylococcus aureus

From the above-prepared enrichment culture, a loop full of culture was taken and transferred aseptically on Mannitol salt agar and incubated at 37 °C for 24 hours. After incubation, the colonies were subjected to confirmation by a hemagglutination test.

Composition of Mannitol Salt Agar Media

- Pancreatic digest of gelatin : 5.0 gms
- Beef extract: 1.0 gms
- Agar: 15.0 gms
- D-Mannitol: 10.0 gms
- Peptic digest of animal tissue: 5.0 gms
- Sodium chloride : 75.0 gms
- Phenol red: 25 mgs

- Distilled Water: 1000 ml

2.8. Gas Chromatography– Mass Spectroscopical Analysis[5]

Methodology

Samples were prepared with respective solvents for GC-MS analysis.

- Agilent Technologies: GC-MS
- GC System: 7820 A
- MSD: 5977 E
- Column :DB-5
- Detector : MS
- Flow rate: 1.2
- Carrier gas : Helium
- Over Temp: 100 ^{o}C -270 ^{o}C(10 ^{o}C/min)

3. Results and discussion

3.1. ICP-OES Analysis of *Kanduparangi chooranam*

Sample ID: *Kanduparangi chooranam* - (Wt : 0.50100 g)

Table 3 ICP –OES analysis of *Kanduparangi chooranam*

S.No	Elements Symbol	Wavelength (nm)	Concentration
1.	As	188.979	BDL
2.	Cd	228.802	BDL
3.	Hg	253.652	BDL
4.	Pb	220.253	BDL

3.1.1. Inference

ICP-OES results of *Kanduparangi chooranam* from the table. 3. showed that the heavy toxic metals like As, Cd, Hg and Pb were Below Detection Limit (BDL).

3.2. Total Microbial Contamination Test of *Kanduparangi chooranam*

Table 4 Results of the Total Microbial Contamination Test of *Kanduparangi chooranam*

Sl. No	Test Particulars	Colony Counts (CFU/ g)	Limits Value (CFU/g)
1.	Total Viable Aerobic Bacterial Count	9.2 x 10^2	1 x 10^5
2.	Total Viable Fungal Count	8.3 x 10^2	1 x 10^3

3.2.1. Inference

The total viable aerobic bacterial count and total viable fungal count of *Kanduparangi chooranam* from the table.4. showed that 9.2x 10^2 and 12.3 x 10^2 respectively detected level of contamination were under the limits value of safety as per WHO guidelines.

3.3. Specific Microbial Contamination Test of *Kanduparangi chooranam*

Table 5 Results of Specific Microbial Contamination Test of *Kanduparangi chooranam*

Sl. No	Test for Specified Pathogens	Colony Counts (CFU/ g)	Limits Value (CFU/g)
1.	*Escherichia coli*	No growth	-
2.	*Salmonella* sp.	No growth	-
3.	*Pseudomonas aeruginosa*	No growth	-
4.	*Staphylococcus aureus*	No growth	-

3.3.1. Inference

Results of specific microbial contamination test of *Kanduparangi chooranam* from the table.5. showed that there was no growth of the following microorganisms, *Salmonella* species, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*.

3.4. Gas Chromatography– Mass Spectroscopical Analysis of *Kanduparangi chooranam*

Table 6 Results of GC –MS analysis of *Kanduparangi hooranam*

Sl. No	Chemical Compounds	Inference
1.	Aflatoxin B_1	Not detected
2.	Aflatoxin B_2	Not detected
3.	Aflatoxin G_1	Not detected
4.	Aflatoxin G_2	Not detected
5.	Organo-pesticide residues (Chlorine, Phophorus & Pyrithroids)	Not detected

3.4.1. Inference

Results of GC-MS analysis of *Kanduparangi chooranam* from the table.6. revealed that there was no trace of chemical compounds of Organo-pesticide residues (Chlorine, Phophorus & Pyrithroids) and Aflatoxins B_1, B_2, G_1 and G_2.

4. Conclusion

The results of this study showed that the mono-herbal Siddha formulation *Kanduparangi chooranam* that was prepared from purified roots of *Pygmaeopremna herbacea* in a proper way as per the Siddha classical method, is useful for treatment purposes in the following diseases like vatha, pitha and kaba disorders, bronchial asthma, fever, mental disorders, sinusitis, burning pain, chronic rheumatism, general body pain, fever with chills and hallucinations, which is devoid of toxic heavy metals, microbes, pesticides and aflatoxins.

Compliance with ethical standards

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Disclosure of conflict of interest

The author hereby declares that he has no conflicts of interest to disclose.

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