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Standardization and Anti-Microbial Evaluation of Siddha formulation *Shaya Chooranam*

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Abstract

The advantage of traditional knowledge in herbal drug development becomes relevant and useful only when the traditional processes are strictly adopted. It is well known that the medicinal activities of herbal drugs are due to the presence of various active principles or phytoconstituents. Siddha system of medicine offers greater advantage of using huge herbal wealth as primary ingredients in most of the formulations. The importance of some formulations were been documented earlier but there are large number of them remain un-explored with respect to safety and efficacy aspect. Hence the main aim of the present investigation is to standardize and to establish the monograph for the formulation *Shaya Chooranam* (SC) as per AYUSH regulatory guidelines. The results obtained from standardization and physiochemical analysis clearly reveals that the loss on drying value was 26.27%, total ash value was 2.92%, in which the water soluble ash is 16.4% and acid insoluble ash is 0.74 %.The alcohol soluble extractive value was 28.93% and water soluble extractive was 37.8%. The result of the phytochemical analysis indicates that the formulation SC shows the presence of flavonoids, glycosides, triterpenoids, phenols, tannins, saponins and carbohydrates. Results of sterility test reveals that there was no growth was observed after incubation period further test for specific pathogen reveals the absence of specific pathogens (*E-coli, Salmonella, Staphylococcus aureus* and *Pseudomonas aeruginosa*).It was observed form the results of *In-vitro* anti-microbial assay that the formulation SC possess significant antimicrobial activity against *Enterococcus fecalis, S.aereus* and exerts moderate effect against *Klebsiella*. From the data obtained from the results of the present investigation it was evident that the formulation SC complies with the regulatory standard and also possess significant anti-microbial activity against the tested microbes which could be due to presence of biologically active phytocomponents present in the formulation.

Keywords: Siddha, (K) *Shaya Chooranam*, Herbal drugs, Standardization, Physiochemical analysis, Antimicrobial activity.

1. Introduction

In recent days there is growing evidence on emergence of alternate complimentary therapy towards ailment of several dreadful diseases, further there is a huge hike in the market for herbal supplement were observed. Hence the need for more rigorous clinical and scientific research on herbal and traditional medicine is strongly advocated for larger acceptances and visibility. Traditional herbal medicines have a long history of use and are generally considered to be safer than synthetic drugs. Traditional medicine-inspired approaches remain important especially for the management of chronic diseases as well as to facilitate natural product drug discovery [1, 2].

Combinations of herbal medicines or phytochemical actives are found to be beneficial in certain diseases when given along with modern synthetic drugs [3]. However, during concurrent use with modern medicines some potential adverse reactions have been reported [4, 5]. Herbal medicines when co-administered with synthetic drugs may result in herb-drug interactions influencing bioavailability leading to adverse events [6].

Herbal medicine includes herbs, herbal materials (like plant parts) or preparations, processed and finished herbal products, active ingredients. In recent years, a huge resurgence of the use of herbal product due to the side effects of modern drugs, failure of modern therapies for against chronic diseases, and microbial resistance. It is estimated that nearly 75% of the plant based therapeutic entities used worldwide were included from traditional/folk medicine. In India, approximately 70% of modern drug are discovered from natural resources and number of other synthetic analogues have been prepared from prototype compounds isolated from plants [7-10].Hence the main aim of the present investigation is to standardize and to establish the monograph for the formulation (K) *Shaya Chooranam* as per AYUSH regulatory guidelines.

2. Materials and Methods

2.1. Standardization and Physicochemical Evaluation [11-12]

2.1.1. Percentage Loss on Drying

10gm of test drug was accurately weighed in evaporating dish .The sample was dried at 105^{o}C for 5 hours and then weighed.

Percentage loss in drying = Loss of weight of sample/ Wt of the sample X 100

2.1.2. Determination of Total Ash

3 g of test drug was accurately weighed in silica dish and incinerated at the furnace at the temperature 400 ^{o}C until it turns white in color which indicates absence of carbon. Percentage of total ash will be calculated with reference to the weight of air-dried drug.

Total Ash = Weight of Ash/Wt of the Crude drug taken X 100

2.1.3. Determination of Acid Insoluble Ash

The ash obtained by total ash test will be boiled with 25 ml of dilute hydrochloric acid for 6mins. Then the insoluble matter is collected in crucible and will be washed with hot water and ignited to constant weight. Percentage of acid insoluble ash will be calculated with reference to the weight of air-dried ash.

