

Preparation of Karkataka Taila, an Edible crab Rasayana, and assessment of its toxicological effects on SH-SY5Y cell line and on *Drosophila melanogaster* embryos

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ABSTRACT

Background: Karkataka Taila (KT) is a virgin coconut oil (VCO) based Rasayana formulation that is enriched with the flesh of freshwater edible crab, *Scylla serrata*, used to treat Parkinson's Disease (PD) or Kampavata by local Ayurveda practitioners of Kerala state. There is no scientific study carried out on its toxicological effects so far. **Objective:** To understand the ayurvedic preparation method for KT and assessment of the toxicological effects of the KT and VCO on SH-SY5Y cell lines and *Drosophila melanogaster* embryos. **Materials and methods:** The SH-SY5Y cell lines treated with different concentrations of KT and VCO range from 6.25 µg/ml to 100 µg/ml and *Drosophila melanogaster* embryos fed with food containing different concentrations of KT and VCO, ranging from 0.005 % to 10 %. **Results:** KT and VCO did not show any significant cytotoxicity effect on SH-SY5Y cell lines up to a dose concentration of 25 µg. But, at 50 µg and 100 µg concentrations, KT has shown a cytotoxic effect and it was higher than the VCO. The toxicological analysis in *Drosophila* has shown that the survival rate of the KT treated group at concentration ranges from 0.005 % to 10 % is significantly decreased from 78.8 % to 27.7 %, compared to the control group, whereas in VCO treated group, at 0.005 % to 10 %, the survival rate has decreased from 76.2 % to 66 %, which is marginally higher than the KT treated group. **Conclusion:** Our findings revealed that as the concentration of Rasayana in the medium increases, there is a noticeable adverse effect on the percentage viability in SH-SY5Y cell lines and in the number of offspring in *Drosophila*. The effect of vehicle, VCO, at the same concentration has shown a protective effect on cell lines and flies. It can be concluded that the toxic effect has been observed only at higher concentrations of KT and at the lower concentration, the toxic effect has been minimal.

Key words: *Drosophila melanogaster*, SH-SY5Y, Rasayana, Toxicology, Virgin coconut oil.

INTRODUCTION

Ayurveda is the science of life or an art of living and it is the most indigenous system of medicine in India, and it is being practiced for over 6000 years.¹ The primary goal of Ayurveda is to maintain good health and disease prevention through the treatment regimen.² In the last decade, there was a significant shift towards the AYUSH system of medicines viz., Ayurveda, Yoga, Unani, Siddha, and Homeopathy from the modern system of medicine.³ In 2021, during the COVID-19 pandemic, to treat and prevent, the physicians recommended the use of immunomodulatory agents from AYUSH medicines, maybe containing a single or polyherbal formulations like AYUSH-64 from Ayurveda,⁴ Arsenic album from Homeopathy,⁵ and Kabasura Kudineer from Siddha.⁶ In Ayurveda, Rasayana or rejuvenating therapy is one of the unique branches and is believed to be useful to overcome challenging diseases in the modern era. According to Charaka, the father of Ayurveda, Rasayana is a method of obtaining the best qualities of various dhatus (tissues). Susruta mentions that Rasayanas are capable of pacifying all afflictions.⁷ It ensures appropriate nourishment, growth, and increased function of all dhatus. Also, it has an impact on both the body and the mind at one single time, reducing the effects of early aging on both and

improving the body's illness resistance.⁸ In Ayurveda, regular consumption of Rasayana is believed to improve memory, learning ability, and concentration. The Rasayana drugs are reported to act as an antioxidant, anti-inflammatory, neuroprotective, immunostimulant, and adaptogenic.⁹

