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Quality Control, Safety Evaluation, and Microbial Assessment of Siddha Formulation *Athiyadhi Kashayam*

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KEYWORDS:

Athiyadhi Kashayam, Siddha formulation, Aflatoxin, Pesticide residue, Sterility test

ABSTRACT:

Athiyadhi Kashayam (AYK), is a potent Siddha formulation which is widely used as an anti-diabetic medicine in treating diabetes. For clinical application ensuring its safety, quality and microbial integrity is essential. The current study aimed to determine the physicochemical characteristics, elemental composition, safety profile and microbial quality of AYK. The SEM-EDX analysis showed the presence of essential elements like oxygen, sodium, magnesium, iron, calcium and zinc indicating its mineral composition. The Aflatoxin analysis carried out using TLC revealed the absence of aflatoxins (B1, B2, G1, G2). The Pesticide residue analysis demonstrated that all the tested compounds were below quantification limits, which is in line with AYUSH standards. Microbial evaluation including sterility testing showed no detectable bacterial or fungal growth and specific pathogen analysis revealed the absence of pathogens such as E. coli, Salmonella, Staphylococcus aureus, and Pseudomonas aeruginosa. From the above findings it is confirmed that AYK meets safety and quality standards and is free from microbial contamination and toxic residues supporting its safe beneficial application in Siddha system.

1. Introduction

Traditionally over the decades, Siddha medicine is well established in South India particularly in Tamil Nadu. It is attributed to the ancient Siddhars, who highlighted holistic approach by integrating spirituality, lifestyle, diet, and pharmacotherapy for treating diseases. The basic principle of Siddha medicine follows three vital humors – *Vatham, Pitham* and *Kabham* which regulates the physiological and pathological processes in the human body. The imbalance of these humors leads to pathological conditions. Siddha medicines focus not only on disease management but also on rejuvenation, restoration of systemic balance and preventive care(*1 et al. 2019; Deep et al. 2013; Dr.R.S. Lekshmi1, Dr.K. Jawaharrani2, Dr.J.Srilekha3 2023; Muthiah et al. 2019; Saha et al. 2015).

Among many potential Siddha formulations, the *Athiyadhi Kashayam* which is composed of *Ficus racemosa, Cassia auriculata, Cassia fistula, Syzygium cumini, Salacia reticulata.* It was formulated based on the literature available from *Mega Nivarana Bodini Ennum Neerizhivu Maruthuvam* (Hakkim P.M. Abdulla Sayabu 1998; Meenakshi C1* 2024). From our previous studies it is evaluated that, AYK has antioxidant, anti-diabetic activity through *in silico* and *in vivo* studies

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(Meenakshi et al. 2025; Meenakshi C 2024; Meenakshi C1* 2024).

Although Siddha formulations are of natural origin, they are not inherently free from environmental contaminants such as aflatoxins, pesticide residues, and microbial pathogens. These contaminants represent a critical concern in practicing the traditional medicine like Siddha which may compromise both safety and therapeutic efficacy(B et al. 2022; Sasikala L, Janani A M 2024).

Aflatoxins, a set of extremely toxic secondary metabolites, produced by fungi such as *Aspergillus flavus* and *Aspergillus parasiticus*, constitute one of the most toxic groups of naturally occurring mycotoxins. These metabolites have often been linked to inappropriate storage of herbal raw materials, especially under high humidity and temperature. The health risks associated with aflatoxin include hepatotoxicity, immunosuppression, mutagenicity, and carcinogenicity, for which aflatoxin B_1 is classified as a human carcinogen of Group I. From the perspective of Siddha medicine, excessive consumption of these contaminated Siddha medicine preparations could result in cumulative toxicity, for which routine screening is essential.

Likewise, pesticide residues are another significant source of contamination. Medicinal plants used in Siddha medicine are often grown in areas where they are likely to be contaminated with agrochemicals like organophosphates, organochlorines, and pyrethroids. Accumulation of these residues can occur during harvesting and processing stages, which can finally result in the formulation. Long-term exposure to pesticide residues can lead to neurotoxicity, endocrine toxicity, reproductive toxicity, and carcinogenesis. Therefore, it is imperative to determine pesticide residues in herbal medicines to ensure compliance with international safety standards.