Acid insoluble Ash = Weight of Ash/Wt of the Crude drug taken X 100

2.1.4. Determination of Water Soluble Ash

The ash obtained by total ash test will be boiled with 25 ml of water for 5 mins. The insoluble matter is collected in crucible and will be washed with hot water, and ignite for 15mins at a temperature not exceeding 450^{o}C. Weight of the insoluble matter will be subtracted from the weight of the ash; the difference in weight represents the water soluble ash. Calculate the percentage of water-soluble ash with reference to the air-dried drug.

Water Soluble Ash = Weight of Ash/Wt of the Crude drug taken X 100

2.1.5. Determination of Alcohol Soluble Extractive

About 5 g of test sample will be macerated with 100 ml of Alcohol in a closed flask for twenty-four hours, shaking frequently during six hours and allowing it to stand for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish, and dry at 105^{o}C, to constant weight and weigh. Calculate the percentage of alcohol-soluble extractive with reference to the air-dried drug.

Alcohol sol extract = Weight of Extract/ Wt of the Sample taken X 100

2.1.6. Determination of Water Soluble Extractive

About 5 g of the test sample will be macerated with 100 ml of chloroform water in a closed flask for twenty-four hours, shaking frequently during six hours and allowing it to stand and for eighteen hours. Filter rapidly, taking precautions against loss of solvent, evaporate 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish, and dry at 105ºC, to constant weight and weigh. Calculate the percentage of water-soluble extractive with reference to the air-dried drug.

Water soluble extract = Weight of Extract/ Wt of the Sample taken X 100

2.2. Qualitative Phytochemical Analysis [13]

Test for alkaloids:

Mayer's Test: To the test sample, 2ml of mayer's reagent was added, a dull white precipitate revealed the presence of alkaloids.

Test for coumarins:

To the test sample, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of yellow color.

Test for saponins:

To the test sample, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of Saponins.

Test for tannins:

To the test sample, ferric chloride was added, formation of a dark blue or greenish black color showed the presence of tannins.

Test for glycosides- Borntrager's Test

Test drug is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests. To 2 ml of filtered hydrolysate, 3 ml of choloroform is added and shaken, choloroform layer is separated and 10% ammomia solution is added to it. Pink colour indicates presence of glycosides.

Test for flavonoids:

To the test sample about 5 ml of dilute ammonia solution were been added followed by addition of few drops of conc. Sulfuric acid. Appearance of yellow color indicates the presence of Flavonoids.

Test for phenols:

Lead acetate test: To the test sample; 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

Test for steroids:

To the test sample, 2ml of chloroform was added with few drops of conc. Sulphuric acid (3ml), and shaken well. The upper layer in the test tube was turns into red and sulphuric acid layer showed yellow with green fluorescence. It showed the presence of steroids.

Triterpenoids

Liebermann–Burchard test: To the chloroform solution, few drops of acetic anhydride was added then mixed well. 1 ml concentrated sulphuric acid was added from the sides of the test tube, appearance of red ring indicates the presence of triterpenoids.

Test for Cyanins

A. Aanthocyanin:

To the test sample, 1 ml of 2N sodium hydroxide was added and heated for 5 min at 100ºC. Formation of bluish green colour indicates the presence of anthocyanin.

Test for Carbohydrates - Benedict's test

To the test sample about 0.5 ml of Benedict's reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic colored precipitate indicates the presence of sugar.

Proteins (Biuret Test)

To extracts 1% solution of copper sulphate was added followed by 5% solution of sodium hydroxide, formation of violet purple colour indicates the presence of proteins.

2.3. Sterility Test by Pour plate Method

About 1ml of the test sample was inoculated in sterile petri dish to which about 15 mL of molten agar 45°C were added. Agar and sample were mixed thoroughly by tilting and swirling the dish. Agar was allowed to completely gel without disturbing it. (About 10 minutes). Plates were then inverted and incubated at 37° C for 24-48 hours. Grown colonies of organism was then counted and calculated for CFU

2.4. Test for Specific Pathogen

One part of the test sample was admixed with 9 mL of sterile distilled water and the test sample was directly inoculated in to the specific pathogen medium (EMB, DCC, Mannitol, Cetrimide) by pour plate method. The plates were incubated at 37°C for 24 - 72h for observation. Presence of specific pathogen was identified by their characteristic color with respect to pattern of colony formation in each differential media.