Crabs are the most common macrofauna in coastal areas, and are found in variety of species and even groups. Some of these crabs have poisons in the form of specific protein compounds.¹⁰ India has approximately 96 species of freshwater crabs out of 1476 species throughout the world.¹¹ *Scylla Serrata* Forskal is an edible freshwater crab in the Portunidae family. It is spread evenly throughout the mangrove forest regions of the Indo-West-Pacific.¹² It is rich in proteins, carbohydrates, minerals, fatty acids, and vitamins.¹³⁻¹⁶ Traditionally it is used by the tribes of Sundarban, West Bengal state in India, to cure pulmonary tuberculosis, urticaria, skin burns, dropsy, body swelling, bone fracture, asthma, insomnia, rickets, measles, epilepsy, and diabetes.¹⁷⁻²⁰ The Ayurvedic preparation of crab by name, Karkataka (crab) Taila (oil) is found to be effective internally and externally in the treatment of knee joint ligament injury.²¹ Karkataka Bhasma is traditionally used to treat constipation, headache, chronic cough, tuberculosis, and neurological disorders. The *Scylla serrata* flesh has been used in

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Ayurveda, Siddha, and Unani system of medicine for alleviating Vata dosha and treating neurological disorders.²²

Parkinson's disease (PD) in Ayurveda is known as "Kampavata" (*Kampa* meaning shaking or tremor and *Vata* is one of the three humors of the body). All the motor and sensory functions in the body are governed by Vata. Major neurological problems come under 'Vata Vyadhis' and Kampavata is one among them. Ayurvedic literature mentions related symptoms, such as rigidity (sthambha), bradykinesia (chestasanga), flexed posture (avanamana), gait abnormalities (gatisanga), monotonous speech (vakvikriti), depression (vishada), impairment in memory (smritihani), and constipation (vibandha) also under Kampavata.²³ One of the Ayurveda treaties, namely, "Basavarajeyam" explained the Kampavata.²⁴ Galen is credited with coining the term "shaking palsy" in the Western medical literature (129–200), later 1817 by Dr. James Parkin (1755–1824), who illustrated PD in a book entitled "An essay on the Shaking Palsy".²⁵ Throughout the world, a total of 10 million people have been affected by PD.²⁶ According to estimates from 2016, India has about 0.58 million people living with PD, with a significant increase in prevalence predicted in the future years.²⁷

In the Kerala state, the local Ayurvedic practitioners use the KT, to treat tremors and palsy even today. Due to the lack of proper scientific validations and concise reports, it becomes less vulnerable in the scientific community. Therefore, based on the preliminary field survey and interaction with traditional healers, we decided, to prepare, and evaluate the formulation systematically and scientifically to prove their claim. The identification of the crab has played a significant role in the preliminary study as the freshwater and marine crabs are found to contain specialized toxic protein, and hence safe handling of crabs is very essential in the screening of active ingredients and toxicological studies of Rasayana using SH-SY5Y cell lines and *Drosophila melanogaster*. The Ayurvedic process used for the preparation of KT is known as Sneha (medicated oil/ghee) Kalpana (pharmaceutical process). The advantage of Sneha Kalpana is that it acts as a vehicle and base and is used either as Taila/grhita, to extract both water- and fat-soluble medicaments from the tissue ingredients, increasing the absorption of the drug in the tissue as well as the shelf life of the formulation.²⁸

Human neuroblastoma cell lines are frequently utilized in the scientific research to evaluate neurotoxicity, oxidative stress, and neurodegenerative disorders. These cell lines are employed in *in-vitro* investigations when neuron-like cells are required.²⁹ *Drosophila melanogaster* is a dipteran insect that belongs to the Drosophilidae family. About a century ago, this insect was introduced as a model in biology, and it was pivotal in the development of research on genetics and related areas. This fly has long been utilized as a genotoxicity model, but it was only recently included as a possible model for researching systemic toxicology or as an alternate model for toxicology research.³⁰ The current investigation aimed at identifying the crab, preparation of the Rasayana by the classical method, preliminary analysis of zoochemical constituents, and toxicological profiling.

MATERIALS AND METHODS

Chemicals

Benedict's reagent, Fehling's reagent A and B, potassium bismuth iodide, potassium mercuric iodide, iodine-potassium iodide, aluminum chloride, ferric chloride, lead acetate, gelatin, analytical grade methanol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich.

