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STANDARDIZATION OF SIDDHA FORMULATION– ADHATODAI KUDINEER

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ABSTRACT

The Purpose of the study was to standardize the Siddha formulation-Adhatodai Kudineer (ADK). Initially, Organoleptic characters like appearance, colour, taste and odour of ADK was noted. ADK was screened for moisture content, total ash value, acid insoluble ash, water soluble extractive value, alcohol soluble extractive value, microbial load, specific pathogen to estimate the quality of study drug. Aqueous extract and ethanol extract of ADK were taken by soxhlet method using 30 gram of sample and 210 ml of solvent. Aqueous extract and Ethanol extract were purified by evaporating solvent through heating in water bath then filtered and used the filtrates for testing. Preliminary Phytochemical evaluation of ADK was carried out following the standard procedure. The results of physiochemical analysis of ADK were found within normal limits. The results of the preliminary phytochemical test showed that alkaloids, carbohydrate glycosides, phytosteroids, phenols, flavonoids, tannins, diterpenoids and quinine are present in aqueous extract of ADK the same phytochemicals were present in ethanol extract of ADK except alkaloids, carbohydrate and tannin. Microbial contamination test showed total bacterial count - 5x10 4 Cfu /g and total fungal count 3x102 Cfu /g. ADK is free from specific pathogen like E.oli, Salmonella, Pseudomonas and Staphylococcus aureus.

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INTRODUCTION

Many plant products have found to play an important role in various diseases. Although, modern medicines are available, herbal medicines have often retained popularity for historical and cultural reasons. Since the usage of these herbal medicines has increased, the issues regarding their safety, quality, and efficacy in industrialized and developing countries are cropped up. (Chauhan Khushbu *et al*, 2011) Ayurveda and Siddha medicines are very effective and have therapeutic value in nature but lack of standardization; it is required to develop the standardization technique. In this study, Adathodai kudineer was selected and screened for standardization technique as per procedures. (Prashant Tiwari *et al* 2011 & Solomon Charles Ugochukwu, 2013) Ingredients of ADK are *Adhatoda vasica , Glycyrrhiza glabra, Piper longum and Abies spectabilis*. (C S Uthamarayan, 1995)

Adhatodai Kudineer has been used as a therapeutic agent in Siddha practice for the treatment of various pathological conditions like common cold, sinusitis, bronchitis, fever, pneumonia, Obstructive lung diseases etc. (Murugesan Mudaliar, 2003). Adhatoda vasica commonly known as vasaka. (Santosh Kumar Singh *et al*, 2014) It is a small evergreen plant with sharp, pointed and lance-like leaves. The leaves are 10-16cm long and greenish brown when dried and bitter in taste. The flowers are bisexual, have large and white petals. It smells like a strong tea. The fruit is a small capsule with small 4 seeds. The entire plant is about 3-4 m in height. The branches are opposite and ascending. The plant has been in use for the past 2000 years in India for medicine. (Anoop Sharma 2016) The strong antibacterial activity was exhibited by most bioactive phytochemicals viz., vasicine of Adhatoda vasica against E. coli and also demonstrated maximum antifungal activity against C. albicans**.** **(**Bharat Singh *et al*,

2013). Glycyrrhiza glabra, also known as Liquorice and sweet wood, belonging to family Leguminaceae. Glycyrrhizin a triterpenoid compound, accounts for the sweet taste of Liquorice root. Gycyrrhiza glabra has the demulcent, expectorant, diuretic, mild laxative, anti-arthritic, anti-inflammatory, (Nirmala , P. *et al* 2011) antibiotic, anti-viral, anti-ulcer, memory stimulant, anti-tussive, (Kumar Anil *et al* 2012 & Yasmeen Jahan *et al*, 2012) aphrodisiac, anti-mytotic, estrogenic, anti-oxidant, anti-caries agent, anti-neoplastic, anti-cholinergic, anti-diuretic, hypolipidemic activity etc. (Kumar Anil *et al* 2012). Survey of literature revealed the presence of alkaloids, lignans, volatile oil and esters in different parts of piper longum. (Chauhan Khushbu *et al*, 2011) *Piper longum* possesses immune-suppressant, anti-asthamatic, (Dhirender kaushik, 2012) stimulant effect, hepato-protective, Hypocholesterolaemic activity, anti-inflammatory, antiameobic and antibacterial etc. (Chauhan Khushbu *et al*, 2011) The methanol extract of leaves of **Abies webbiana** showed the best significant anti-inflammatory activity as compared to that of diclofenac sodium (150 mg/kg p.o). (Siva Sankar Nayak *et al*, 2004)