2.5. Anti-Microbial Assay [14]

The anti-microbial activity of the test drug SC was carried out by disc diffusion method. The concentrations of the test compounds used were of 500, 1000, 2000 and 4000 µg. The target microorganisms were cultured in Mueller–Hinton broth (MHB). After 24 h the suspensions were adjusted to standard sub culture dilution. The Petri dishes containing Muller Hinton Agar (MHA) medium were cultured with diluted bacterial strain. Sabouraud dextrose was utilized for the growth of fungal strains. Disc made of Whatman No.1, diameter 6 mm was pre-sterilized and was maintained in aseptic chamber. Each concentration was injected to the sterile disc papers. Then the prepared discs were placed on the culture medium. Standard drug Streptomycin (10µg), Amphotericin B (20µg) used as a positive reference standard to determine the sensitivity of the microbial species tested and 20 µl of DMSO was used as vehicle control. Then the inoculated plates were incubated at 37° C for 24 h for bacteria and 72 h for fungus. The diameter of the clear zone around the disc was measured and expressed in millimeters as its anti-microbial property.

3. Results

3.1. Physicochemical Evaluation of SC

Results obtained from the physicochemical evaluation of the drug SC reveals that the loss on drying value was 26.27%, total ash value was 2.92%, in which the water soluble ash is 16.4% and acid insoluble ash is 0.74 %.The alcohol soluble extractive value was 28.93% and water soluble extractive was 37.8%. The results were tabulated in Table 1.

Table 1: Physicochemical Analysis of the test drug SC

S.No	Parameter	Mean (n=3) SD
1.	Loss on Drying at 105 °C (%)	26.27 ± 2.47
2.	Total Ash (%)	2.92 ± 0.126
3.	Acid insoluble Ash (%)	0.746 ± 0.122
4.	Water Soluble Ash (%)	16.4 ± 1.2
5.	Alcohol Soluble Extractive (%)	28.93 ± 2.27
6.	Water soluble Extractive (%)	37.8 ± 1.74

The results were represented in triplicate mean± SD

3.2. Qualitative Phytochemical analysis of SC

It is evident that the medicinal activities of polyherbal formulations are due to the presence of various active principles or phytoconstituents. The results of the preliminary phytochemical analysis of the sample SC reveals the presence of bioactive phytocomponents such as flavonoids, glycosides, triterpenoids, phenols, tannins, saponins and carbohydrates which may have tendency to possess several biological activities. The results were tabulated in Table 2 and illustrated in figure 1.