Besides chemical contaminants, microbial pathogens are also an important risk factor associated with herbal medicines. Microbial contamination may occur at various stages of herbal medicines, namely, during the collection, handling, processing, and storage of the herbal drugs. Microorganisms like bacteria (Escherichia coli, Salmonella species), fungi, and yeast are the common contaminants of herbal medicines. Such contaminants may cause spoilage of the herbal drugs, thereby affecting the shelf life of the drugs. In addition, the microorganisms may cause infections, especially among immunocompromised patients. In addition, the microorganisms may also produce toxic metabolites. Therefore, the microbiological quality of Siddha medicines is assessed by conducting tests like total viable count, detection of pathogens, and sterility tests.

With the inclusion of these quality control practices along with physicochemical and elemental analysis, the efficacy and reproducibility of traditional formulations can be ensured. This not only guarantees the safety and efficacy of traditional medicine for patients but also helps in the universal acceptance and scientific validation of Siddha medicine for modern-day healthcare practices.

The objective of the present study was to evaluate the quality, safety, and micro bio status of AYK using SEM–EDX analysis, aflatoxin detection, pesticide residue analysis, and microbiological analysis as per AYUSH and WHO guidelines.

2. Materials and Methods

2.1 Sample

Athiyadhi Kashayam (AYK) was obtained and subjected to various analytical procedures.

2.2 SEM–EDX Analysis

SEM–EDX analysis was performed using an EDAX-TSL (AMETEK) scanning electron microscope at an accelerating voltage of 20 kV. The sample was mounted on carbon tape and sputter-coated prior to analysis to enhance conductivity and image clarity.

2.3 Aflatoxin Analysis

Aflatoxin analysis was carried out using Thin Layer Chromatography (TLC). Standard aflatoxins and test samples were applied to pre-coated TLC plates and developed in a solvent system (chloroform: acetone: isopropyl alcohol, 85:10:5). The plates were examined under UV light at 365 nm (Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025).

2.4 Pesticide Residue Analysis

The sample was extracted using acetone followed by filtration and concentration using a rotary evaporator. The residue was reconstituted in toluene and analyzed for

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pesticide residues including organochlorine, organophosphorus, carbamates, and pyrethroids (Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025).

2.5 Sterility Test (Pour Plate Method)

The sterility of the formulation was evaluated using the pour plate method. The sample was inoculated into sterile Petri dishes followed by addition of molten agar at 45°C. Plates were incubated at 37°C for 24–48 h and extended up to 72 h for fungal observation. Colony forming units (CFUs) were assessed (Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025).

2.6 Test for Specific Pathogens

The sample was inoculated into selective media: EMB agar (*E. coli*), Deoxycholate agar (*Salmonella*), Mannitol salt agar (*Staphylococcus aureus*), Cetrimide agar (*Pseudomonas aeruginosa*). The plates were incubated at 37°C for 24–72 hours and observed for characteristic colony formation (Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025).

3. Results

3.1 SEM–EDX Analysis

Elemental analysis of the Siddha medicine formulation AYK was carried out using EDX to determine the mineral profile and toxicological potential (Fig 1a & 1b). The findings indicated that oxygen (O) was the predominant element in the Siddha medicine formulation, making up 55.55% of the weight percentage and 70.70% of the atomic percentage of the formulation. This is expected since oxygen is a common constituent of organic compounds such as polyphenolic compounds, flavonoids, and carbohydrates.

Among the macro-elements, silicon (Si) (10.21%), calcium (Ca) (9.61%), sodium (Na) (6.67%), and magnesium (Mg) (6.92%) were present in appreciable quantities. The presence of chlorine (Cl) (5.58%) and sulfur (S) (1.72%) further indicates the presence of bioactive compounds, since the presence of sulfur-containing phytochemicals is associated with antioxidant and antimicrobial potential as seen in Table 1. Trace elements such as iron (Fe) (2.74%) and zinc (Zn) (0.14%) were also identified in the formulation. Most importantly, the analysis for potential toxic heavy metals such as arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) was also conducted. These toxic metals were identified to be either absent or present in negligibly low quantities, indicating that the formulation is relatively safe in terms of potential toxic metal contamination. Minor negative or near-zero values for some of the identified metals, such as Cd and Pb, can be considered due to instrumental noise or detection limits associated with EDX analysis. Based on the elemental profile identified in the *AYK* formulation, it is evident that the formulation is safe and relatively free from potential toxic metal contamination (Dr.M.Shopika and Dr.A.Ganesan 2025; Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025; Saranya 2025).

