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Safety Assessment of Siddha Formulation *Athiyadhi kashayam* by Short and Long-term Toxicity Studies in Wistar Rats

***C. Meenakshi*[1*] *and G. Bharathkumar*[2]**

[1]*Lecturer, Govt. Siddha Medical College, Arumbakkam, Chennai (Tamil Nadu), India.*
[2]*Department of Siddha, The Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai (Tamil Nadu), India.*

(Corresponding author: C. Meenakshi)*
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ABSTRACT: Traditional siddha practice is a medical skill that is distinctive in both composition and mechanism. The main reason for the efficacy is that siddha formulations are composed of potentially bioactive plants. The major purpose of the current investigation is to evaluate the safety profile of the siddha formulation *Athiyadhi kashayam* (AK) in both short-term (acute) and long-term (sub-acute) toxicity tests in accordance with the rules established by the OECD. During the course of this inquiry, each of the test animals received a single dosage of 2,000 mg/kg by oral administration, and they were monitored for a period of one week. During the course of the subacute study, experimental rats were given multiple doses of the investigational medication AK (200 and 400 mg/kg/day) over the course of 28 days. The findings of the acute trial revealed that there was no discernible shift in clinical observation after 14 days of close monitoring and observation in rats that had been given the drug. In the subacute investigation, there was no statistically significant difference seen between the animals in the AK treatment group and the animals in the control group in terms of body weight, food/water consumption, haematological observations, or serological findings. Outcome of our study revealed evidence-based data on the safety of the siddha drug AK and proved its safe therapeutic usage on humans.

Keywords: Siddha, Toxicity Study, Athiyadhi Kashayam, Acute, Subacute, Hematology, Serology.

INTRODUCTION

The most extensive collection of materia medica is found in the Indian traditional medicine system. Pharmacotherphy, on the other hand, provides as an alternative therapy for chronic diseases that usually result in undesirable side effects owing to long-term exposure to allopathic medications (Ekor, 2014). While interest in these old systems develops, the advantages are limited due to concerns about toxicity that some of these substances may cause. The Siddha school of medicine largely focuses on historical traditional medicines to treat a wide range of infectious and non-communicable diseases (Sen & Chakraborty 2016).

Herbal products have gained steadily growing popularity over the past decade, and are currently utilised by the population of both developed and developing nations (Yuan *et al.,* 2016). Herbal remedies are complex mixture of organic substances that can originate from any part of a plant (Raynor *et al.,* 2011). Despite having promise in vitro and in vivo efficacy, a considerable proportion of treatment candidates lack suitable preclinical toxicity profiles. For a treatment to enter clinical usage, preclinical toxicity studies are critical in the drug research pipeline.

Preclinical toxicity studies are subdivided into short, mid and long term based on the length of the drug administration. These research employ suggested animal models and approved protocols. Correlating the animal responses to humans is the conclusive aim of the toxicology investigations (Anwar *et al.,* 2022).

One of the key objectives of toxicity testing both in the early (preclinical) and late (clinical) stages of the drug research and development process is to detect potentially hazardous compounds that exist in experimental formulations. Moreover, it will offer a chance for a comprehensive examination of safer and more promising alternatives (Van Norman, 2020). Toxicological research aiming at discovering possible danger factors in experimental drugs prior to human exposure, particularly if harm or sickness emerges after a long latency period. To build the notion of preventative toxicological, a paradigm shift is required (Gundert-Remy *et al.*, 2015).

Acute and sub-acute toxicity studies seek to determine the dose range of the test item, whether taken once or repeatedly, causes death or serious toxicological effects. These investigations provide an additional chance to examine the impact the test substance has on morphology, clinical chemistry, or other factors. They can also provide an early warning of chances of adverse events before proceeding clinical investigations (Kpemissi *et al.,* 2020). In the field of pharmacology, safety pharmacology is a subspecialty that focuses on

the identification and characterisation of pharmacological activities that have an impact on the clinical safety of a medication. The core test battery of safety pharmacology is referred to as the cardiovascular test battery, the central nervous test battery, and the respiratory test battery. The guideline suggests evaluating the effects of a potential drug on the functions of the cardiovascular, central nervous, and respiratory systems (Pugsley *et al.,* 2008).