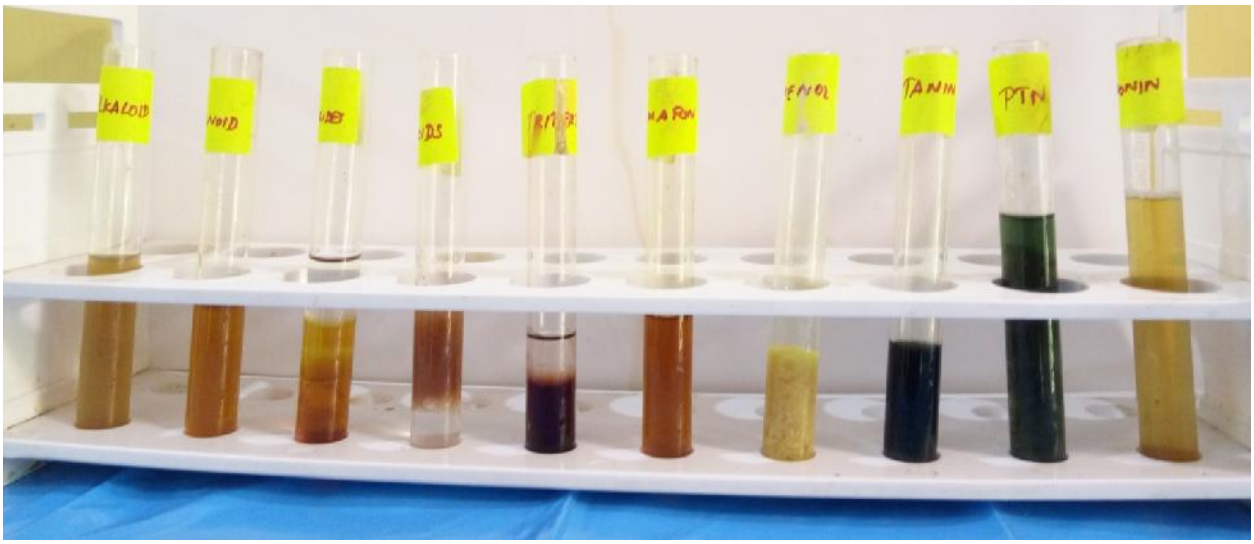


Figure 1: Phytochemical Analysis study report

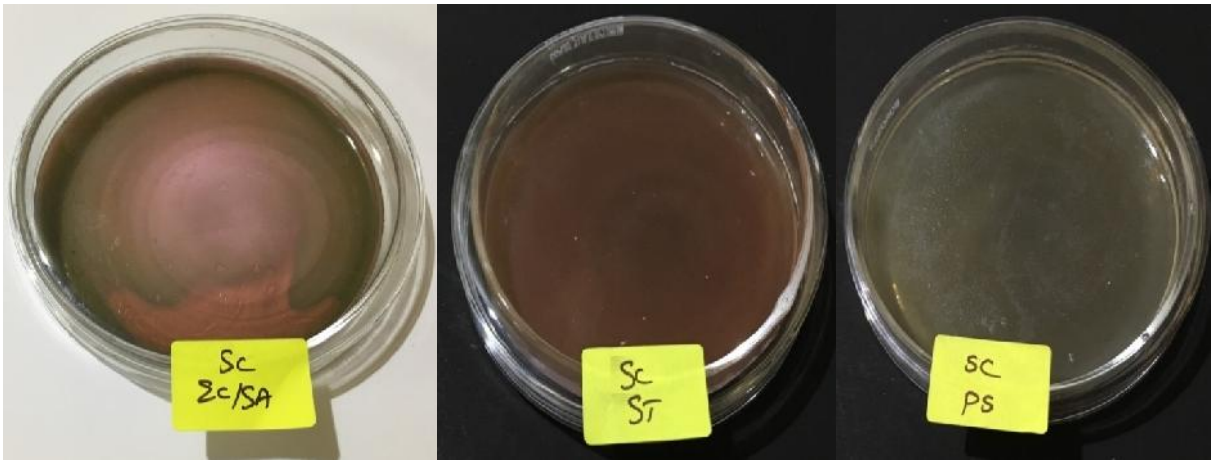
Table 2: Preliminary Phytochemical Analysis of SC

S.no	Test	Observation
1	Alkaloids	-
2	Flavanoids	+
3	Glycosides	+
4	Steroids	-
5	Triterpenoids	+
6	Coumarin	-
7	Phenol	+
8	Tannin	+
9	Protein	-
10	Saponins	+
11	Sugar	+
12	Anthocyanin	-
13	Betacyanin	-

Note: +-> Indicates Presence and - -> Indicates Absence of the Phytocomponents.

3.3. Sterility Evaluation and Test for specific pathogen analysis of SC

There was no growth / colonies were observed in any of the plates inoculated with the test sample. Absence of growth indicates the absence of specific pathogen such as *E.coli, Salmonella, Staphylococcus aureus* and *Pseudomonas aeruginosa.* The results were shown in table 3, 4 and represented in figure 2.



E-coli* / Salmonella Specific Medium** ***Staphylococcus aureus ***Pseudomonas aeruginosa***

Figure 2: Culture plates with medium for Specific pathogens (K) Shaya Chooranam

Table 3: Sterility result Analysis of SC

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

Table 4: Specific pathogen result Analysis of SC

Organism	Specification	Result	Method
E.coli	Absent	Absent	As per AYUSH specification
Salmonella	Absent	Absent	
Staphylococcus aureus	Absent	Absent	
Pseudomonas aeruginosa	Absent	Absent	

3.4. Anti-Microbial Profiling of SC

From the results of the present investigation it was observed that the test drug SC possess significant antimicrobial activity against *Enterococcus faecalis* with the maximum zone of inhibition of about 13 mm at the concentration on 4000µg and with *S. aereus* it exerts maximum zone of 15 mm at the concentration on 4000µg. Further sample SC is moderately effective against *Klebsiella* with the maximum zone of inhibition of about 10 mm at the concentration on 4000µg. The sample SC is not effective against bacteria *Escherichia coli* and fungi *Candida albicans.* The results were shown in table 5 and represented in figure 3 to 7.