Collection and identification of raw materials

Fresh specimens of *Scylla Serrata* F used for the study were collected from Kanayankode River (Part of Korapuzha), Calicut, Kerala. The

sample was taxonomically identified and authenticated at the Kerala University of Fisheries and Ocean studies (KUFOS), Kochi, Kerala. The taxonomic code of the specimen is 2311114001.

Preparation of Karkataka Taila and VCO by the traditional method

Edible crab Rasayana was prepared by the traditional method and the process was known as *Sneha Kalpana*. In Sanskrit, Sneha means oil, and Kalpana means pharmaceutical process (Figure 1). Here, 1 part of Kalka (crab flesh), 4 parts of Sneha (coconut milk), and 16 parts of Dravya (water) were mixed. Coconut milk was prepared by grating the coconut and grinding it mechanically with water. All three ingredients were mixed and boiled in a bronze vessel. The consistency of formulation is very significant in this preparation. First, the consistency would be in the mud stage, then the wax stage, and finally in the sand stage. At the sand stage, the formulation was removed from the fire and filtered immediately to obtain Rasayana. The same procedure was used to prepare VCO without crab flesh (Figure 2).

Qualitative zoochemical analysis of Karkataka Taila and VCO

Rasayana prepared was subjected to qualitative chemical tests for the identification of the nature of zooconstituents present. For the identification of carbohydrates (Molisch test, Fehling test, Barfoed's test, Benedict test, and iodine test), proteins (Biuret test, Xanthoprotein test, and lead acetate test), amino acids (Ninhydrin and Millions test), steroids (Salkowski reaction and Liebermann-Burchard reaction), flavonoids (Shinoda test and alkaline reagent test), saponins (froth test), phenols (ferric chloride test), fats and oils (translucent spot test) chemical test were carried out on KT and VCO.^{31,32}

Physicochemical characterization

Morphological and elemental analysis

Scanning electron microscopy was used to examine the morphology of KT particles. Energy-dispersive X-ray spectroscopy was used for elemental measurement, which was combined with scanning electron microscopy (SEM).

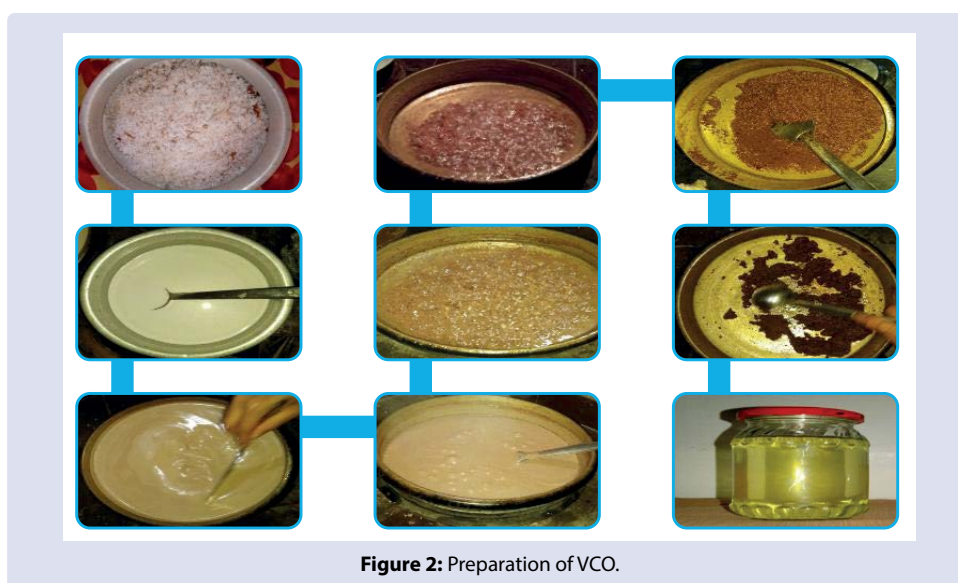
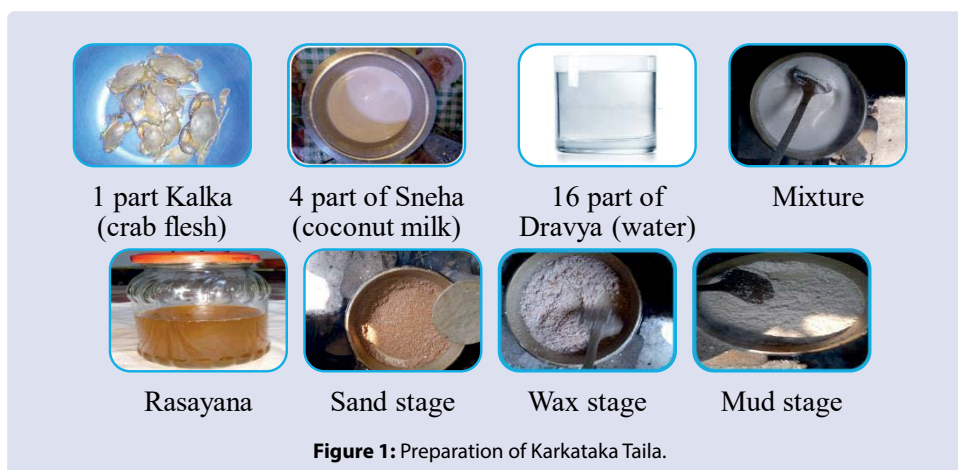
Exposure of KT and VCO to SH-SY5Y cell lines