The safety and long history of usage of Siddha medicine have garnered it widespread interest. To determine whether or not the inherent chemical constituents in a siddha formulation provide any sort of harm to the user's health, toxicity tests are routinely conducted. Before being recommended for use in people, traditional medicines like siddha medications must first prove their safety in preclinical trials (Pavithra & Anbu 2022). *Ficus racemo*sa, *Cassia auriculata*, *Cassia fistula*, *Syzygium cumini*, and *Salacia reticulata* come together in a new siddha preparation called *Athiyadhi kashayam*. Multiple indications have been treated with this compound (Hakkim, 1998). There may still be a lack of sufficient documentation to support claims that this formulation is completely risk-free. Therefore, the major goal of the current study is to conduct acute and subacute toxicity tests on the siddha formulation *Athiyadhi kashayam* (AK) in accordance with OECD rules to evaluate its safety profile.

MATERIALS AND METHODS

Animal: Wistar albino adult rats of both sexes were used in the investigation. The facility air handling unit (AHU) helped ensure that the polypropylene cages the animals were kept in were always filled with clean air. There was light and darkness cycle of 12:12 hours were maintained every day. The room was maintained at a temperature of 22±2 degree Celsius and a relative humidity of between 50 and 65 percent. They were regularly fed pellets and have access to an endless supply of water. All of the animals were acclimated to the laboratory environment for a full week before the start of the study. The experimental technique was approved by the Institutional Animal Ethics Committee at K.K. College of Pharmacy in Chennai, Tamil Nadu, India, with the clearance number KKCP/2020/02.

Acute toxicity Study: All of the test animals in this study were fasted for 8-12 hours with free access to water before they were dosed in accordance with OECD guideline 423 (OECD 423,2002). *Athiyadhi kashayam* (AK) 2000 mg/kg (p.o.) was administered orally to the test animals. Observation were evaluated frequently for the first 14 days to look for signs of toxicity after being exposed for the first 4 to 24 hours. The symptoms of CNS and ANS poisoning were investigated. These symptoms included tremors, convulsions, sleepiness, steric behaviour, coma, and death etc. Perioral weighing was used to critically evaluate the indicators of the aforementioned toxicity in animals given the test medication. Gross necropsy and clinical examinations were performed on all animals at the conclusion of the study.

Sub-Acute Toxicity Study: Animals in this study had been accustomed to their environments for 7 days before receiving therapy, as recommended by OECD guideline 407 (OECD 407, 2008). Ten animals (5 males and 5 females) were randomly assigned to each of the two groups: control and drug-treated. Three groups were created: one acted as a control, while the other two received the experimental medication AK (at doses of 200 and 400 mg/kg/day, respectively, for 28 days). Constant weighing and observation for behavioral changes including feeding behaviour were used to detect signs of toxicity in the rats' central nervous system, cardiovascular system, and autonomic nervous system. At the conclusion of the 28th day, the animals fasted for the night while still having access to water. After the 28th day, the animals went without food but had access to water during the night. The animals were given an overdose of the anaesthetics mentioned in the CPCSEA appendix and sacrificed on day 29.

Haematology and Biochemical analysis: The blood samples underwent analysis through established procedures utilising the automated Mindray Haematology Analyzer 2800. The parameters that were assessed are the count of RBC, WBC, and the level of Haemoglobin (Thomas *et al*., 2017). The study involved the analysis of serum samples for various biomarkers including Total Cholesterol, HDL, LDL, TGL, BUN, Creatinine, SGPT and SGOT (Olayode *et al.,* 2019).

Statistical analysis: One-way ANOVA (GRAPH PAD PRISM 5) will be used for the statistical analysis. The data was summarised as a mean SE. Dunnett's test was used to provide a statistical comparison between the two groups. The cutoff for significance was chosen at a p-value of less than 0.05 (Visweswara, 2007).

RESULTS

Effect of *Athiyadhi kashayam's* on clinical symptoms Acute Oral Toxicity Study. The dose of AK used for acute toxicity study is 2000mg/kg which is higher than the normal therapeutic dose. No mortality observed at this dose level, further no significant change with respect to clinical signs on acute toxicity observed for (24-48 h) and a long period (14 days). The results were tabulated in Table 1.

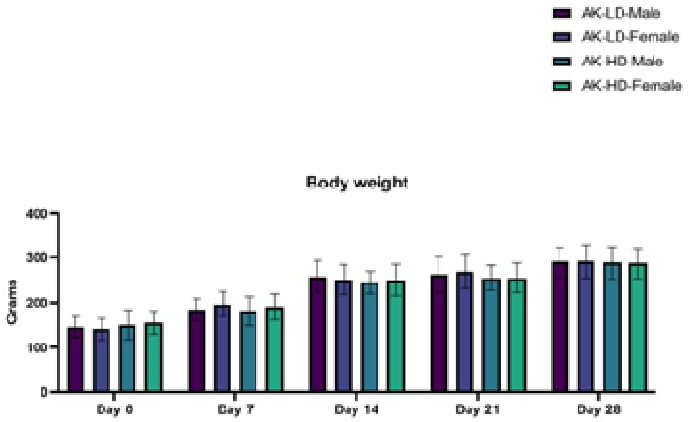
Table 1: Effect of Athiyadhi kashayam on clinical signs in Acute Oral Toxicity Study.

