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Physicochemical Evaluation and Biological standardization of Novel Siddha Formulation Manosilai before and after Purification: A Comparative Analytical Approach

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Abstract

In the process of drug discovery, the current strategy upon which the scientist relies on the alternate source is from natural origin. Due to prevailing side effect on regular usage of allopathic medicine people of developing countries started migrating towards the traditional healing therapy like Siddha. Siddha system of medicine pioneering in emphasizes the biological activity of the various phytocomponents with respect to the etiology and pathophysiology of various dread full diseases emerging in humans and animals. It is evident that there are some metallic preparations used in Siddha as potency of acting as analgesics, anti-microbial, immune modulators, Hepato, neuro and nephron protectant. Siddha system of medicine is one of the oldest traditional healing therapies known to the mankind since several centuries. It has numerous novel formulations which benefit the society for various ailments. As per the AYUSH guideline Siddha system of medicine has standard protocol starting from collection of raw material, purification, formulation and biological evaluation. The main aim of the present investigation is to carry out the physicochemical evaluation of the drug Manosilai and to make the comparative analysis of the Manosiali before and after purification. The results obtained from physicochemical analysis clearly reveals that the Loss on drying at 105^{o}C of Manosilai before and after purification was found to be 0.4481 and 0.2953 %w/w. Total Ash value found to be 4.088 and 3.892 %w/w similarly acid insoluble value of Manosilai before and after was found to be 0.0809 and 0 %w/w. Water Soluble Extractive value was found to be 1.028 and 0.9300 %w/w. Similarly, alcohol soluble extractive value was 0.2795 and 0.5559 % w/w. Results of microbial analysis reveals the absence of pathogenic organism including bacteria and fungi. Further with respect to the toxins there is an absence of aflatoxin B1, B2, G1 and G2was observed in both before and after purification. It is essential for a formulation to be free from pesticide residues in order to ensure that the Manosilai was subjected to GC MS and LC MS analysis and the results clearly indicates that pesticide level is below the limit of quantification. It was concluded from the results of the present investigation that the drug Manosilai complies with the standards described by the AYUSH.

Keywords: Siddha system, Manosilai, Physicochemical analysis, Microbial analysis, Aflatoxin, Pesticide level

1. Introduction

Standardization of Siddha formulations is an essential factor in order to assess the quality, purity, safety and efficacy of formulation based on the concentration of their active principles. It is very important to establish a system of standardization for every Siddha drugs in the market, since the scope for variation in different batches of medicine is enormous [1]. Over the past two decades, interest in traditional medicines has increased considerably in many parts of the world [2]. The Indian systems of medicine in general, and Ayurveda and Siddha in particular, which originated several centuries ago, are holistic approaches to healthcare.

Standardization and quality assurance of metallic preparations is a major problem. Batch-to-batch variations may occur in their efficacies due to variation in the biological functional group exist. Thus, standardization is important for the establishment of consistent biological activity, chemical profile and a quality assurance program for production and manufacturing of Siddha preparations.

Medicines under the Indian Systems of Medicine (ISM) are required to be manufactured in sanitized environs following Good Manufacturing Practices (GMP) norms, duly laid down by the Government of India. The Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) is the regulator of the ISM manufacture in the country. Among other things, AYUSH is involved in evolving pharmacopoeial standards for Indian Systems of Medicine and Homoeopathy drugs. Minerals are essential for the proper function and structure of a cell. However, the human body cannot absorb most of the minerals in their natural (inorganic) form [3].

Siddha medicines are mostly polyherbal, but may also include metals, chemicals, and/or animal products. The common Siddha preparations are Bhasma (calcined metals and minerals), Churna (powders), Kashaya (decoctions), Lehya (confections), Ghrita (ghee), Taila (oil), and Mezhugu (wax). Manosilai is a kind of novel Siddha raw drug consist of comprises of arsenic as a major component. Siddha practitioners prescribe Manosilai as a therapy for different dreadful diseases. However, scientific evidence on standardization aspect of Manosialia is very limited Hence the main aim of the present investigation is to carry out the physicochemical evaluation of the drug Manosilai and to make the comparative analysis of the raw drug Manosiali before and after purification.

