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In vivo Safety Profiling of Nilavembu Kudineer and Kabasura Kudineer Chooranam in Zebrafish Embryos

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Siddha medicine is one of the most ancient medical systems of India in which Nilavembu Kudineer Chooranam (NKC) and Kabasura Kudineer Chooranam (KKC) were commonly used preparations for various ailments.
Objective: This study aimed to assess the teratogenic effects and determine the LC50 values of Nilavembu Kudineer Chooranam (NKC) and Kabasura Kudineer Chooranam (KKC) using zebrafish embryos as a model.
Methodology: The study was conducted as per the OECD Test Guideline 236 using zebrafish embryos. Each group consisted of 20 embryos, with two replicates per drug. The aqueous extracts

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of NKC and KKC were prepared and for NKC, a dose range 750–12,000 µg/mL, while for KKC, it was 10–640 µg/mL were used as test concentrations. The developing embryos were monitored for mortality, hatchability, and developmental abnormalities at 5, 24, 48, 72 and 96 hours post-fertilization (hpf).

Results: The LC50 value for NKC at 96 hpf was determined to be 2208 µg/mL, with no evidence of teratogenicity. For KKC, no mortality or teratogenic effects were observed at the tested concentrations.

Conclusion: The current findings demonstrates that NKC produces 50% mortality at the concentration of 2208 µg/mL, dose dependent hatchability and no developmental abnormalities up to 96 hpf. Whereas, KKC produced no mortality, teratogenicity and 100% hatchability at the tested concentrations up to 96 hpf.

Keywords: Zebrafish; Nilavembu Kudineer Chooranam; Kabasura Kudineer Chooranam; teratogenicity.

1. INTRODUCTION

Herbal medicine remains essential and widely practiced in many developing countries. In India, 70% of the population relies on traditional herbal remedies for their primary healthcare needs (Bodeker, 2005). The Siddha medicine, one of the oldest medicinal system in the world is been widely used in the southern states of India. They have gained popularity in the treatment of various infectious and highly contagious diseases like chikungunya, dengue fever, and swine flu (Meenakumari et al., 2021). The Siddha system of medicine, which dates back centuries, has been practiced by Tamil vaithiyars (native healers of Tamil Nadu), who promoted an impeccable lifestyle that continues to influence societies in Tamil Nadu (Patwardhan and Hooper, 1992). Among Indian traditional medicine systems, Siddha holds a holistic position, pioneering treatments for both communicable and non-communicable diseases (Elakkiyaa et al., 2020).

Among Siddha formulations, *Nilavembu Kudineer Chooranam* (NKC) is a classical polyherbal remedy prescribed for viral fevers, associated pain, and immunity enhancement to prevent the recurrence of viral infections. It consists of 15 ingredients in equal proportions and its detailed composition is mentioned in Table 1 (Mekala and Murthy, 2020). It is commonly recommended as a prophylactic during monsoons and as an adjunct therapy for patients with viral fevers (Amruthavalli et al., 2020). Classical Siddha texts recognize it as a first-line therapy and a general remedy for fevers caused by unidentified microbial infections (Noble and Shi, 2012). In desi chickens, this preparation reported to stimulate both humoral and cell-mediated immunity (Kavinilavan et al., 2017). *Andrographis paniculata* (nilavembu), one of the ingredients in NKC has shown effect against Newcastle disease virus in chickens (Nagajothi, 2018).

Another notable Siddha preparation, *Kabasura Kudineer Chooranam* (KKC), is a polyherbal decoction comprising 15 medicinal herbs, the detailed composition is mentioned in Table 2 (Noble and Shi, 2012). In the name kabasura kudineer chooranam, kaba refers to kapha dosha, which indicates fever caused by an excessive buildup of kapha (mucus and phlegm). Asura signifies herbs that relieve these symptoms, while kudineer means decoction and choornam refers to powder. This preparation is reported to have anti-inflammatory, antipyretic, and antibacterial properties (Saravanan et al., 2018). The Siddha manuscript, “Citta Vaittiyattirattu” mentions the use of it to treat common respiratory issues like flu, dry and wet cough and fever (Harini et al., 2022). It is known for its effectiveness against respiratory infections and possesses anti-inflammatory, analgesic, antiviral, antifungal, antioxidant, hepatoprotective, antipyretic, anti-asthmatic, and immunomodulatory properties. Due to its antiviral and immune-boosting effects, the Tamil Nadu government has recommended *Kabasura Kudineer* as a therapeutic remedy for COVID-19 management (Taskeen et al., 2022).