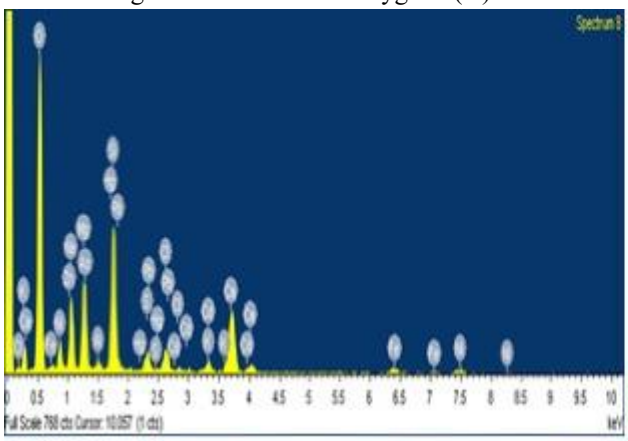

Fig 1a. SEM-EDX analysis of the sample AK

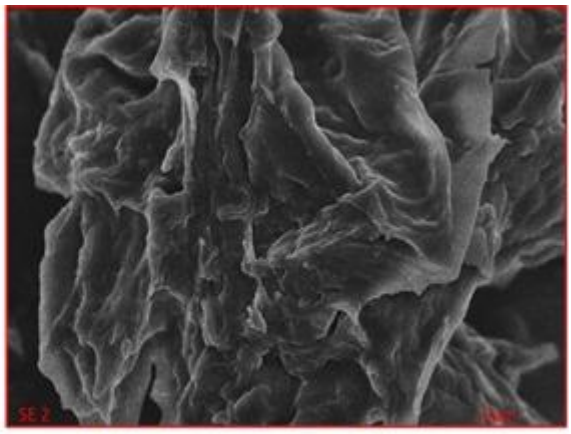

Fig 1b. SEM image of AK – Cluster

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Table 1. Elemental Peak Table obtained from SEM-EDX showing the relative weight percentage and atomic percentage of major and trace elements

Element	App Conc	Intensity Comp	Weight %	Weight % Sigma	Atomic %
O K	1.47	1.0562	55.55	1.33	70.70
Na K	0.15	0.8969	6.67	0.45	5.91
Mg K	0.13	0.7476	6.92	0.38	5.80
Si K	0.22	0.8680	10.21	0.42	7.40
S K	0.04	0.8963	1.72	0.21	1.09
Cl K	0.05	0.7893	5.58	0.24	1.38
K K	0.03	1.0443	1.17	0.20	0.61
Ca K	0.22	0.9805	9.61	0.43	4.58
Fe K	0.06	0.8191	2.74	0.48	1.00
Zn L	0.00	0.4328	0.14	0.91	0.04
As L	0.00	0.9746	0.00	0.60	0.00
Cd L	-0.01	0.7383	-0.48	0.37	-0.09
Hg M	0.00	0.7414	0.23	0.76	0.02
Pb M	0.00	0.7192	-0.07	0.91	-0.01

Total Weight % = 100.00

3.2 Aflatoxin Analysis

Aflatoxin content in AYK formulation was detected using Thin Layer Chromatography (TLC) and compared with AYUSH specified permissible limits. Aflatoxins B1, B2, G1 and G2 are secondary metabolites of certain fungal species belonging to Aspergillus. The presence of them in Siddha formulations poses serious health risks to consumers. Aflatoxins are known to cause hepatotoxicity and carcinogenic effects in humans.

In the present study, no spots were observed in the test sample under UV light corresponding to the standards of Aflatoxin B1, B2, G1, and G2. In contrast, the standard samples of Aflatoxin B1, B2, G1, and G2 showed distinct fluorescent spots at their respective Rf values. This indicates that the levels of Aflatoxin B1, B2, G1, and G2 in the test sample are below the detection limit of the method employed in the analysis (Table 2).

The results of the analysis clearly prove that the formulation meets the permissible levels of AYUSH, which are 0.5 ppm each for Aflatoxin B1 and G1, and 0.1 ppm each for Aflatoxin B2 and G2. From the results of the analysis, it can be concluded that *Athiyadhi Kashayam* is free from Aflatoxin and can be safely consumed by the people in terms of mycotoxin levels (Dr.M.Shopika and Dr.A.Ganesan 2025; Janani AM 2025; Khan, S., Alam, S., Siddiqui 2025; Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025; Saranya 2025).