The SH-SY5Y cell line was obtained from the National Centre for Cell Sciences (NCCS) in Pune, India. In a 25 cm² tissue culture flask, the cell line was cultured in Dulbecco's Modified Eagles medium (DMEM) containing 10 % fetal bovine serum (FBS), L-glutamine, sodium bicarbonate, and an antibiotic solution containing penicillin (100U/ml), streptomycin (100g/ml), and amphotericin B (2.5g/ml). Cell lines were cultured at 37°C in a humidified 5 percent CO₂ incubator (NBS Eppendorf, Germany). In 96-well plates, cell viability was determined using the MTT assay method. The KT and VCO were suspended in cell culture media and exposed to the SH-SY5Y cell line at various concentrations from 6.25 µg to 100 µg.³³

Exposure of KT and VCO to *Drosophila melanogaster* embryo

Drosophila melanogaster stock and culture

The Department of Zoology, University of Mysore, Manasagangotri, Mysore, India provided *D. melanogaster* wild-type (Oregon K) flies. The flies were maintained and reared in *Drosophila* Laboratory, on standard *Drosophila* medium containing 10 g agar, 100 g wheat, 100 g jaggery, 7.5 ml propionic acid (antibacterial and antifungal agent), and yeast at constant temperature and humidity (22–24°C; 60–70 % relative humidity) under 12 h dark/light cycle conditions.



Preparation of Delcour cup for embryo collection

A Delcour cup was prepared by mixing 2 g sucrose and 2 g agar. The ingredients were boiled in 100 ml of distilled water. After removal from the fire, the solution was mixed with 3.5 ml of ethanol and 2.5 ml of acetic acid. Poured into the plastic cup and made into a convex surface. Waited for solidification for 1 hour. Small depression was made throughout the cup to lay the eggs by flies. The yeast paste was placed in the center of the cup for attracting flies.

Collection of embryos

The male and female flies were separated by giving anesthesia to the flies and transferred 200 females and 100 males each to bottles. The flies were fully fed with yeast and kept overnight. The next day all the flies were transferred into the plastic bottle (Figure 7). The Delcour cup containing yeast was kept down and a plastic bottle containing flies was kept above, covered properly, and kept overnight. The next day embryos were collected without damage by the use of a small brush and blade along with the media. *Drosophila* embryos were transferred to either media only (Group 1) or with the following additives; Group 2) 0.005 % of KT/100 ml of media, Group 3) 0.01 % of KT/100 ml of media, Group 4) 0.05 % of KT/100 ml of media, Group 5) 0.1 % of KT/100 ml of media, Group 6) 1% of KT/100 ml of media, Group 7) 5 % of KT/100 ml of media, and Group 8) 10 % of KT/100 ml of media. The concentrations of KT in culture media were ascertained from the dose calculated from

the human dose. For each treatment, there were 7 culture vials, each vial containing 50 embryos (total of 350 embryos/treatment). After 14 days, the number of flies that emerged was counted in every day. The KT in the range of 0.05 % to 10 % of media has shown toxicity in flies. So, we performed the toxicity studies of VCO at 0.005 % to 10 % as well to evaluate whether the toxicity was due to the KT or VCO.

