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Acute Toxicity Profiles of *Siddha* Medicine *Sivanarvembu kuzhi thylam* on Rats

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Siddha medicated oil *Sivanarvembu kuzhi thylam* is used in the management of various skin diseases such as leucoderma, leprosy, eczema, infected wounds, insect bites and boils. Ingredients of this formulation are roots of *Indigofera aspalathoides,* tubers of *Corallocarpus epigaeus* and seeds of *Celastrus paniculatus.* The present study aims to prepare the oil and to evaluate its safety and toxicity profiles by performing heavy metal analysis using Atomic Absorption Spectroscopy (AAS) through *in vitro* assays and carrying out *in vivo* acute toxicity studies in female Wistar rats. AAS results revealed that the heavy metals were present only in the below detectable level. *In-vitro* assay results clearly showed that the aqueous extract of selected drugs showed no toxicity in the healthy PBMCs at various concentrations. It was also observed that PBMCs showed an increase in cell proliferation at higher concentrations of each extract which also suggests that the selected drug may have a positive effect on cell survivability. No mortality was observed in the treated group of rats throughout the observation period of 14 days in acute toxicity studies. Based on the results of the research study, it is concluded that SVKT will not produce any toxic effects. . The acute oral LD_{50} of test substance was observed to be greater than 2000 mg/kg b.w. Based on the results of the research study, it is concluded that SVKT does not produce any toxic effects.

Keywords: *Sivanarvembu kuzhi thylam,* Atomic Absorption Spectroscopy, *in vitro*, *in-vivo*, acute toxicity, Wistar rats.

The traditional Siddha system is one of the most ancient systems of Indian medicine, and unique to the southern part of India.Siddhars are the founders of this system, and they classified Siddha medicines according to the mode of administration into two categories 32 types of internal medicines and 32 types of external medicines[1]. The common preparations of internal medicines include kudineer (decoctions), churnam (medicated powders), legium (medicated jam),nei (medicated ghee), mathirai (pills), vadagam (sweet pills), rasayanam (medicated Granules),parpam (calcined metals and minerals) and chenduram (red oxide)[2].

Thylam is one among them and is used both internally and externally. *Thylam* is a medicated oil preparation and is prepared by boiling the decoction, juice, milk and pastes of herbal drugs with oils as mentioned for a specific

period and filtered. It retains its medicinal value for one year[1].

Some of the *thylam* are prepared by a process termed "*kuzhi thylam*" in siddha terminology. In this process the constituent drugs are crushed coarsely and charged into an earthen pot, the bottom of which is perforated with some holes through which iron wires are passed and drawn out and tied to converge. The mouth of the vessel is covered with a lid and sealed. A pit is dug into which the pot will just fit in. At the bottom of the pit, a collecting vessel is placed and centered. The pot is placed in it so that the convergent wires are pointing to the mouth of the collection vessel. Cow dung cakes are arranged around the pot and they are set to fire. The set up is dismantled after it is completely cooled down and the oil is collected in the vessel at the bottom and is taken for further use[2&3].

In the present study a polyherbal medicated oil *"Sivanarvembu kuzhi thylam"* (SVKT) is selected which is used for skin/dermal problems, infected wounds, insect bites and boils[4]. The present study aims to prepare the oil and evaluate the heavy metal limits and also carry out *in-vitro* and *in-vivo* acute toxicity profiles in female wistar rats. It has three ingredients namely roots of *Indigofera aspalathoides,* tubers of *Corallocarpus epigaeus* and seeds of *Celastrus paniculatus*[2].

In the present scenario mercury, lead, cadmium and arsenic are common heavy metals in all edible products. These are toxic to human beings if we are exposed to longer periods[5]. To prove the safety of SVKT - heavy metal analysis was performed using Atomic Absorption Spectroscopy (AAS) for the oil and its main ingredient *Indigofera aspalathoides (Sivanarvembu choornam* –SVC). In this work, *in vitro* and *in vivo* acute oral toxicity profiles were also determined with a view to assess the non-toxic nature of the selected formulation.