The absence of Aflatoxin in the formulation can be attributed to the proper procurement, handling, and storage of the raw herb materials, as well as good manufacturing practices.

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Table 2. Detection of aflatoxins (B_1, B_2, G_1, and G_2) in *Athiyadhi Kashayam* using Thin Layer Chromatography (TLC) compared with AYUSH permissible limits.

Aflatoxin	*Sample*	*AYK AYUSH Specification Limit*
B1	*Not detected - Absent*	*0.5 ppm (0.5mg/kg)*
B2	*Not detected - Absent*	*0.1 ppm (0.1mg/kg)*
G1	*Not detected - Absent*	*0.5 ppm (0.5mg/kg)*
G2	*Not detected0 Absent*	*0.1 ppm (0.1mg/kg)*

3.3 Pesticide Residue Analysis

The issue of pesticide residue in herbal formulations is a major safety concern, as medicinal herbs can be contaminated with agrochemicals during their cultivation, collection, and storage. In the present investigation, the pesticide residues of various classes, including organochlorine, organophosphorus, carbamate, and pyrethroids, were estimated in AYK, and the results were compared with AYUSH permissible limits.

In the analysis, all pesticide residues were found to be below the quantification limit (BQL) in the sample mentioned in Table 3. Organochlorine pesticides such as alpha, beta, gamma, and delta benzene hexachloride (BHC), DDT, and endosulfan were not detected in the sample. Organophosphorus pesticides such as malathion, chlorpyrifos, and dichlorvos; carbamate pesticide such as carbofuran; and pyrethroid pesticide such as cypermethrin were found to be below the quantification limit in the sample (Dr.M.Shopika and Dr.A.Ganesan 2025; Janani AM 2025; Khan, S., Alam, S., Siddiqui 2025; Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025; Saranya 2025).

The absence of detectable pesticide residues suggests that the formulation has conformed to the AYUSH safety standards, which have established limits ranging from 0.1 mg/kg to 3 mg/kg depending on the specific chemical compounds. These results suggest that the raw materials used for the preparation of AYK were probably grown under controlled conditions with minimum exposure to pesticides or were processed well enough to remove any trace of contaminants.

Table 3. Pesticide residue analysis of *Athiyadhi Kashayam* (AYK) showing levels of organochlorine, organophosphorus, carbamate, and pyrethroid pesticides in comparison with AYUSH permissible limits.

Category	**Pesticide**	**Result (AYK)**	**AYUSH Limit (mg/kg)**
Organochlorine Pesticides	Alpha BHC	BQL	0.1
	Beta BHC	BQL	0.1
	Gamma BHC	BQL	0.1
	Delta BHC	BQL	0.1
	DDT	BQL	1
	Endosulfan	BQL	3
Organophosphorus Pesticides	Malathion	BQL	1
	Chlorpyrifos	BQL	0.2
	Dichlorvos	BQL	1
Carbamates	Carbofuran	BQL	0.1
Pyrethroids	Cypermethrin	BQL	1

3.4 Microbial load analysis

The microbial quality of herbal preparations is one of the most important parameters to be considered for ensuring

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the safety, efficacy, and shelf life of herbal formulations. The present study aims to evaluate the microbial load and specific pathogenic organisms of *Athiyadhi Kashayam* using standard guidelines as prescribed by AYUSH. The results are depicted in Fig 2 and Table 4.

The total bacterial count and total fungal count of *Athiyadhi Kashayam* were found to be absent. This shows that the microbial load is well within the acceptable limits as prescribed by AYUSH, i.e., not more than 10^5 cfu/g for bacteria and 10^3 cfu/g for fungi. The absence of microbial growth shows that the preparation, processing, and storage of the herbal formulation have been carried out under stringent hygienic conditions (Dr.M.Shopika and Dr.A.Ganesan 2025; Janani AM 2025; Khan, S., Alam, S., Siddiqui 2025; Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025; Saranya 2025).

This is particularly important in herbal formulations, as the low or absent microbial load is important in ensuring that there is no spoilage of the product and that the product does not contain harmful bacteria and fungi, which will act as impurities and cause a loss of efficacy of the product. Some of the fungi that contaminate herbal medicines have the potential of producing mycotoxins, which are harmful and can cause health risks.