Statistical analysis

To determine whether the difference between treatments was significant, a one-way analysis of variance with Turkey's post hoc test was used, with the difference considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Explanation of the preparation of Karkataka Taila and VCO by the traditional method

Traditional Ayurvedic healers followed a monotonous procedure for the preparation of KT. In the preparation, they were using freshwater crab flesh. Compared to marine crabs these crabs have the adaptability to change to the varying environmental conditions. The Ayurvedic healers collect the crab as fresh as possible to get a significant effect. Further, these crabs were alive up to 3 to 4 days after collection. This feature made these crabs specific for these Rasayana. Further, during the preparation, a constant fire was maintained using wood. Proper

stirring was maintained in one direction without forming any lumps. The consistency of Rasayana was very important. First, the formulation was in the mud stage, then wax, and finally sand stage. Once it reached the sand consistency, the Rasayana was removed from the fire and filtered immediately.

In preliminary zoochemical analysis, KT showed the presence of amino acids and proteins. In addition, the KT was found to contain fats and lipids; phenols, and saponins and this could be due to the VCO. VCO was found to contain all the mentioned phytoconstituents except amino acids and proteins.

Morphological and elemental analysis

To investigate the morphology of KT, SEM analysis was performed (Figure 3) and the result revealed that at low magnification, a highly

smooth crystal surface was seen. The SEM analysis revealed the Rasayana preparation resulted in the formation of nanocrystals. To confirm the elemental composition EDAX was performed and EDAX has shown the presence of various elements. During the EDAX measurement, different areas were focused and the corresponding peaks were shown (Figure 4). Details of the four EDAX spectra values measured of the Rasayana in atomic and weight % have been listed (Table 1).

Cell viability study

Human neuroblastoma SH-SY5Y cell lines were used in the cell culture study to understand the toxicological effects of KT and VCO by the MTT assay method (Figure 5 and Figure 6). SH-SY5Y cell lines were treated with different concentrations of KT and VCO, to assess the percentage of cell viability. The KT and VCO treatment ranged from

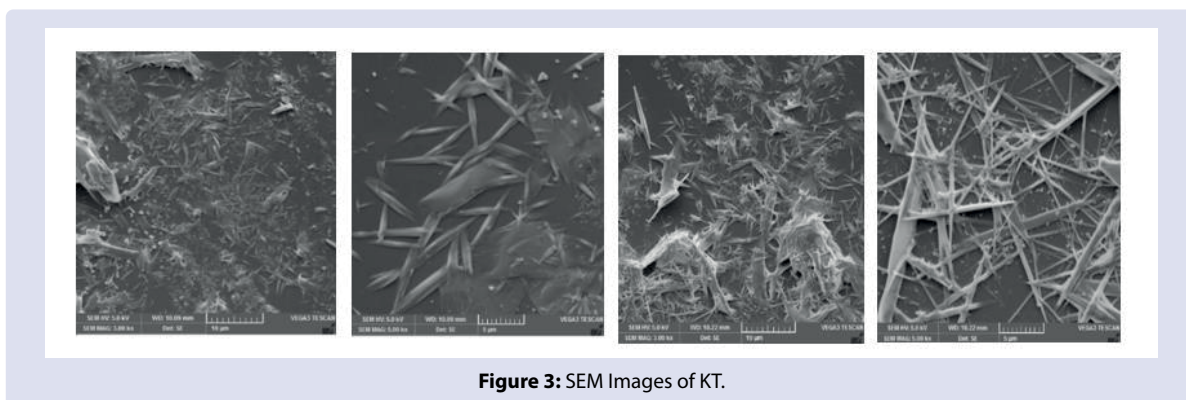


Figure 3: SEM Images of KT.