Sr. No.	Parameters	Observation in Control Group	Observation in Test Group Control
1.	Body Mass	No significant deviations found	No significant deviations found
2.	Assessments of posture	No significant deviations found	No significant deviations found
3.	Convulsion	No signs of abnormalities	No signs of abnormalities

	Limb paralysis		
4.	Body composition	No significant deviations	No significant deviations
5.	ANS responses	No significant deviations	No significant deviations
6.	Sensitivity reaction	No significant deviations	No significant deviations
7.	Movement and Locomotion	No significant deviations	No significant deviations
8.	Muscle gripness	No significant deviations	No significant deviations
9.	Urination	No significant deviations	No significant deviations
10.	Pinna Reflex	No significant deviations	No significant deviations
11.	Mortality	None	None
12.	Morbidity	None	None

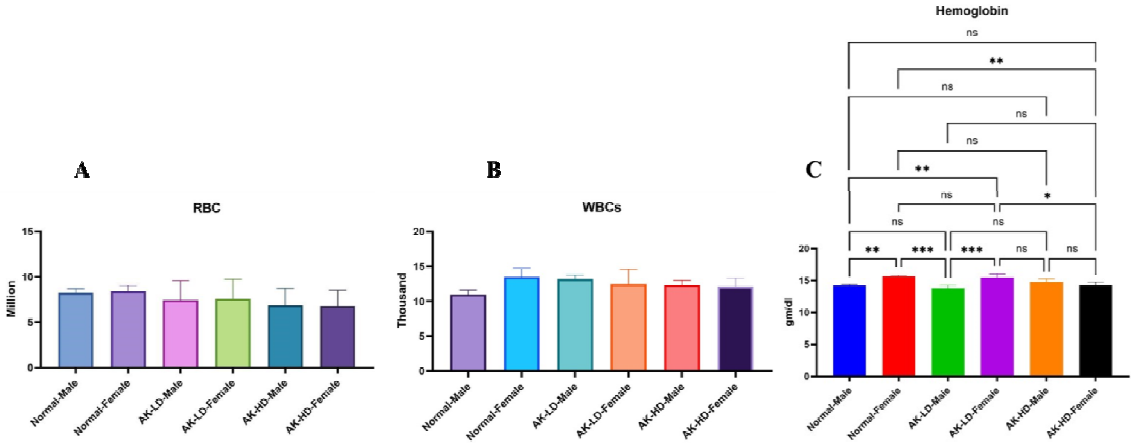
Effect of *Athiyadhi kashayam* on Body mass and Feeding behaviour in sub-acute toxicity. In safety studies body mass is a primary assessor, outcome of the study provokes no evidence of severe toxicity throughout the 28 days of continuous oral treatment with AK at both dosages, as measured by the body weight of the rats submitted to the sub-acute toxicity investigation. Fig. 1 depicts the findings. There was no statistically significant difference in the eating behaviour of either male or female rats given AK at either the low or high dosage levels, according to the results of the examination of their food and water intake.

Sub-acute oral toxicity investigation of *Athiyadhi kashayam* on rat haematology. In sub-acute toxicity study, AK at 200 and 400 mg/kg in female and male rats for 28 days did not significantly change blood parameters like RBC, WBC, and Hb compared to control group rats (Fig. 2).



Note: LD represents low dose and HD represent high dose treatment groups

Fig. 1. Body mass of the experimental Rats in Sub-acute oral toxicity study.

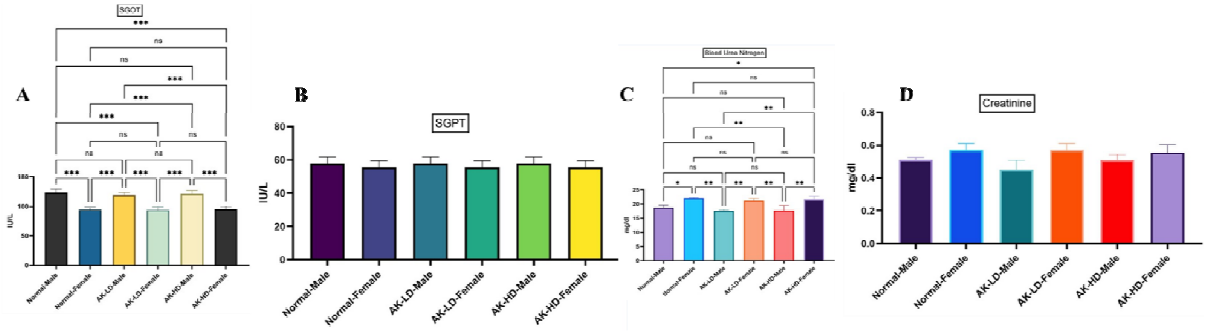


Note: ns represents the value of non-significance, ** represents P< .001 and *** represent P value < .0001