Despite the proven pharmacological efficacy and clinical benefits of these formulations, ensuring their safety is crucial to maximizing their therapeutic potential. The validation of many Siddha formulations remains limited, even as global interest in Siddha medicine continues to grow. Reliable safety assessments are essential. Traditional toxicity screening methods, which involve animals such as rodents, dogs, and

Table 1. Composition of *Kabasura kudineer chooranam*

S. No.	Botanical Name	Local Name (Tamil)	Part
1	*Anacyclus pyrethrum* (L.) Link	Akkirakaram ver	Root
2	*Andrographis paniculata* Burm.f.Nees	Nilavembu	Whole plant
3	*Clerodendron serratum* (L.) Moon	Siruthekku	Root
4	*Coleus aromaticus* Benth	Karpuravalli	Leaf
5	*Costus speciosus* (J.Koenig) Sm.	Koshtam	Root
6	*Cyperus rotundus* L.	Korai kilangu	Root tuber
7	*Hygrophila auriculate* Schumach	Neermulli ver	Root
8	*Justicia adhatoda* L.	Adathodai	Leaf
9	*Piper longum* L.	Thippili	Fruit
10	*Sida acuta* Burm.f.	Vattatthiruppi ver	Root
11	*Syzygium aromaticum* (L.) Merr. & L.M.Perry	Ilavankam	Flower bud
12	*Terminalia chebula* Retz.	Kadukkai	Fruit rind
13	*Tinospora cordifolia* (Thunb.) Miers	Seendhil	Stem
14	*Tragia involucrate* L.	Sirukanchori ver	Root
15	*Zingiber officinale* L.	Sukku	Rhizome

Table 2. Composition of *Nilavembu kudineer chooranam*

S. No.	Botanical Name	Local Name (Tamil)	Part
1	*Andrographis paniculata* Burm.f. Nees	Nilavembu	Whole plant
2	*Chrysopogon zizanoides* (L.) Roberty	Vettiver	Root
3	*Cyperus rotundus* L.	Koraikilangu	Root tuber
4	*Plectranthus vettiveroides* (Jacob) Singh and Sharma	Vilamichai ver	Root
5	*Piper nigrum* L.	Milagu	Fruit
6	*Mollugo cerviana* (L.) Ser.	Parpadagam	Whole plant
7	*Santalum album* L.	Sandanam	Bark
8	*Trichosanthes cucumerina* L.	Paipudal	Whole plant
9	*Zingiber officinale* Roscoe	Sukku	Rhizome

rabbits, are expensive, time-consuming, and labor-intensive (Han et al., 2018). A promising alternative for *in vivo* toxicity testing is the use of zebrafish (*Danio rerio*) embryos. This model is versatile and well-suited for high-throughput analysis, offering advantages typical of *in vitro* approaches, such as low cost, high sensitivity, and rapid testing durations (Elakkiyaa et al., 2020). Zebrafish is one among the OECD-recommended test species outlined in Test Guideline 236 (Fish Embryo Acute Toxicity Test) and Test Guideline 215 (Fish, Juvenile Growth Test). Zebrafish assays align with the 3R principles of animal studies- Reduction, Replacement, and Refinement- and provide a refined research model. The transparency of zebrafish embryos during early developmental stages allows for detailed observations (Van der Laan et al., 2012, Cassar et al., 2020). Therefore, the objective of this present study is to evaluate the safety profile of *Nilavembu Kudineer Chooranam* (NKC) and *Kabasura Kudineer Chooranam* (KKC) using zebrafish embryos as a model.