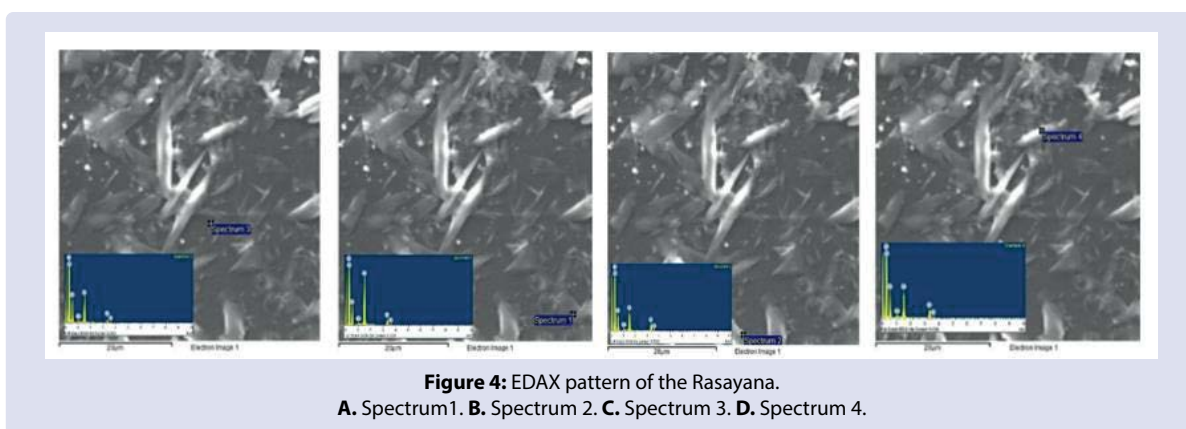


Figure 4: EDAX pattern of the Rasayana.
A. Spectrum 1. B. Spectrum 2. C. Spectrum 3. D. Spectrum 4.

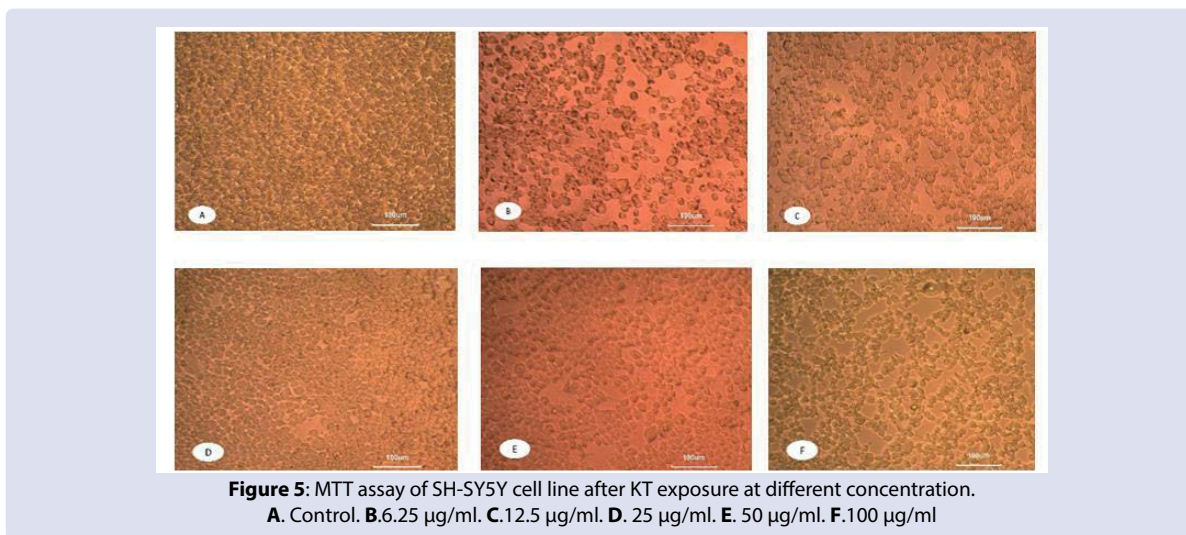


Figure 5: MTT assay of SH-SY5Y cell line after KT exposure at different concentration.
A. Control. B.6.25 µg/ml. C.12.5 µg/ml. D. 25 µg/ml. E. 50 µg/ml. F.100 µg/ml