Table 5: Anti-Bacterial Evaluation of SC with Zone of Inhibition

Test Organism	Zone of Inhibition in mm					
	DMSO	500 µg	1000 µg	2000 µg	4000 µg	Streptomycin 10µg
Escherichia coli	-	-	-	-	-	**20**
S. aereus	-	**11**	**11**	**14**	**15**	**23**
Enterococcus fecalis	-	**10**	**10**	**12**	**13**	**27**
Klebsiella pneumoniae	-	-	-	**09**	**10**	**20**
Candida albicans	-	-	-	-	-	**Amphotericin B 20µg 10**

Figure 3: Effect of SC against *Escherichia coli*

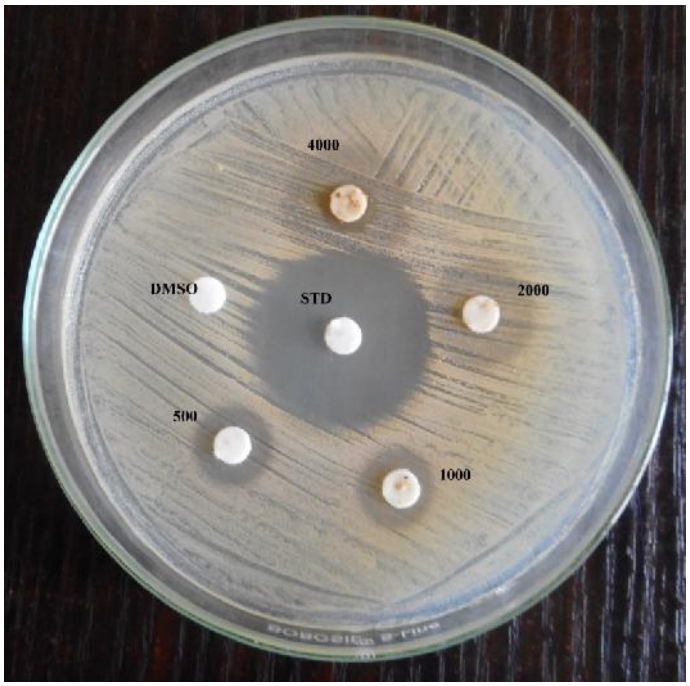


Figure 4: Effect of SC against *Staphylococcus aureus*

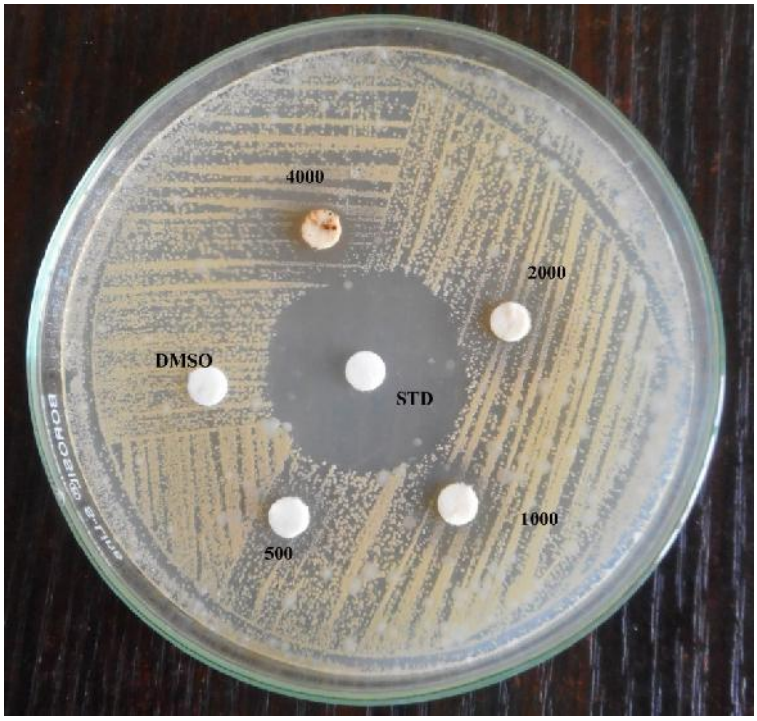


Figure 5: Effect of SC against *Enterococcus faecalis*

Figure 6: Effect of SC against *Klebsiella*

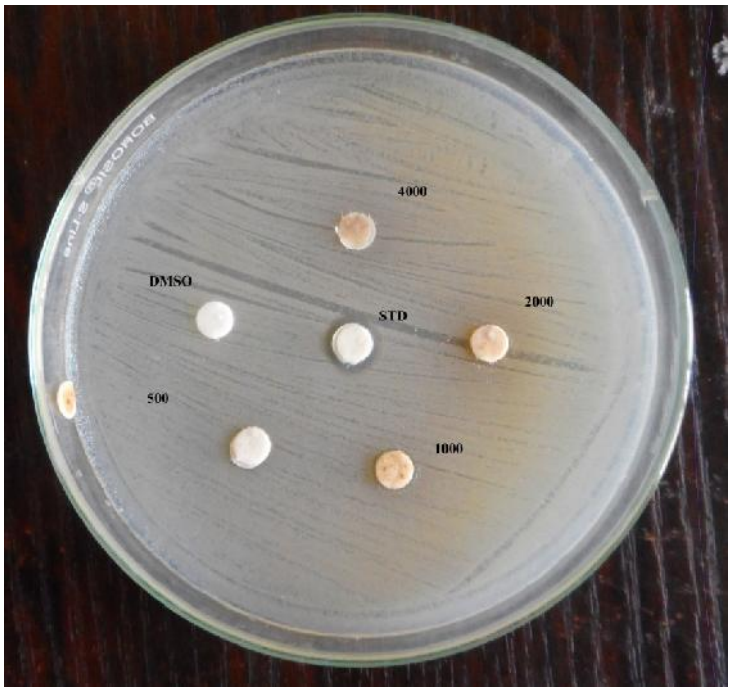


Figure 7: Effect of SC against *Candida albicans*

4. Discussion

Polyherbal preparation or natural products show an important role in diseases prevention and treatment through the enhancement of antioxidant activity, inhibition of bacterial growth, and modulation of genetic pathways. The therapeutic role of number of herbal drugs in disease management is still being enthusiastically researched due to their less side effect and affordable properties. It has been accepted that drugs based on allopathy are expensive and also exhibit toxic effect on normal tissues and on various biological activities. It is a largely accepted fact that numerous pharmacologically active drugs are derived from natural resources including medicinal plants [15, 16].

Siddha system of traditional medicine has numerous formulations which are utilized for the treatment of disease in mankind since several years. Till now there are several formulations which need to be standardized and evaluated for its potency. The results obtained from standardization and physiochemical analysis clearly reveals that the loss on drying value was 26.27%, total ash value was 2.92%, in which the water soluble ash is 16.4% and acid insoluble ash is 0.74 %.The alcohol soluble extractive value was 28.93% and water soluble extractive was 37.8%.