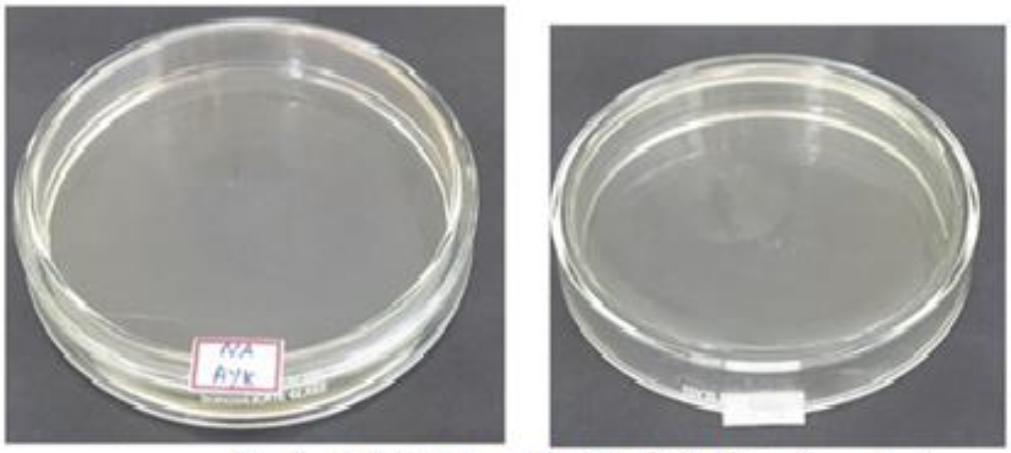

Fig 2. Petriplates of Microbial Load analysis

Table 4. Microbial Load Analysis of *Athiyadhi Kashayam*

Test	Result	Specification	As per AYUSH/WHO
Total Bacterial Count	Absent	NMT 10^5 CFU/g	As per AYUSH specification
Total Fungal Count	Absent	NMT 10^3 CFU/g	As per AYUSH specification

Specific pathogen test:

The formulation was checked for the presence of particular pathogenic microorganisms such as *Escherichia coli, Salmonella species, Staphylococcus aureus, and Pseudomonas aeruginosa* (Fig 3a-d). These pathogens are usually responsible for contamination caused by improper handling, contaminated water supply, and improper processing conditions.

The results indicated that all the pathogenic microorganisms were absent in AYK (Table 5). This indicates that the formulation is safe for use, and it follows the AYUSH safety specification. The absence of *Escherichia coli* indicates that there is no faecal matter in the formulation. The absence of Salmonella species indicates that the formulation is free from pathogens that cause severe gastrointestinal infections. Furthermore, the absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* indicates that the formulation is free from opportunistic pathogenic microorganisms that cause skin infections, respiratory infections, and systemic infections in immunocompromised individuals (Dr.M.Shopika and

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Dr.A.Ganesan 2025; Janani AM 2025; Khan, S., Alam, S., Siddiqui 2025; Raghavi Marimuthu, Abarna Balasubramani, Karpagambal Ramamoorthy, Suresh Ramasamy, Haritha Seshan, Sathish Adithya Rajathinakaran 2025; Saranya 2025).