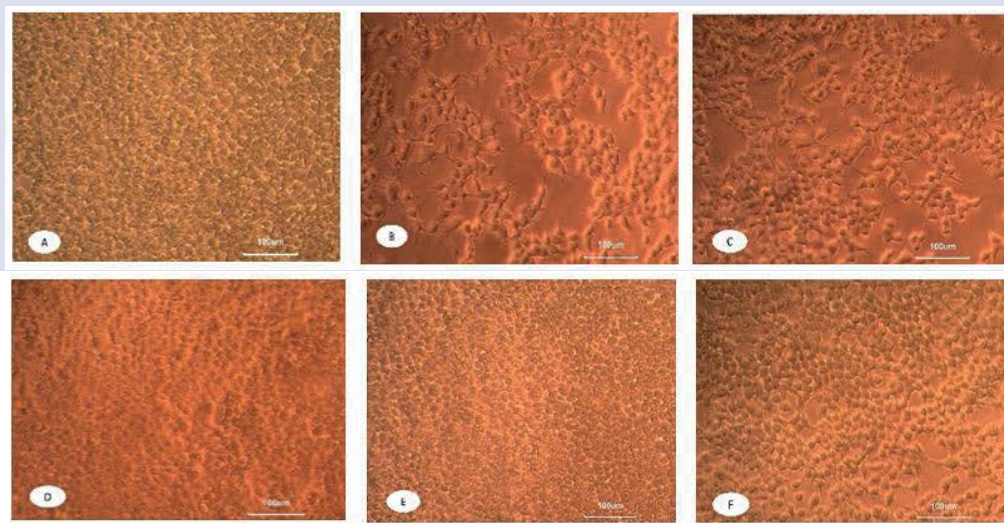


Figure 6: MTT assay of SH-SY5Y cell line after VCO exposure at different concentration. A. Control. B. 6.25 µg/ml. C. 12.5 µg/ml. D. 25 µg/ml. E. 50 µg/ml. F. 100 µg/ml



Figure 7: Toxicological analysis of KT and VCO using *Drosophila embryo*.

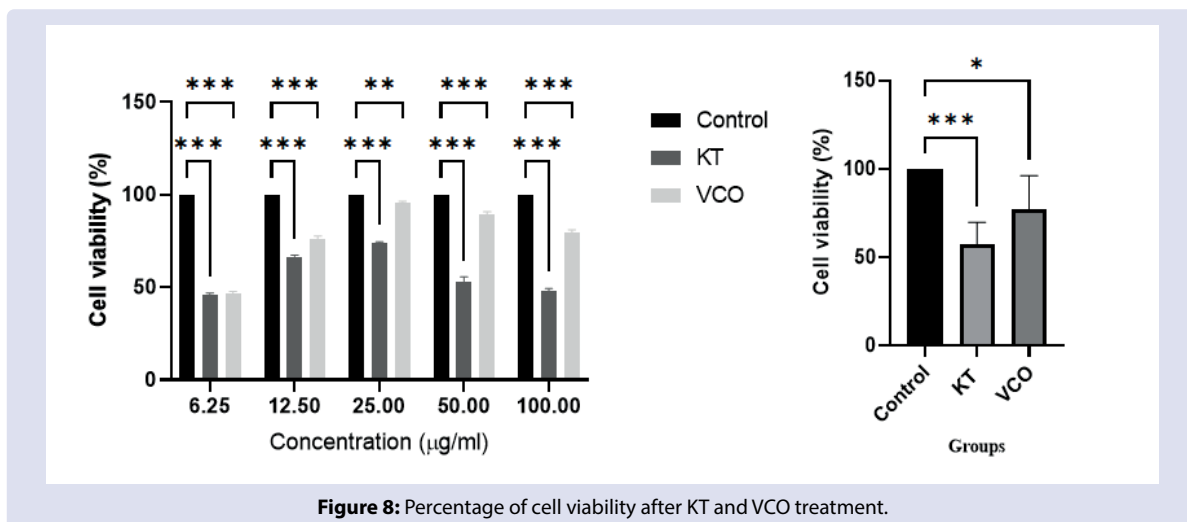


Figure 8: Percentage of cell viability after KT and VCO treatment.