MATERIALS AND METHODS

Plant drugs used as ingredients

Roots of *Indigofera aspalathoides,* tubers of *Corallocarpus epigaeus* and seeds of *Celastrus paniculatus*[2] (Figure 1) were procured from the local herbal market Thanjavur, Tamil Nadu, India, identified and authenticated in the NABL accredited Pharmacognosy lab of CARISM, SASTRA University.

Indigofera aspalathoides

Corallocarpus epigaeus

Celastrus paniculata

Fig. 1. Herbal ingredients of SVKT

In the Siddha system, *Indigofera aspalathoides* is called as *sivanarvembu* or *ghanthaari.* It acts as a stimulant and demulcent. It is used for treating all types of skin diseases including leprosy, fissure of palms and soles itching, fungal infection of the skin, insect bites and chronic wounds and also makes the skin shining. It is also used in treating asthma and flatulence. This root relieves the tooth pain, mouth ulcer and swelling[6]. It has anti-inflammatory activity and cures psoriasis disease which was proven by Baskar et.al, (2015) in their *in vivo* research[7]. This plant has anti diabetic activity[8]. Besides, its anticancer potential was also proved through the in-*silico* studies by Krishnasamy et.al, 2015[9]. It is also the main ingredient of a *Siddha* formulation namely *Makavallati ilakam*[2]. Earlier studies reported its antibacterial activity[10] and very high anti-inflammatory activity[11].

The second drug is *Celastrus paniculatus* seeds. In Siddha, it called as *vazhuluvai, kanguni, mallkanguni* and *athiparicham*. It acts as an aphrodisiac, stimulant, alterative, diaphoretic and as nervine tonic. It is used for treating diarrhea, dysentery, amebic colitis, irritable bowel syndrome, enteritis, cough, hemiplegia, joint pain, pricking pain of the limb and also used to heal chronic wounds. A review article explains that it can act as an antiviral, antibacterial, insecticidal, anti-inflammatory, anti-spermatogenic, sedative, anti-fatigue, analgesic, hypolipidemic, arthritogenic, antirheumatic, aphrodisiac, emetic, laxative and also as a nervine tonic[12]. Herbal oil extracted from this seed is called *Jyotishmati* oil in Ayurveda. It has a medicinal effect on the Central Nervous System. It is employed to treat acute and chronic immobilization stress. The oil obtained from the seeds possesses sedative and anticonvulsant properties[13]. It is the main ingredient in many *Siddha* formulations like *Irasakanti meluku*, *Idi vallathi melugu, Karudankilangu enney* and *Makavallati ilakam*[2].

The third ingredient is the tuber of *Corallocarpus epigaeus*. In Tamil, it is called a*gasakarudan kizhangu, kollancovai* and *pei–seenthil.* It has alterative and tonic properties. It is useful in anemia, body pain, internal body heat, itching, eczema and herpes zoster. It acts as an antidote for heavy poison. A research paper explained that this tuber has antifungal activity[13]. Its anti diabetic activity was proved with ethanol extract by Gnananath et al., (2013)[14]. An *In vivo* screening proved its analgesic, anti-inflammatory and anti-pyretic activities[15]. It is one of the ingredients in a Siddha herbo – metallic formulation of *rasaganthi melugu*[16].