Fig. 2. Toxicity investigation of Athiyadhi kashayam : (A) RBC, (B) WBC and (C) Haemoglobin.

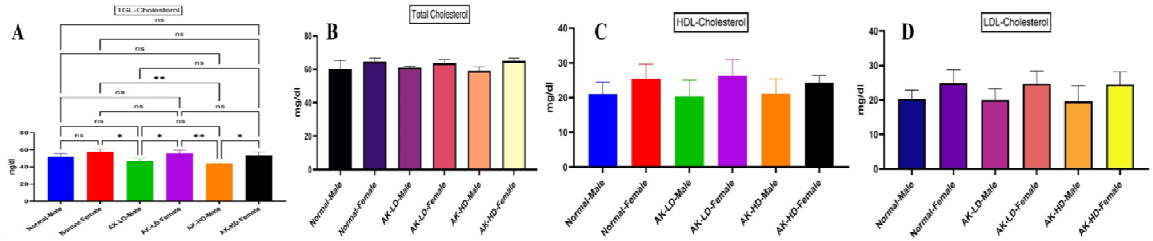
Serology investigation of *Athiyadhi kashayam* in Sub-acute oral toxicity study. In sub-acute toxicity study, female and male rats were treated with AK at 200 and 400 mg/kg for 28 days did not significantly change serological parameters like SGOT, SGPT, BUN, and serum creatinine indicating the wide safety margin of the test drug (Fig. 3).

Effect of *Athiyadhi kashayam* on Lipid profile of rats in Sub-acute oral toxicity study. Administration of AK both the dose level advocates a significant decrease ($p>0.05$) in serum triglyceride level in female rats and a non-significant difference in male rats compared to their control group. A non-significant difference was observed in the serum total cholesterol, HDL and LDL level of sample belongs to female and male rats treated with AK compared to control rats.



Note: ns represents the value of non-significance, ** represents P< .001 and *** represent P value < .0001

Fig. 3. Serology profile of the experimental Rats in Sub-acute oral toxicity study: (A) SGOT, (B) SGPT, (C) BUN and (D) Creatinine.



Note: ns represents the value of non-significance, ** represents P< .001 and *** represent P value < .0001

Fig. 4. Serology profile of the experimental groups: (A) Triglyceride, (B) Total Cholesterol, (C) HDL and (D) LDL.

DISCUSSION

The siddha system of medicine is an age-old practice that has been significantly compensating for the healthcare need of the people for several centuries. The philosophy of siddha therapy is centered primarily on balance among three fundamental humors such as vata, pitha, and kaba. According to siddha philosophy a shift in the balance of the tridoshas can lead to metabolic changes in the body that invites potential health problems (Sivaraman *et al.,* 2019).

Phytomedicine, often known as herbal therapy, has long been considered the most popular alternative medical practise worldwide. This is due to the fact that in both developing and developed countries where conventional or modern drugs are widely used, people who rely on plant-based remedies as an alternative to diseased conditions make up a larger percentage of the world's population (around 80%; Tugume & Nyakoojo 2019). Although many people use herbal therapies, concerns have been raised about their safety due to illnesses and deaths associated with side effects like hepatotoxicity and nephrotoxicity, and only a small fraction of these therapies have been evaluated through clinical trials of varying stages (Amadi & Orisakwe 2018).

Preclinical research relies on acute toxicity studies to categorise and determine doses for experimental drugs. In addition to mortality, biological impacts such study time, behavioural reactions, body weight, and recovery in animals who survived the exposure are important for determining acute toxicity. Acute toxicity study provides the LD50 value, therapeutic index, and pharmaceutical safety (Erhirhie *et al.,* 2018). Acute toxicity studies of AK up to 2000 mg/kg showed no fatality. Bodyweight changes, skin colour change, faecal consistency, gait analysis, urine analysis, sensory responses, animal behaviour abnormalities, and neuromuscular coordination might indicate poisoning. Drug-treated rats and controls had similar behaviour and body weight. Thus, 2000mg/kg siddha formulation AK is harmless.