2. MATERIALS AND METHODS

2.1 Preparation of NKC and KKC Decoction

The commercial preparations of *Nilavembu Kudineer Chooranam* (NKC) and *Kabasura Kudineer Chooranam* (KKC) were purchased from the local pharmacy. The decoction of KKC was prepared by boiling 5 g of KKC powder in 240 mL of water, reducing it to one-fourth of its volume (60 mL), and then filtering it (Natarajan et al., 2020). To prepare the aqueous extract of NKC, 25 g of coarse NKC powder was boiled with 800 mL of water until it was reduced to 125 mL and then filtered. A stock solution with a concentration of 50 mg/mL was then prepared for further studies (Shanmugapriya et al., 2019).

2.2 Zebrafish Husbandry

Adult zebrafish were reared in a mixture of dechlorinated tap water and reverse osmosis (RO) water at a ratio of 4:1. The rearing environment was maintained at a temperature of

29°C, with a pH range of 7.6 to 8.4. Water hardness was controlled between 50 and 100 mg/L, and electrical conductivity was maintained between 360 and 520 µS. The fish were fed earthworm flakes twice daily as their standard diet. Additionally, one week before the breeding period, they were provided with freeze-dried, protein-rich worms (Westerfield, 2007).

2.3 Breeding

For breeding, a specially designed transparent plastic breeding tank was used, consisting of two nested bread boxes, with the inner box having a window mosquito net at the bottom. Two pairs of male and female zebrafish were introduced into the breeding tank at a 2:1 ratio. The fish remained in the breeding tank overnight, and the following morning, upon exposure to the first flash of light, the female zebrafish laid eggs. These eggs were externally fertilized by sperm released by the male zebrafish (Westerfield, 2007). The live eggs, identified by their small, transparent, and round shape, were carefully collected using a Pasteur pipette and transferred to Petri dishes filled with embryo water (prepared by dissolving 0.06 g of ocean salt and 100 µL of methylene blue in 1 L of RO water). Only normally dividing, spherical embryos at 5 hours post-fertilization (hpf) were selected for the study (Truong et al., 2011).

2.4 Toxicity Assessment of NKC and KKC

At 5 hpf, zebrafish embryos were exposed to various concentrations of NKC (750, 1500, 3000, 6000, and 12,000 µg) and KKC (10, 20, 40, 80, 160, 320, and 640 µg), along with a control group. Each group consisted of 20 embryos with three replicates. The percentage of livability, hatchability, and abnormalities in zebrafish embryos/larvae treated with different concentrations of NKC and KKC in groups I to VI was observed at 5, 24, 48, 72, and 96 hpf. The LC50 was determined using probit analysis (da Silva Jr et al., 2023).

3. RESULTS AND DISCUSSION

3.1 Mortality, Hatchability and Abnormality Assessment

Though the KKC and NKC were commonly used for the treatment of various communicable and non-communicable diseases, their developmental toxicity is not yet explored. Hence, in the present study zebrafish embryos were used as the model to assess the developmental toxicity of them. The study was conducted as per OECD Test Guidelines 236 using zebrafish embryo. Mortality and hatchability were recorded from 5 hpf to 96 hpf. Embryos hhwere examined for developmental abnormalities, including a curved body axis, craniofacial malformations, pericardial oedema, yolk sac oedema, and whole-body oedema at 24, 48, 72, and 96 hpf. The observed parameters in the NKC- and KKC-treated groups are presented in Tables 3 and 4, respectively. The developmental stages of the zebrafish embryos were shown in Fig 2.

3.2 Nilavembu Kudineer Chooranam (NKC)

Investigation of teratogenic effects of NKC in zebrafish embryos at concentrations ranging from 10 µg to 640 µg, reported no mortality and teratogenicity (Shanmugapriya et al., 2019). Based on these findings, the present study tested NKC lethality at concentrations ranging from 750 µg to 12,000 µg. The NKC-treated groups exhibited dose-dependent mortality (Fig. 3). The LC50 value was determined using probit analysis, a graphical method for calculating LC50 values, where the log dose (X-axis) was plotted against the probits of mortality (Y-axis) for various concentrations (Zahir et al., 2021). The LC50 value at 96 hpf, as determined by probit analysis, was 2208 µg/mL (Fig. 1). No developmental abnormalities were observed at any dose level of NKC at the dose range of 10–640 µg/mL range aligning with our current study (Shanmugapriya et al., 2019).