Microbial contamination in herbal drugs is increases health hazard to human. Microbial load higher than WHO norms may be harmful as they can produce toxic substance like aflatoxins which may cause harm to the human heath instead of curing the disease. There are innumerable reports of mycotoxin contamination of herbal drugs are available which are harmful. The bacterial and fungal contamination can be reduce to appreciable extend by following the proper method of collection, storage and packaging and decontamination prior to formulation. Herbal drugs usually contain plenty of bacteria and fungi, often originating from soil and atmosphere, it generally contaminate the drugs. The determination of *E. coli* and other entero bacteria moulds may indicate the quality of production. The presence of microbial contaminant in non sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines.(Nandna Khurana *et al*, 2010) It is need of hour to estimate the microbial contamination of the herbal products.

In this study Organoleptic characters, physiochemical Parameters and Phytochemical test for alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, proteins and amino acids, diterpenes, quinines, gum and mucilage of Adathodai kudineer were done. Analysis of microbial load and Specific Pathogen test were performed for ADK.

MATERIALS AND METHODS

Adhatodai kiudineer was collected from Outpatient Department of The TN Dr MGR Medical University, Chennai and used for Standardization test. Initially, organoleptic characters, Physicochemical evaluation, preliminary phytochemical screening, analysis of microbial load and Specific Pathogen test were done by following the standard procedure.

Evaluation of Organoleptic Characters

Organoleptic characters refer to the evaluation of formulations by appearance, colour, odour, taste, etc. Organoleptic evaluation of ADK was carried out using traditional and standard techniques.

Physiochemical Analysis

Physicochemical evaluation of the study drug was done following the standard procedure. (Lavanya, B. 2016., WHO 1998) Three samples screened for loss on drying, total ash value, acid insoluble ash, water soluble ash to estimate the quality of study drug.

Determination of Total Ash values

The ash remaining following ignition of sample is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash. The total ash method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

Procedure: 4 gm of sample weighed, placed evenly in a previously ignited and tarred silica dish. Ignited in a muffle furnace at 600° C until it turned white in color. It indicated the absence of carbon.

Percentage of Total ash= $\frac{weightoftheashX100}{weightofthesampletaken}$

Determination of acid insoluble ash

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Procedure: Added to the ash 15 to 25 ml of the hydrochloric acid and boiled for 10 minutes, covering the dish with a watch glass to prevent sputtering. Allowed to cool and filtered and contents of the dish through the ash less filter paper. Washed the filter paper in hot water until the washings are free from hydrochloric acid, as tested by silver nitrate solution and returned it to the dish. Evaporated carefully on the water bath and ignited in the muffle furnace at 550° C $\pm$ 25° C for 1 hour. The dish was allowed to cool in the desiccators and weighted.

Percentage of Acid insoluble ash=

$$\frac{weightoftheacidinsolubleresidueweightX100}{weightofthesampletaken}$$

Determination of water soluble ash

Total ash 1g was boiled for 5min with 25ml water and insoluble matter collected on an ash less filter paper was washed with hot water and ignited for 15min at a temperature not exceeding 450^0C in a muffle furnace. Difference in weight of ash and weight of water.

Water soluble extractive value

5g of sample is taken in 250 ml iodine flask. Add 100ml of water and then keep it in shaker for about 6 hrs and then it is left to stand for whole night, and then it is filtered with 4 size filter paper.