2. Materials and Methods

2.1. Source of raw drugs:

Manosilai was purchased from a well reputed indigenous drug shop at Chennai. Goat's urine was collected from anverthikanpet village. Ulunthu (*Phaseolusmungo* Linn) was purchased from country grossary shop at Thambram, Chennai, Tamil Nadu, India. All raw drugs were authenticated Prof.P.Jayaram, Plant Anatomy Research Centre, Chennai, Tamil Nadu, India.

2.2. Purification of raw drugs [4]

Raw drugs are purified as mentioned in *Agathiyar Vaithiya Kaviyam*.

2.3. Formulation of *Manosilai*

Manosilai was made into small pieces and make in to a bundle, the above bundle was boiled with goat's urine by using thula appliances and then the bundle was removed and kept in black gram boiled water followed by this bundle was opened and dried.

2.4. Organoleptic Investigation

The macroscopical evaluation of Manosilai was performed as per the methods of Khandelwal [5]. Organoleptic characters such as color and texture were studied

2.5. Physico-chemical analysis [6]

2.5.1 Determination of pH

About 5 g of test sample was dissolved in 25ml of distilled water and filtered the resultant solution is allowed to stand for 30 mins and the subjected to pH evaluation using pH meter.

2.5.2. Percentage Loss on Drying

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

2.5.3. Determination of Total Ash

3 g of test sample was accurately weighed in silica dish and incinerated at the furnace a temperature 400ºC 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.

2.5.3. Determination of Acid Insoluble Ash

About 0.5gm of the ash obtained by total ash test boiled with 25 ml of dilute hydrochloric acid for 6mins. Then the insoluble matter collected in crucible and was washed with hot water and ignited to constant weight. Percentage of acid insoluble ash calculated with reference to the weight of air-dried ash

2.5.4. Determination of Alcohol Soluble Extractive

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

2.5.5. Determination of Water Soluble Extractive

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

2.5.6. Biochemical analysis of Basic and acidic radicals

Biochemical analysis of the trial drug Manosilai was subjected for qualitative analyses of cations and anions as per the procedure described by Asokan and sofowora et al [7] [8].

2.6. Determination of Aflatoxins [9]

Standard: Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2. Solvent: Standard samples were dissolved in a mixture of chloroform and acetonitrile (9.8:0.2) to obtain a solution having concentrations of 0.5 µg/ ml each of aflatoxin B1 and aflatoxin G1 and 0.1 µg/ml each of aflatoxin B2 and aflatoxin G2.

Test solution: Concentration 1 µg/ ml Standard aflatoxin was applied on to the surface to pre-coated TLC plate in the volume of 2.5µL, 5µL, 7.5µL and 10µL. Similarly, the test sample was placed and allow the spots to dry and develop the chromatogram in an unsaturated chamber containing a solvent system consisting of a mixture of chloroform, acetone and isopropyl alcohol (85: 10: 5) until the solvent front has moved not less than15 cm from the origin. Remove the plate from the developing chamber, mark the solvent from and allow the plat to air- dry. Locate the spots on the plate by examination under UV light at 365 nm.

2.7. Estimation of Pesticide Residue

Pesticide value of sample was estimated by means of AOAC 2007. 01 by GCMS/ LC MS MS. Sample Preparation The acetate buffered Queechers sample preparation method was applied. After homogenization with a house hold mill a 15gm portion of the homogenized sample was weighed into a 50 ml poly tetra fluro ethylene tube (PTFE) and 100 ml of surrogate standard solution in acetonitrile was added followed by 15 ml of acetonitrile containing 1% acetic acid. Then 6 gm of MgSO4and 2.5 gm sodium acetate trihydrate were added. Then centrifuge the sample at 4000 rpm. Then transferred the supernatant and filtered with PTFE filter. Then sample was transferred to auto sample vials and the extracts were evaporated to dryness under a steam of Argon. The analysis done by Gas chromatography, Liquid chromatography coupled to tandem mass spectroscopy with triple quadruple mass analyzer GC- MS/ LCMS MS [10].

2.8. Determination of Microbial load [11]

Total bacterial count Strains for evaluation *E.coli, Salmonella typhimurium, Staphylococcus aureus, Enterobacter*. By using pour plate method.

2.8.1. Microbial count

Test drug was prepared at the concentration of 10- 100 µg/ ml and dissolved in 100 ml of peptone water. Using the standard loop which carried 0.01 ml of the extract solution was inoculated in nutrient agar, blood agar and McConkey agar plates. The plates were incubated at 37º C overnight and the colonies were

counted. The organisms were identified using biochemical tests such as Indole test, Triple sugar iron (TSI), Urease, Citrate, Mannitol Motility and phenyl pyruvic acid.