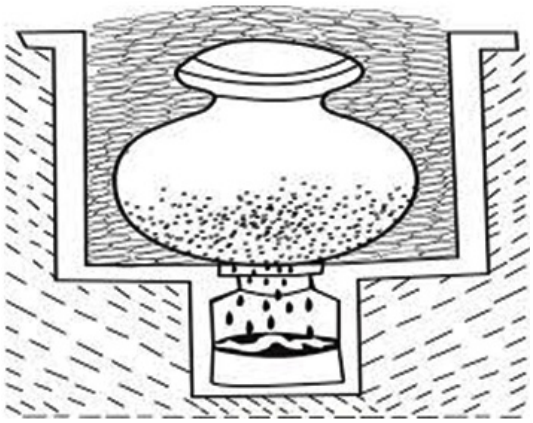

Fig. 2. The set-up model of *kuzhi thylam* preparation

Preparation of the oil

All the ingredients were powdered coarsely using a lab mill and then mixed as per the procedure given in *the Siddha* formulary of India and Formulary of Siddha medicine. *Indigofera* whole plant and *Celastrus seeds* were ground with *Corallocarbus* tuber juice and cakes were made and kept aside for semi drying. An earthen pot with few holes in the bottom was taken and thin wires were bent such that they converge and these converged wires were placed in the holes of the bottom of the earthen pot a few inches away from the base. This set pot was filled with the semi-dried cakes prepared using herbal ingredients. The mouth part was sealed with a suitable earthen plate using mud clay dipped gada cloth. The oil was prepared

Table 1. Heavy metal analysis by atomic absorption spectrometry(AAS)

Sample name	Lead (ppm)	Cadmium (ppm)	Mercury (ppm)	Arsenic (ppm)
SVKT	BDL	BDL	BDL	BDL

through the destructive distillation of the plant ingredients and collected by placing a collecting vessel in the center of the pit (Figure 2). Heat was applied only to the pot by igniting cow dung cakes arranged around and above the pot. After cooling removed the upper pot and collected the oil in the bottom vessel. Oil had blackish brown color and fire smoke smell.

Atomic Absorption Spectroscopy (AAS)

It is a heavy metal analyzing instrument according to Beer-Lambert's law, the model is Analyst 400 (for flame), HGA 900 (for graphite furnace), Perkin Elmer. If a solution is aspirated into a flame, a vapor that contains atoms of the metal may be formed. Some of the gaseous metal atoms may be raised to an energy level which is sufficiently high to permit the emission of radiation characteristic of the metal. However, a much larger number of atoms will normally remain unexcited or ground state. These ground state atoms are capable of absorbing radiant energy of their specific resonance wavelength. The extent of absorption will be proportional to the number of ground-state atoms present in the flame. Based on this concept presence of heavy metal is calculated.

In vitro toxicity study

MTT assay procedure

In vitro cytotoxicity was evaluated for the aqueous extract of SVKT and the main ingredient SVC (dry powder of *Indigofera aspalathoides*) employing MTT assay as per standard textual procedures of Mossman T, (1983)[19]. This assay was performed against freshly collected healthy Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were treated with various concentrations of selected drugs and incubated for 24 h at 37 °C in humidified 5% CO_2. After 24 hours, 10 ìL aliquots of serial dilutions of plant powder and oil aqueous extract (1000-1.95 ìg/ml) were added to cells and incubated for 48 h. Cell viability was assessed through the MTT assay. Briefly, 25 ìL of MTT (5 mg/mL) was added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilized with 100 ìL of a solution containing 50% N, N-dimethylformamide and 20% Sodium dodecyl sulfate. The optical density of each well was measured using the Epoch microplate spectrophotometer (BioTek, USA) set at 590 nm filter.

Acute Toxicology Study Design

The acute oral toxicity of SVKT was performed as per OECD-425 guidelines[20]. The study was conducted after approval by the Institutional Animal Ethical Committee, SASTRA Deemed University. (IAEC Approval Number: 310 /SASTRA/IAEC/RPP). Five healthy female Wistar rats of age 8 to 12 weeks, which were nulliparous and non-pregnant were chosen for the conduct of the present study. All the experimental animals were maintained under standard conditions (Temperature: 22±3°C and relative humidity: 50 to 70%) during the experimental period. The animals were provided with standard rodent pellet feed (Altromin, Germany) and RO water *ad libitum* and were acclimatized for seven days before the conduct of the study.