Take 10ml from that filtrate into 250ml weighed beaker. It is kept in oven at 110^0 c for one hr and then it is cooled and weighed.

Percentage of water soluble extractive

$$= \frac{W_2g - W_1g}{\text{Sample wt}} \times \frac{100}{10} \times 100$$

W_1= Beaker Weight
W_2= dried sample weight

Alcohol soluble extractive value

2.5g of sample is taken in 250ml iodine flask. Add 50ml of ethanol and then keep it in shaker for about 6 hrs and then it is left to stand for whole night, and then it is filtered with 4 size filter paper.Take10ml from that filtrate into 250ml weighed beaker. It is kept in oven at 110^0c for one hr and then it is cooled and weighed.

Percentage of alcohol soluble extractive

$$= \frac{W_2g - W_1g}{\text{Sample wt}} \times \frac{50}{10} \times 100$$

W_1= Beaker Weight

W_2= dried sample weight

Estimation of pH

Procedure: The pH meter is an electrical device that determines the acidity (or) basicity of aqueous solutions, one of most commonly monitored parameters. To use a pH metre, the pH electrode is first calibrated with standard buffer solutions with known pH values that span the range being measured.

Sample preparation: 5gm of sample is taken in beaker and 100 ml of water is added to it. This solution is measured for pH using pH meter.

pH Measurement: To make a pH measurement, the electrode is immersed into the sample solution until a steady reading is reached.

The preliminary phytochemical screening Test

The preliminary phytochemical screening test was carried out for each extracts of the samples as per the standard procedure. (Kumar Anil *et al* 2012)

Preparation of Extract: The extract was prepared by using Soxhlet apparatus. 30 gram coarse powder of Adhatodai Kudineer, 180 ml of water were taken and 90 degrees Celsius temperature was maintain for 8 hrs for preparation of aqueous extract. The same procedure was followed for taking ethanol extract of ADK. But the temperature was maintained in 30 degrees Celsius. The extracts obtained with each solvent were filtered through whatman filter paper no:1. The extracts were kept in water bath set at 60 degrees Celsius and allowed to evaporate the solvent. Finally, the concentrated extracts were collected separately and kept in air tight container in refrigerator until further use. Each extraction was taken by dissolving 1 gm of extract with 40 ml of distilled water and then filtered and used the filtrates for testing Preliminary Phytochemicals. For both aqueous extract and ethanol extract the following test has been carried out and results were incorporated in result section.

Detection of alkaloids

Extract was dissolved individually in diluted hydrochloric acid and filtered.

Mayer's test

2 ml of extract was treated with few drops of Mayers' reagent; formation of yellow coloured precipitate indicates the presence of alkaloids.

Wagner's test

2 ml of filtrate was treated with Wagner's reagent. Formation of brown /reddish precipitate indicates the presence of alkaloids.

Detection of carbohydrate

Extract was dissolved individually in 5 ml of distilled water and filtered. The filtrates were used for test.

Molisch's test

2 ml of filtrate was treated with few drops of alcoholic Alpha naphthol solution in a test tube. Formation of the violet ring at the junction indicates presence of carbohydrates.

Benedict's test

Filtrate was treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Detection of Glycosides

Liebermann's test

2ml of extract was treated with 2ml chloroform and 2ml of acetic acid, Violet colour change into blue and green indicates presence of Glycosides.

Detection of Saponins

Froth test: Extract was diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 centimetre layer of foam indicates the presence of Saponins.

Foam test

0.5gram extract was shaken with 2 ml of water. If foam produced persists for 10 minutes, it indicates the presence of Saponins.

Detection of phytosterols

Salkowski's test

Extracts was treated with chloroform and filtered; the filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand for few minutes. Golden yellow colour indicates the presence of triterpenes.