A study on sub-acute repeated dose toxicity is typically conducted for a duration of at least 28 days. The administration of the test substance occurs on a daily basis over a defined duration via the oral route at a designated time. The biological effects of phytopharmaceuticals can be assessed through oral toxicity testing in rats, as per the principle that pharmacology is essentially a form of toxicology at a reduced dosage (Alkahtani *et al.,* 2022). At lower, non-lethal doses, toxic substances can exhibit intriguing pharmacological properties. Conducting toxicity screening on rodents is a crucial step in assessing the safety profile of herbal preparations. During our

investigation on subacute toxicity, it was observed that rats administered with AK at the doses of 200 and 400 mg/kg did not exhibit any noteworthy alteration in their body weight. The chosen toxicity doses for the study encompass the standard therapeutic range of the experimental drug in human subjects. The results of our 28-day repeated oral dose investigation indicate that subjects administered with the experimental drug AK did not exhibit any clinical signs of toxicity, morbidity, or mortality across the treatment groups. This suggests that the AK formulation was considerably safe at the examined doses throughout the observation period. Red blood cell manufacturing occurs in the bone marrow, where the hematopoietic system is sensitive to harmful chemicals as this system is a vital indication of human and animal health (Abid & Mahmood 2019). AK at 200 and 400 mg/kg in female and male rats for 28 days did not significantly modify RBC, WBC, or Haemoglobin levels compared to control group rats.

Liver damage produced by a chemical agent can be quantified using biochemical indicators like SGOT and SGPT. Liver enzymes is localised in the cytoplasm and is released into the blood when cellular liver injury has taken place. Both SGOT and SGPT are enzymes that are released into the bloodstream when the liver is injured, namely when the mitochondria of liver cells are damaged (Gowda et al., 2009). Increase in liver enzymes becomes a critical marker of hepatic cell membrane damage. Results from the serological analyses showed that repeated oral administration of the test drug KV at both dose levels did not alter the cellular and enzyme levels of the treated rats compared to those of the control group.

Kidney damage caused by drugs is a common problem in the pharmaceutical industry. Serum creatinine values, along with blood urea nitrogen (BUN) levels, have been found to be useful in diagnosing acute renal injury. The ability to detect drug-induced kidney impairment at an earlier stage is a major benefit of novel biomarkers in clinical trials. Muscle-derived creatinine has diagnostic use as a functional marker of renal damage. Released directly into the bloodstream in response to damage, BUN and creatinine serve as a more reliable early indication of drug-induced kidney toxicity (Griffin et al., 2019). In our current study, treatment with AK at 200 and 400 mg/kg for 28 days results in no significant change in BUN and serum creatinine levels, indicating the test drug's broad safety margin. Elevated cholesterol levels might be the cause of drug induced anabolism in hepatocytes or innervation of metabolic dysfunction mediated by the trial drug. Rise in lipid level contributes to cardiovascular disorder hence it become essential to validate the lipid profile as one of the serious bench mark in ascertaining the safety of the AK formulation. Compared to their control group, female rats' blood triglyceride levels decreased significantly ($p>0.05$) after AK administration. Male rats' levels did not change. AK-treated female and male rats had similar blood total cholesterol, HDL, and LDL levels to control rats.

CONCLUSIONS

Siddha, an ancient medical system, is continually providing credible leads for the discovery of therapeutically effective bioactive components from herbs. Therefore, traditional knowledge supported by modern science is necessary to extrapolate the safety profile of the siddha formulations. Acute toxicity testing showed that a dosage of 2000 mg/kg of the trial medication KV had no influence on the animals' normal physiological functioning or behavioural. There were no reported fatalities over the 14-day duration of the trial. Sub-acute toxicity study findings support no obvious changes in body weight, food intake, water consumption, haematological, or serological profiles of the rats. The current study's findings supplied evidence-based data on the safety nature of the siddha medicine *Athiyadhi kashayam*, and they also gave justification for the secure clinical use on people with previous suggested validations.

FUTURE SCOPE

1. This study was primarily focused on evaluating the safety profile of the polyherbal siddha formulation *Athiyadhi kashayam*.
2. Further clinical trial is needed to determine the safety range of *Athiyadhi kashayam* before making a clinical recommendation.

Authors Contribution: The author C. Meenakshi made an immense contribution in study design, Experimental analysis, Data collection and in preparation of the manuscript. The author G. Bharathkumar contributed immensely in data validation, statistical interpretation, result analysis and in preparation of the manuscript.

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Conflict of Interest. None.

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