Additionally, in another study, no mortality or toxic signs were noticed in an acute toxicity study of NKC in mice at doses up to 2000 mg/kg (Anbarasu et al., 2011). NKC demonstrated no significant treatment-related toxicity in a 90-day oral toxicity study in Sprague-Dawley rats, with the no observable effect level (NOEL) established at 200 mg/kg, supporting its safety for potential long-term therapeutic use at this dose (Sukkur et al., 2024). In current study, NKC produced dose dependent decrease in hatchability and the unhatched embryos did not survive. These findings indicate that NKC is not teratogenic at doses up to 12,000 µg/mL; however, mortalities were observed at concentrations starting from 750 µg/mL in zebrafish embryos.

Table 3. Observed parameters in NKC treated zebrafish embryos

Parameters	Concentration (µg/ml)					
	Control	750	1500	3000	6000	12000
Hatchability	100%	89%	68%	47%	19%	0%
Mortality	0%	12%	34%	56%	82%	100%
Curved body axis	NSA	NSA	NSA	NSA	NSA	NSA
Craniofacial malformation	NSA	NSA	NSA	NSA	NSA	NSA
Pericardial oedema	NSA	NSA	NSA	NSA	NSA	NSA
Yol sac oedema	NSA	NSA	NSA	NSA	NSA	NSA
Whole body oedema	NSA	NSA	NSA	NSA	NSA	NSA

NSA – No Such Abnormality

Table 4. Observed parameters in KKC treated zebrafish embryos

Parameters	Concentration (µg/ml)							
	Control	10	20	40	80	160	320	640
Hatchability	100%	100%	100%	100%	100%	100%	100%	100%
Mortality	0%	0%	0%	0%	0%	0%	0%	0%
Curved body axis	NSA	NSA	NSA	NSA	NSA	NSA	NSA	NSA
Craniofacial malformation	NSA	NSA	NSA	NSA	NSA	NSA	NSA	NSA
Pericardial oedema	NSA	NSA	NSA	NSA	NSA	NSA	NSA	NSA
Yolk sac oedema	NSA	NSA	NSA	NSA	NSA	NSA	NSA	NSA
Whole body oedema	NSA	NSA	NSA	NSA	NSA	NSA	NSA	NSA