Detection of phenols

Ferric Chloride test: 2 ml of extracts was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins

Gelatin test

To the 2 ml extract, 1% of gelatine solution containing sodium chloride was added; formation of white precipitate indicated the presence of tannins.

Detection of flavonoids

Alkaline reagent test

Extract was treated with few drops of 10% sodium hydroxide, formation of intense yellow colour then on addition of diluted hydrochloric acid it becomes colourless, it indicates the presents of flavonoids.

Lead acetate test

Extract was treated with few drops of lead acetate solution; yellow colour precipitate indicates presence of flavonoids.

Detection of Proteins and Aminoacids

- Xanthoproteic Test: The extract was treated with few drops of concentrated Nitric acid. Formation of yellow colour indicates the presence of proteins.
- Ninhydrin Test: To the extract, 0.25 % ninhydrine reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Detection of diterpenes

Copper Acetate test

Extract was dissolved in water and treated with 3-4 drops of copper Acetate solution; formation of emerald green colour indicates the presence of diterpenes.

Detection of gum and mucilage

The extract was dissolved in 10 ml of distilled water and to this 2ml of absolute alcohol with the constant stirring white cloudy precipitate indicates the presence of gum and mucilage.

Detection of Quinones

Extract was treated with concentrated HCL and observed for the formation of yellow precipitate or yellow discolouration.

Determination of Microbial contamination

The limits of microbial contamination are: total aerobic bacteria 105cfu/g, yeasts and moulds 103 cfu/g, *Enterobacteria* and other Gram negative organisms 103cfu/g and *E. coli* and Salmonella should be absent. (Adenike Okunlola *et al*, 2007) It is essential that limits be set for microbial contamination and the European Pharmacopoeia now gives non-mandatory guidance on accepTable limits.

Total Bacterial Count and Total Fungal count: Herbal drugs usually contain plenty of bacteria and fungi, often originating from soil and handling, and it generally contaminate the drugs.

Procedure for serial dilution: 1ml of the preparation is then serially diluted three times using 1 in 10 dilution, i.e 1ml of product in 9ml of distilled water in first test tube, 1ml of mixture of first test tube in 9 ml distilled water for second test tube, 1ml of mixture from second test tube in 9ml distilled water for third test tube to inactivate the preservative in the product. 1 ml of the diluted preparation in the third test tube is then added to each Petri dish labelled for test experiment. Different agar medium is liquefied by placement in a heated water bath and 20ml of each poured into the respective Petri dishes and allowed to set. The petri dishes are the incubated at 37^{o} C for 72 hours.

Colony Forming Unit: The number of growth or colony-forming units on each plate is counted and recorded.

Determination of specific Pathogen

The determination of *E. coli* and other entero bacteria moulds may indicate the quality of production.

Procedure: For powders, 1gquantities was dissolved in 9 mL of sterile distilled water. Serial dilutions were made and viability assessed using the pour plate method. The plates were incubated at 37oC for 24h. The plate was placed on a colony counter and the number of colony forming units was taken. The microbial content was taken as the mean of duplicate determinations. The media utilized were Nutrient agar, For detection of fungal growth in the samples, Sabouraud dextrose agar was poured into the plate and allowed to set and 1ml aliquot of each sample was spread on the surface and the plates were incubated at 270C for 72 h. The viable aerobic bacterial count and the viable count for moulds (dry surface method) and the absence (or presence) of *Escherichia coli* and *Staphylococcus aureus* were assessed using well established methods (Kumar Anil *et al* , 2012)

RESULTS

Organoleptic Characters

Organoleptic evaluation of ADK was carried out using traditional and standard techniques. (Santosh Kumar Singh, *et al*, 2014) And Organoleptic Characters of ADK was tabulated in Table1.