2.8.2. Total Bacterial Count:

About 1ml of the test solution contains test drug at fixed concentration as diluted with minimum of 1/ 10th dilution ratio was poured on to each petri dish (Petri dishes 9-10 cm in diameter) aseptically, add to each dish 15- 20 ml of sterilized agar medium, previously melted and kept below 45ºC, and mix. Growth of bacteria was detected using soybean- casein digest agar medium. After the agar solidifies, plates were been incubated at least for 2 days at 30-35ºC. The number of colonies was counted and the values were expressed as CFU.

2.8.3. Total Fungal Count

About 1ml of the test solution contains test drug at fixed concentration as diluted with minimum of 1/ 10th dilution ratio was poured on to each petri dish (Petri dishes 9-10 cm in diameter) aseptically, add to each dish 15- 20 ml of sterilized agar medium, previously melted and kept below 45º C, and mix. Growth of fungi was detected using potato- dextrose agar, plates were been incubated at least for 2- 3 days at 20- C. The number of colonies was counted and the values were expressed as CFU.

3. Results

3.1. Results of Physicochemical evaluation of Manosilai

Organoleptic property of the Manosilaijustifies the genuinity of the finished product with respect to its color and fine powdered texture. Loss on drying at 105°C of Manosilai before and after purification was found to be 0.4481 and 0.2953 %w/w. The results obtained from the physicochemical evaluation revels that the total ash value of Manosilai before and after purification was found to 4.088 and 3.892 %w/w. In which acid insoluble value of Manosilai before and after purification was found to be 0.0809 and 0 %w/w. Water Soluble Extractive value of manosilai before and after was found to be 1.028 and 0.9300 %w/w. Similarly alcohol soluble extractive value was 0.2795 and 0.5559 % w/w.pH of the formulation Manosilai before and after purification was found to be 7.54 and 7.79. The results were tabulated in Table 1.

Table 1: Results of Physicochemical evaluation of Manosilai Before and After Purification

S.No	Physico-chemical parameer	Before purification % in w/w (mg/g)	After purification % in w/w (mg/g)
11	Appearance	Dark orange colour fine powder	Light orange colour fine powder
22.	pH at 25º C (1% w/w solution)	7.54	7.79
23.	Loss on Drying at 105ºC	0.4481 %w/w	0.2953 %w/w
24.	Total Ash	4.088 %w/w	3.892 %w/w
35.	Acid Insoluble Ash	0.0809%w/w	0
26.	Water Soluble Extractive	1.028 %w/w	0.9300 %w/w
27.	Alcohol Soluble Extractive	0.2795 %w/w	0.5559 %w/w

3.2. Biochemical Analysis of Manosilai

The results of the biochemical analysis of the test sample Manosilai reveals that the sample before purification shows negative for solubility and ash test and shows positive for action of heat and flame test. The drug after purification shows positive for solubility, heat and flame test and negative for ash test. Test for acid radicals indicates the presence of trace functional group in the formulation Manosilai. The results obtained from the present investigation shows that the formulation Manosilai before and after purification shows the presence of carbonate and sulphides and other radicals such as chloride, sulphate, phosphate, nitrates and borates are absent. Basic radicals are most consistent for mediating the enzymatic action in biological system in particular with copper. The results obtained from the present

investigation shows that the formulation Manosilai before and after purification shows the presence of copper, calcium, potassium, mercury and arsenic. Other basic radicals such as zinc, Iron, lead, aluminum, sodium and magnesium are absent. The results were tabulated in table 2.

Table 2: Results of Biochemical Analysis of Manosilai before and after purification