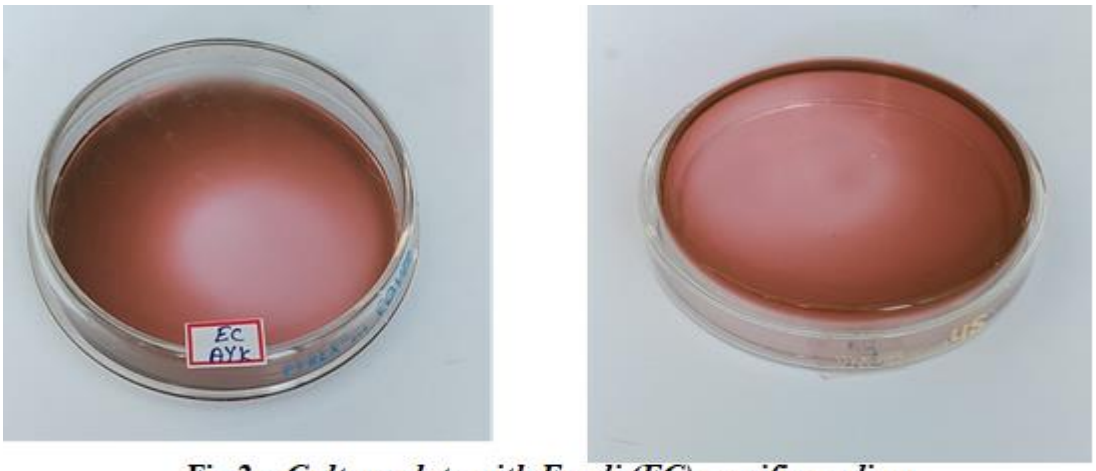

Fig 3a. ***Culture plate with E-coli (EC) specific medium***

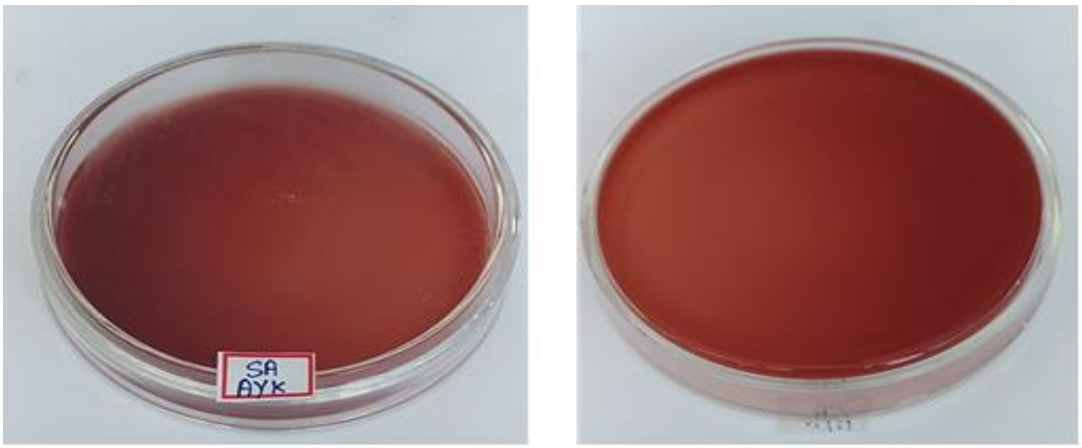

Fig 3b. ***Culture plate with Salmonella (SA) specific medium***

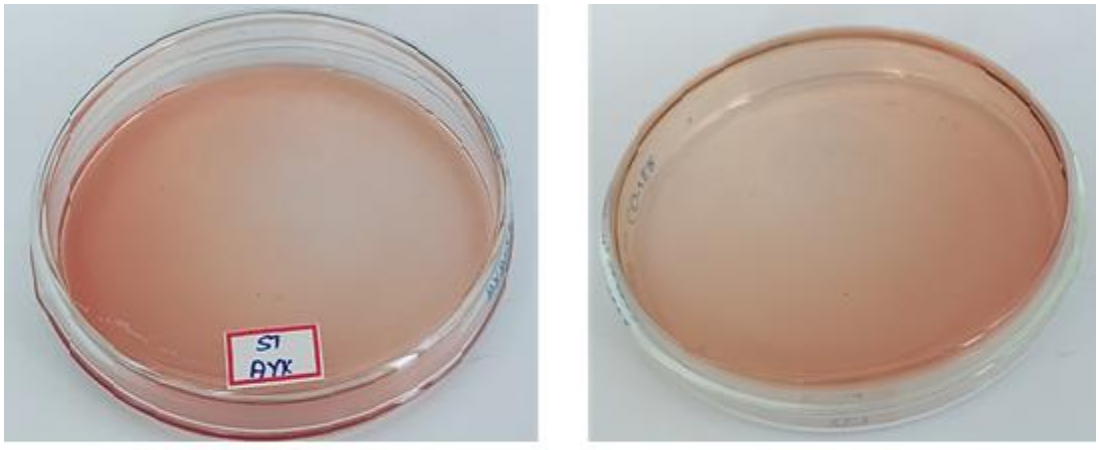

Fig 3c. ***Culture plate with Staphylococcus Aureus (ST) specific medium***

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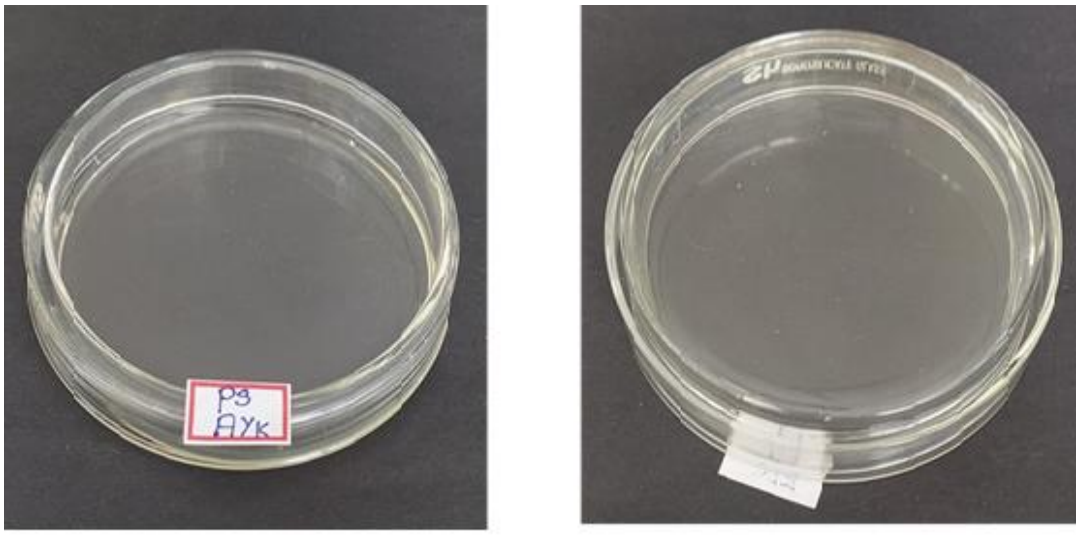

Fig 3d. ***Culture plate with Pseudomonas Aeruginosa (PS) specific medium***

Table 5. Specific Pathogen (Sterility) Test of *Athiyadhi Kashayam*

Organism	Specification	Result	Method
Escherichia coli	Absent	Absent	As per AYUSH
Salmonella	Absent	Absent	
Staphylococcus Aureus	Absent	Absent	
Pseudomonas Aeruginosa	Absent	Absent	

4. Conclusion

The current study represents a comprehensive evaluation of the safety, quality, and elemental profile of *Athiyadhi Kashayam*, as per AYUSH guidelines. The analysis for aflatoxin content confirmed the absence of these toxic compounds, thereby ensuring that the formulation is free from any risk of hepatotoxic and carcinogenic effects due to mycotoxin contamination.

In addition, the pesticide residue analysis confirmed that all the tested pesticide residues, including organochlorine, organophosphorus, carbamate, and pyrethroid classes of pesticide residues, were found to be below the quantification limit (BQL) and within permissible limits, thereby ensuring minimal risk of toxic chemical exposure.