Table 1. Organoleptic Characters of ADK

S.No	Organoleptic characters	Result
1	Appearance	Coarse powder
2	Color	Green
3	Taste	Bitter with Sweet taste
4	Odour	Pleasant

Physiochemical parameters

Results of analysis of Physiochemical parameters of Adathodai Kudineer was tabulate in Table.2

Table 2. Physiochemical parameters of Adathodai Kudineer

S. No	Physiochemical Parameters	Values
1	Moisture content	3.22%
2	Total ash content	7.50%
3	Acid insoluble ash	3.34 %
4	Water soluble ash	3.43 %
5	Water soluble extractive value	3.26 %
6	Alcohol soluble extractive value	0.98 %
7	pH value	7.11

Phyto-chemical analysis

Results of preliminary Phyto -chemical analysis of Aqueous and Ethanol extract of *Adhatodai Kudineer* was tabulated in Table.3

Table 3. Phyto-chemical analysis of Aqueous and Ethanol extract of Adhatodai Kudineer

S.No	Phytochemicals	Test Name	Aqueous extract of ADK	Ethanol extract of ADK
1	Alkaloid	Mayer's test	-	-
		Wagner's test	+	_
2	Carbohydrate	Molisch's test	-	-
		Benedict test	-	-
3	Glycoside	LibermannBurchard's test	-	-
4	Saponins	Froth test	+	+
		Foam test	+	+
5	Phytosterol	Salkowski's test	+	+
6	Phenols	Ferric chloride test	+	+
7	Tannins	Gelatin test	-	-
8	Flavonoid	Alkaline Reagent test	+	+
		Lead acetate test	+	+
9	Protein and amino acid	Xanthoproteic test		
10	Diterpenes	Copper acetate test	+	+
11	Gum and mucilage	Extract + alcohol	-	-
12	Quinones	NAOH + Extract	+	+

Microbial contamination test

Results of the microbial analysis of ADK were tabulated in Table 4.

Table 4. Results of Microbial Contamination Test

S.NO	Microbial contamination test	Results
1	Bacterial count	$5x10^4$ Cfu /g
2	Fungal count	$3x10^2$ Cfu /g

Specific Pathogen test

The Analytical screening of ADK showed that the product is free from specific pathogen like E.oli, Salmonella, and Pseudomonas and Staphylococcus aureus. Results were presented in Table 5.

Table 5. Results of Specific Pathogen Test

S.NO	Specific pathogen	Results
1	*E. coli*	Absent
2	*Salmonella*	Absent
3	*Pseudomonas*	Absent
4	*staphylococcus*	Absent

DISCUSSION

For the siddha formulations Adhatodai Kudineer (ADK), reports of organoleptic, preliminary phytochemicals, physio chemical, microbial load, specific pathogen screening tests are not available.

Therefore discussion is made with study related to single drugs ie ingredients of the ADK. Preliminary phytochemical screening of extract of Adhatoda vasica, it showed the presence of Saponin, Alkaloids, Tannins, Flavonoids, Steroids, Carbohydrates, Vitamin C, Cardiac Glycoside and Reducing sugar groups. (Santosh Kumar *et al* , 2014) Preliminary phytochemical screening of extract of Adhatoda kudineer is similar to above results except for tannins, carbohydrates, vitamin C, cardiac glycosides and reducing sugars. The Petrolium ether extract of Piper longum contain alkaloids, carbohydrates. Flavonoids, glycosides and steroids. (Dhirender kaushik *et al*, 2012) Survey of literature revealed the presence of alkaloids, lignans, volatile oil and esters in different parts of piper longum.[1], But in this study, volatile oil, esters, lignans, carbohydrates, glycosides are absent in the phytochemical analysis; this variation may be due to present of $1/4^{th}$ of *Pipper longum* in the ADK.

In the present study specific pathogenic bacteria are absent in ADK formulation. It is as per WHO norms. Total bacterial load is higher in *Glycerrhiza glabara*, (Nandna Khurana *et al* 2010) but in this study, microbial load is within the limits in ADK. So it proves that ADK is free from microbial contamination. The findings of this study also highlighted the safety of the Adhatodai Kudineer. The information obtained from preliminary phytochemical screening will be use full in finding out the reality of the drugs.

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