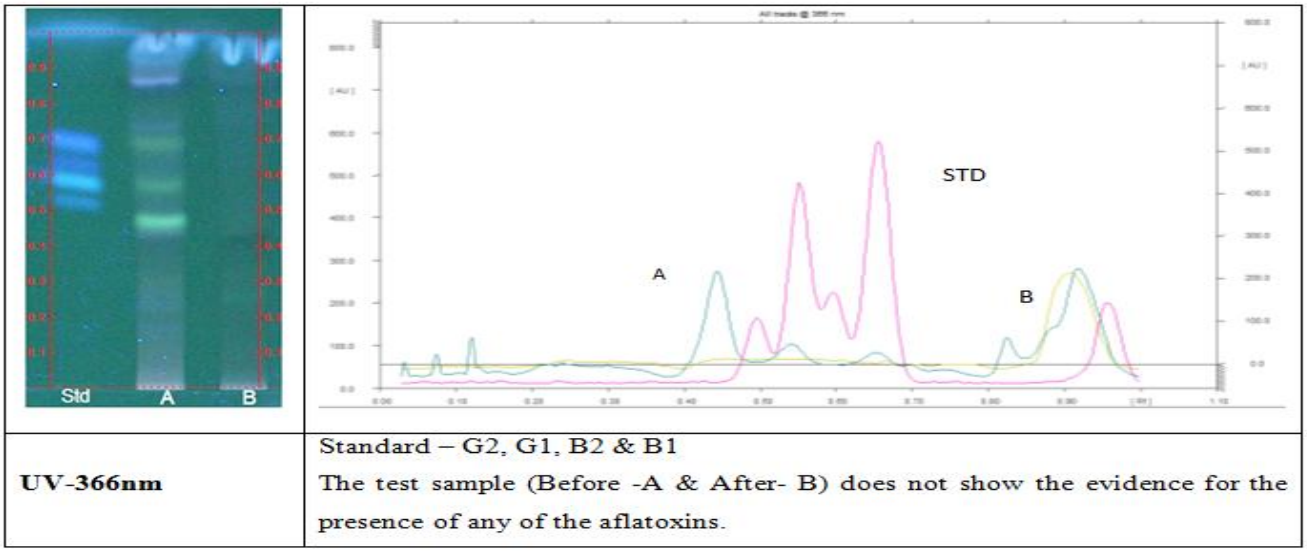
S.No	Experiment	Before Purification	After Purification
1	Solubility	_	+
2	Action of Heat	+	+
3	Flame Test	+	+
4	Ash Test	_	_
	Test for acid radicals		
1	Test for Sulphate	_	_
2	Test for chloride	_	_
3	Test for phosphate	_	_
4	Test for carbonate	+	+
5	Test for nitrate	_	_
6	Test for Sulphide	+	+
7	Test for fluoride & oxalate	_	_
8	Test for nitrite	_	_
9	Test for borate	_	_
	Test for basic radicals		
1	Test for lead	_	_
2	Test for copper	+	+
3	Test for aluminium	_	_
4	Test for iron	_	_
5	Test for zinc	_	_
6	Test for calcium	+	+
7	Test for magnesium	_	_
8	Test for ammonium	_	_
9	Test for potassium	+	+
10	Test for sodium	_	_
11	Test for mercury	+	+
12	Test for arsenic	+	+
	Miscellaneous		
1	Test for starch	_	_
2	Test for reducing sugar	+	+
3	Test for the alkaloids	+	+
4	Test for tannic acid	_	_
5	Test for unsaturated compound	_	_
6	Test for amino acid	_	_
7	Test for type of compound	_	_

+ Presence and - Absence

3.3. Detection of Aflatoxins in Manosilai before and after purification

The results of aflatoxin analysis of the test formulation Manosilai before and after purification reveals the absence of deadly mycotoxins such as alflatoxin B1, B2, G1 and G2. This justifies the purity of drug and its application on human usage. The results were tabulated in Table 03 and figure 1.

Figure 1: Densitometric chromatogram of Manosilai Vs Standard Aflatoxin at UV-366nm



UV-366nm	Standard – G2, G1, B2 & B1 The test sample (Before -A & After- B) does not show the evidence for the presence of any of the aflatoxins.

Table 3: Result of Aflatoxin analysis of raw drug Manosilai before and after purification

Toxin	Manosilai Before Purification	Manosilai After Purification
Aflatoxin B1	ND	ND
Aflatoxin B2	ND	ND
Aflatoxin G1	ND	ND
Aflatoxin G2	ND	ND

ND- None Detected

3.4. Results on Estimation of Pesticide Residue of Manosilai before and after purification

It is essential for a formulation to be free from pesticide residues in order to ensure this formulation Manosilai before and after purification was subjected to GC MS and LC MS analysis and the results clearly indicates that there is no trace of pesticide were observed in both of these samples and there is a trace level of Hepachlor Epoxide an organochlorine pesticide was observed of about 1.779mg/kg for before and 0.413mg/kg for Manosilai after purification samples. The results were tabulated in Table 04.