The microbial quality analysis for AYK confirmed the absence of total bacterial and fungal load, and sterility testing confirmed the absence of specific pathogenic organisms such as *Escherichia coli, Salmonella spp., Staphylococcus aureus, and Pseudomonas aeruginosa*. This ensures that the formulation is safe, devoid of any microbial contamination, and prepared under proper hygienic conditions.

In addition, the SEM-EDX analysis confirmed the presence of essential macro- and micro-elements such as calcium, magnesium, sodium, silicon, iron, and zinc, which could have contributed to its therapeutic value. The absence of toxic heavy metals such as arsenic, cadmium, mercury, and lead is a significant finding, thereby ensuring its safety profile.

Overall, the results confirm the safety of AYK by reiterating the fact that the formulation meets the existing safety standards. In addition, the present study highlights the significance of standardization of the Siddha herbal formulation to increase the international acceptance of the herbal formulation.

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Authors contribution

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Conceptualization, Validation and formal analysis of the study were performed by Dr.S.Balamani, The original draft was prepared by Dr.M. Ramani, while writing, editing and methodology were handled by Dr.C.Meenakshi, Visualization and supervision were provided by Dr.G.Bharathkumar, and Publication support was assisted by Dr.S.Selvakumar. All authors have reviewed and approved the final version of the manuscript for publication.

Conflict of Interest

The authors declare no conflict of interest.

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