SVKT was suspended in distilled water, administered to all the animals at a dose of 2000 mg/kg body weight and were observed for 14 days. All the animals were fasted overnight before drug treatment. The test substance was administered by oral gavages using syringe and stainless steel ball - tipped oral gavage needle. During the observation period, all the animals were observed twice every day for mortality. Bodyweight of each animal was recorded just prior to the test substance treatment (Day 0), Day 7 and 14 using an electronic animal weighing balance (Sartorius AG, Germany). The feed intake for individual animals were recorded daily for the entire study period.

All the animals were observed individually after the treatment of the test substance during the entire observation period for the presence of any signs of toxicity including alopecia, catalepsy, chromodacryorrhea, clonic, coma, convulsion, diarrhea, dullness, excessive grooming, change in gait, hyperactivity, lacrimation, nasal discharge, nasal irritation, piloerection, polyuria, prostration, repetitive circling, respiratory distress, salivation, scaling, tonic, tremor and uro-genital staining. After 14 days of observation period, all the animals were euthanized using carbon-di-oxide and were subjected to gross pathology.

RESULTS

AAS results

The AAS results (Table 1) revealed that the levels of heavy metal in the herbal oil SVKT

was below the detectable limit (BDL). This type of AAS method was previously used by *Bhattacharjee* et al., 2017 for determining heavy metal contents in plants [21].One more *Siddha* research paper declared that heavy metal limits in BDL .

***In vitro* toxicity study**

The results clearly showed that the aqueous extract of selected drugs SVKT and SVC showed no toxicity to healthy PBMCs at different concentrations used. It was also observed that PBMCs showed an increase in cell proliferation at higher concentrations. Figure.3 explains the toxicity of SVKT and SVC on normal PBMC.

***In vivo* Acute oral toxicity study (AOT)**

Acute oral toxicity study for SVKT was done in female rats at dose of 2000 mg/kg body weight as a single administration following OECD guideline 425[20]. In the fourteen days of study period we observed the animal death, weekly body (Table 2), Daily feed intake was measured and remained unaffected (Table 3), noted any signs of toxicity such as changes in respiration, circulation, autonomic and central nervous system, behavioral pattern were observed during the entire observation period.

Table 2. Weekly Mean Body Weight Changes In Rats

Animal ID	Sex	Body Weight(g)		
		Day 0	Day 7	Day 14
4319	Female	202.66	204.85	217.34
4320	Female	193.21	195.45	197.15
4321	Female	196.41	196.21	204.81
4322	Female	195.30	200.89	202.47
4323	Female	193.18	187.36	190.35
	Mean	196.15	196.95	202.42
Standard deviation		3.89	6.57	10.02

DISCUSSION

AAS results

From this results we concluded that SVKT has no any heavy or toxic metal. Table.1 clearly shown this. One more Siddha research paper declared that heavy metal limits in BDL[22] .

***In vitro* toxicity study**

It was also observed that PBMCs showed an increase in cell proliferation at higher concentrations of each extract which also suggests that selected drugs may have a positive effect on cell survivability. In these results compared to SVC and SVKT has high cell proliferation activity. This type of in vitro study was carried out previously by Christian G J et.al., 2016 in the *Siddha* medicine *Kariuppu mezhugu*[23].

***In vivo* Acute oral toxicity study (AOT)**

No mortality was observed in any treated rats during the experimental period and all animals survived up to 14 days after the administration of SVKT[24]. It was observed from the present study that a slight increase in weekly body weight