NSA – No Such Abnormality

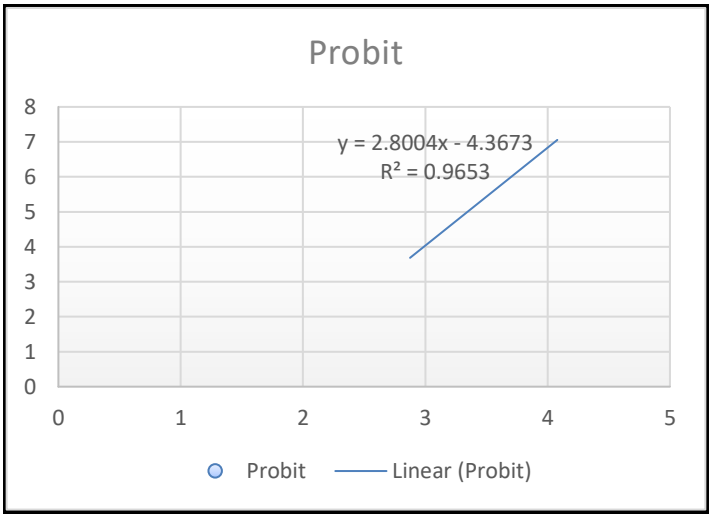

Fig. 1. Probit analysis to find the 96 hpf LC50

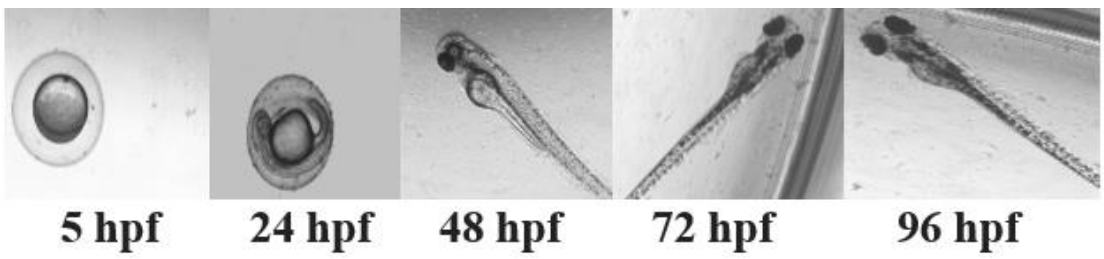

Fig. 2. Developmental stages of zebrafish embryos treated with KKC

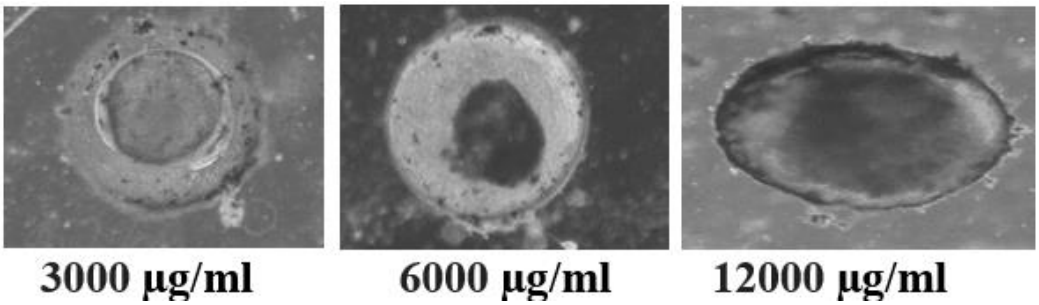

Fig. 3. Dead embryo at 24 hpf treated with 3000 µg/ml of NKC

3.3 Kabasura Kudineer Chooranam (KKC)

Zebrafish embryos were exposed to KKC at concentrations ranging from 10 µg/mL to 640 µg/mL. No mortality or developmental abnormalities were observed at any tested concentration up to 96 hpf. All groups exhibited 100% hatchability at 96 hpf. In a study it was found that KKC, at doses up to 1.5 mL/kg, exhibited no toxic effects in rats (Muthuramu and Yessu, 2020). In another study, *in silico* toxicity screening of 37 phytoconstituents of KKC revealed no carcinogenic or tumorigenic properties (Kiran et al., 2022). In a study as per OECD TG 407- Repeated dose 28-day Oral Toxicity studies of drugs in rodents, KKC at doses of 200mg, 400mg, 800mg, showed no mortality or no abnormal effects were noticed. The feed and water intake, average body weight gain, hematology, serum biochemistry and histopathology were normal and reported that KKC is safe for recurrent use (Parvatharajakumaran et al., 2023). The KKC has promising anti-inflammatory and antioxidant activities (Shruthi et al., 2021), thereby supports embryonic survival and development. Several in silico, in vitro, in vivo, and clinical investigations showed the potential of herbal medication to treat and prevent COVID (Shivangani et al., 2023) and decrease in viral load was noticed in asymptomatic COVID-19 patients who received KKC for a period of 7 days (Natarajan et al., 2020). Therefore, the present study confirms that KKC does not cause developmental toxicity or mortality at doses up to 640 µg/mL in zebrafish embryos.

4. CONCLUSION

Developmental toxicity was assessed for NKC and KKC at dose ranges of 750 µg/mL to 12,000 µg/mL and 10 µg/mL to 640 µg/mL, respectively. Embryos were monitored for mortality, hatchability, and developmental abnormalities up to 96 hpf. The LC50 value for NKC at 96 hpf was determined to be 2208 µg/mL, with no evidence of teratogenicity. No mortality or teratogenic effects were observed for KKC at the tested concentrations. Therefore, the polyherbal formulations NKC and KKC do not exhibit teratogenic effects at the tested doses in zebrafish embryos.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc. have been used during the writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology.

Details of the AI usage are given below:

1. ChatGPT was used for spelling and Grammer check of the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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