Table 4: Results of Pesticide Residue Analysis of Manosilai before and after purification

S.No	Parameter Test	Before Purification	After Purification
Organochlorine Pesticides			
1	Alpha HCH	ND	ND
2	HCB	ND	ND
3	Beta – HCH	ND	ND
4	Gamma – HCH	ND	ND
5	Delta –HCH	ND	ND
6	Heptacholr	ND	ND
7	Aldrin	ND	ND
8	Hepachlor Epoxide	1.779	0.413
9	Chlordane (cis& trans)	ND	ND
10	Endosulfan (alpha)	ND	ND
11	Endosulfansulphate	ND	ND
12	O,p' &p,p'-DD	ND	ND
13	Dieldrin	ND	ND
14	O,p' & DDD	ND	ND
15	Endrin	ND	ND
16	Endosulfan – Beta	ND	ND
17	O,p' &p,p' DDT	ND	ND
18	Mehoxychlor	ND	ND
Organophosphorous Compounds			
19	Phorate	ND	ND
20	Methyl parathion	ND	ND
21	Malathion	ND	ND
22	Chlorphyrifos	ND	ND
23	Ethion	ND	ND

ND- None Detected

3.5. Results of Microbial load of Manosilai before and after purification

Microbial contamination signifies more dreadful diseases in humans and its aggravates the co-infection. The result of microbial analysis of study drug Manosilai before and after purification reveals the absence of bacterial and fungal strains. The results of the study further proves the absence of pathogens such as *E.coli* ,*Enterobacter*, *Salmonella typhimurium* and *Staphylococcus aureus*. The results were tabulated in Table 05.

Table 5: Results of Microbial Load Analysis of Manosilai before and after purification

S.No.	Parameters	Reference Limits as per WHO (2007)	Results		
			Manosilai (Before Purification)	Manosilai (After Purification)	Remarks
1	***Total Bacterial Count (TBC)***	10^5CFU/gm	Absent	Absent	Within permissible limits
2	***Total Fungal Count (TFC)***	10^3CFU/gm	Absent	Absent	
3	***Enterobacter***	10^3	Absent	Absent	
4	***Escherichia coli***	10	Absent	Absent	
5	***Salmonella Spp***	None	Absent	Absent	
6	***Staphylococcus aureus***	None	Absent	Absent	

4. Discussion

Indian system of medicine is one of the oldest and well known documented health traditions in the world. Drug discovery based on traditional information is a key path towards the discovery of new drug. Now a day's reverse pharmacology is an approach where discovery of leads/formulations is based on the documented clinical experiences and scientific observations through series of studies. Reverse pharmacology based on traditional knowledge concentrate on the reversing routine 'laboratory-to-clinic' development to 'clinics-to-laboratories'. Safety is considered as most significant point remains and the effectiveness becomes a matter of validation. This process is highly useful to find better and safer leads [12].

Standardization expression is used to describe all measures which are taken during the manufacturing process and quality control leading to a reproducible quality. It's also include the herbal drugs preparation to a define content of a constituent or a group of substance with known therapeutic activity respectively by addition of excipients or by mixing herbal drugs preparation. In other words, it's ensuring that every Siddha formulation has correct ingredient in correct amount and will induce intended therapeutic effect [12,13].

Organoleptic characters such as color and appearance are unique for each and individual preparation as I denote the purity and genunity of the test drug. In the present investigation the appearance of Manosilai before purification was found to be Dark orange color whereas after purification the color turned Light orange. pH of the preparations was one of the most important characters as it is considered to be the significant limitation factor for the formulation to dissolve and to cross the various biological layers to reach the target organ. pH of the formulation Manosilai before purification was 7.54 and after purification it was 7.79. It clearly denotes the greater solubility factor of the prepared formulation.

The loss on drying test used to determine the measure of amount of water and volatile matter present in a sample when the sample is dried under the specified conditions. Moisture is one of the major factors responsible for the deterioration of the drugs and formulations. Low moisture content is always desirable for higher stability of the drugs. The percentage of loss on drying of raw drug Manosilai before and after purification was changed from 0.4481 % w/w to 0.2953%w/w. The drastic change in loss on drying from before to after purification process depicts the extensive shelf life of the drug [14].