Now-a-days, there is a constant need to explore their medicinal uses and also to conduct phytochemical and bioactivity studies to prove their therapeutic properties. To know any information about any medicinal plant, there is a necessary to go through all the available texts of siddha and also the previous reviews from recent research. Phytochemical investigations and biological reviews on the plants will lead to the valuable information which can help the scientists to know more advanced knowledge about these plant species. The result of the phytochemical analysis indicates that the formulation SC shows the presence of flavonoids, glycosides, triterpenoids, phenols, tannins, saponins and carbohydrates.

Siddha system of medicine pioneers among other traditional therapies that belong to south East Asia especially in treating dreadful infectious diseases. Formulations have been prepared as per Vedic literatures and processed for emphasizing its efficacy before administering the same for clinical use. The ever increasing resistance of pathogens to antibiotics as well as the undesirable side effects of certain antimicrobial agents has necessitated the discovery of novel bioactive compounds [17]. There has been an increasing interest in medicinal plants as a natural alternative to synthetic drugs. It was observed form the results of In-vitro anti-microbial assay that the formulation SC possesses significant antimicrobial activity against *Enterococcus faecalis*, *S. aereus* and exerts moderate effect against *Klebsiella*.

5. Conclusion

There has been an increasing interest in herbal preparations as a natural alternative to synthetic drugs. Many reports have been published in recent years on the antimicrobial activity of herbs and other polyherbal formulations derived from plants against etiological agents of infectious diseases and food-borne pathogens. From the data obtained from the results of the present investigation it was evident that the formulation SC complies with the regulatory standard and also possess significant anti-microbial activity against the tested microbes which could be due to presence of biologically active phytocomponents present in the formulation.

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