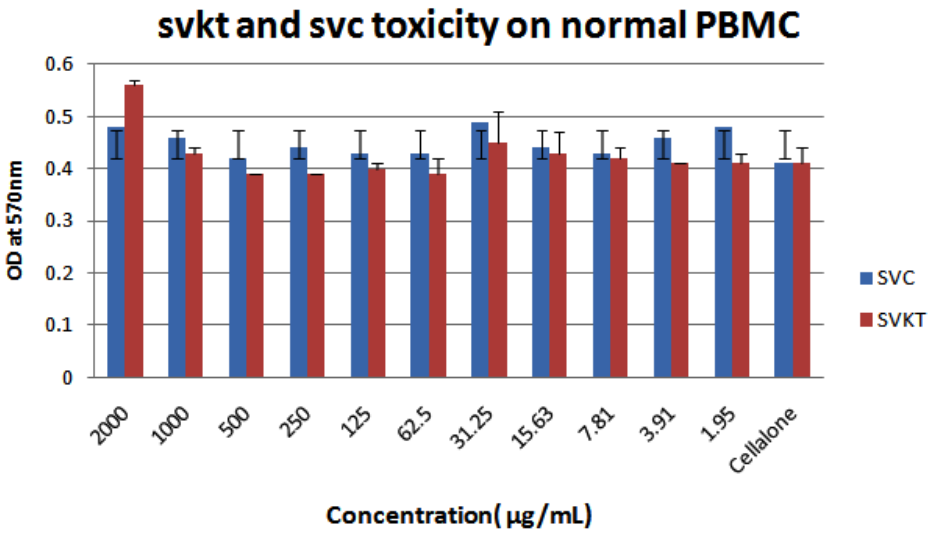

Fig. 3. SVKT and SVC toxicity on normal PBMC

Table 3. Daily feed intake

Animal ID	Sex	Day 0	1	2	3	4	5	6	7	8	9	10	11	12	13
4319	F	5.69	12.86	14.25	9.90	12.12	11.28	11.95	12.68	11.44	16.21	11.54	9.91	13.90	12.15
4320	F	12.35	17.32	12.51	14.52	7.95	10.07	11.45	12.96	11.06	9.04	13.24	8.64	9.43	12.91
4321	F	11.18	11.78	11.73	12.96	12.06	9.11	16.48	16.22	12.40	11.22	12.37	11.47	12.49	16.00
4322	F	11.58	13.06	11.77	12.22	11.03	11.05	15.33	11.16	13.48	12.10	13.26	10.41	9.57	16.48
4323	F	10.38	12.97	13.75	9.65	10.47	6.83	13.73	9.41	13.85	8.34	11.50	10.87	10.10	13.69
Mean		10.24	13.60	12.80	11.85	10.73	9.67	13.79	12.49	12.45	11.38	12.38	10.26	11.10	14.25
SD		2.64	2.14	1.15	2.07	1.70	1.81	2.15	2.52	1.22	3.11	0.87	1.07	1.99	1.91

was found in SVKT treated animals on Day 14 (Table.2). Daily feed intake was measured and remained unaffected throughout the experimental period (Table.3). No visible signs of toxicity such as changes in respiration, circulation, autonomic and central nervous system, behavioral pattern were observed during the entire observation period.

No test compound-related findings were observed at necropsy. All gross observations were agonal (abnormal pattern of breathing) in nature and bore no relation to treatment with the test substance. All animals survived until the end of the study. With this data, we concluded that SVKT did not produce any toxicity to humans. A similar *in vivo* toxicological study was done on a Siddha medicine *Vajjiravalli chooranam* and concluded that herbal medicines have a non-toxic effect even if used long time for therapeutic purpose [25].

CONCLUSION

To confirm the therapeutic safety of SVKT, the acute toxicity test was carried out. AAS results revealed that the toxic metals are present only below the detectable level. SVKT was orally given at a higher dose of 2 gm/kg to the wistar rats. Single-dose oral administration of the test substance did not produce any adverse toxic effect on the body weight changes, feed and the gross anatomy of treated female wistar rats. The acute oral LD_{50} of the test substance was observed to be greater than 2000 mg/kg b.w. Clinically SVKT has used by the Siddha medicine practionares and its therapeutic effects is already proven one. This research work is a small step to prove this in scientifically.

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Abbreviations used

SVKT-*sivanarvembu kuzhi thylam;* WHO-World Health Organisation; AAS-Atomic Absorption Spectroscopy; SVC – sivanarvembuchoornam; PBMC-Peripheral blood mononuclear cells; IAEC-Institutional Animal Ethics committee; BDL-Below Detectable Limit; PPM-parts per million; OECD- Organization for Economic Co-operation and Development; SD-Standard Deviation; LD-Lethal Dose.

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