The Ash limit tests are to determine the measure the amount of the residual. A high ash value is an indication of contamination, substitution, adulteration or carelessness in preparing the drug and the less Total ash value indicates the purity of the drug. The Total ash values of Manosilai for before and after purification process was 4.088 %w/w and 3.982 %w/w respectively. As the Total ash value is much reduced in after purification, it implies that the inorganic constituents are much reduced after purification. The acid - insoluble ash limit test is to measure the amount of ash insoluble to diluted hydrochloric acid. Acid-insoluble ash value of Manosilai before and after purification was 0.0809 %w/w and 0 %w/w respectively. This indicates the greater physiologic availability of the drug and also indicates the purity of the drug after purification.

Extraction value determines the number of active constituents in a given amount of the formulation when extracted with a solvent media such as water and alcohol. The water soluble and alcohol soluble extract values provides indication of the extent of polar and non-polar compounds respectively. The extract value of water is changed from 1.028%w/w to 0.930 %w/w during purification. It indicates that water solubility is slightly decreased after purification. The alcohol soluble extractive value of the formulation changed from 0.2795 %w/w to 0.5559 %w/w. There is a reduction in alcohol extract value was observed in after purification, which indicates that the alcohol solubility is increased. Hence it is concluded that alcohol not is better solvent of extraction than water [15].

The results of the biochemical analysis of the test sample Manosilai reveals that the sample before purification shows negative for solubility and ash test and shows positive for action of heat and flame test. The drug after purification shows positive for solubility, heat and flame test and negative for ash test. Test for acid radicals indicates the presence of trace functional group in the formulation Manosilai. The results obtained from the present investigation shows that the formulation Manosilai before and after purification shows the presence of carbonate and sulphides and other radicals such as chloride, sulphate, phosphate, nitrates and borates are absent. Basic radicals are most consistent for mediating the enzymatic action in biological system in particular

with copper. The results obtained from the present investigation shows that the formulation Manosilai before and after purification shows the presence of copper, calcium, potassium, mercury and arsenic. Other basic radicals such as zinc, Iron, lead, aluminum, sodium and magnesium are absent.

Development of standards for plant-based drugs being a challenging task, it needs innovative and creative approaches. At each and every step of standardization viz; identification, organoleptic, pharmacognostic, physicochemical, phytochemical, presence of xenobiotics, microbial load and toxicity needs special attention because of complex nature of plant based medicines and the inherent variability of their constituents [16].

Microbial contamination signifies more dreadful diseases in humans and its aggravates the co-infection. The result of microbial analysis of study drug Manosilai before and after purification reveals the absence of bacterial and fungal strains. The results of the study further proves the absence of pathogens such as *E.coli* ,*Enterobacter*, *Salmonella typhimurium* and *Staphylococcus aureus*.

Pesticides are used extensively throughout the world. In the United States, more than 18,000 products are licensed for use, and each year > 2 billion pounds of pesticides are applied to crops, homes, schools, parks, and forests. Environmental Protection Agency (EPA) sets a maximum legal residue limit (called a tolerance) for each treated food. The tolerance is the residue level that triggers enforcement action. That is, if residues are found above that level, the commodity will be subject to seizure by the government. Prolong exposure of even low-level pesticides leads to alteration in reproductive cycle and other neurological dysfunction in humans [17].

It is essential for a formulation to be free from pesticide residues in order to ensure this formulation Manosilai before and after purification was subjected to GC MS and LC MS analysis and the results clearly indicates that there is no trace of pesticide were observed in both of these samples and there is a trace level of Hepachlor Epoxide an organochlorine pesticide was observed of about 1.779mg/kg for before and 0.413mg/kg for Manosilai after purification samples.

The metabolites are called mycotoxin which literally means “fungal poison thereby referred to as mycotoxin.” Aflatoxins are the deadliest mycotoxins, they are produced by Aspergillus species and are known to be one of the deadliest carcinogens due to detrimental effects they can exert on their consumers, and this is also confirmed by the International Agency for Research on Cancer (IARC). There are five different types of aflatoxins that exist in nature; they are aflatoxin B (AFB1), aflatoxin B (AFB2), aflatoxin G (AFG1), aflatoxin G (AFG2), and aflatoxin M (AFM1), respectively. The categories of foods they contaminate are cereals and cereals’ products; herbs and spices; nuts and oil seeds [18]. The results of aflatoxin analysis of the test formulation Manosilai before and after purification reveals the absence of deadly mycotoxins such as alflatoxin B1, B2, G1 and G2. This justifies the purity of drug and its application on human usage.

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