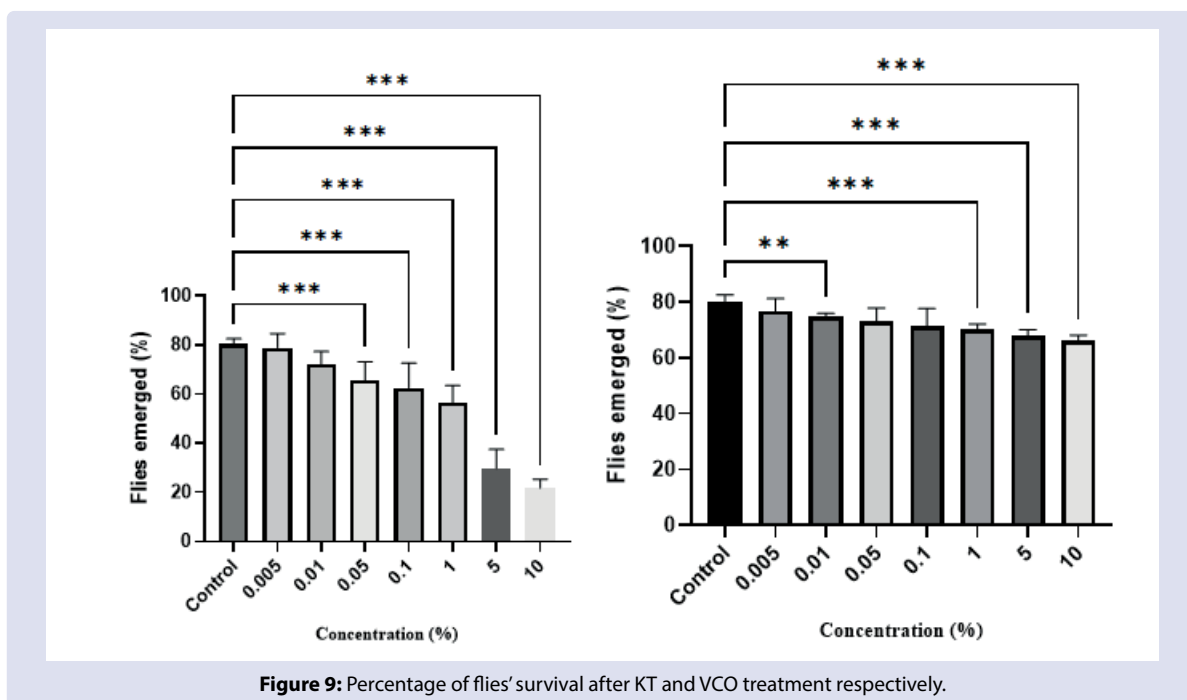


Figure 9: Percentage of flies' survival after KT and VCO treatment respectively.

Table 1: EDAX weight ratio of Rasayana nanocrystals (four spectrums focused on four distinct areas).

KT	C		O		Na		Al		K	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
Spectrum 1	55.05	68.30	20.95	19.51	0.46	0.30	16.98	9.38	6.57	2.50
Spectrum 2	55.51	68.22	24.51	22.61	0.57	0.36	8.74	4.78	10.67	4.03
Spectrum 3	61.03	71.08	25.90	22.64	0.35	0.21	9.39	4.87	3.33	1.19
Spectrum 4	52.73	65.13	27.92	25.89	0.54	0.35	8.80	4.84	10.00	3.79

6.25 µg (46.2 % and 46.5 % cell viability respectively), 12.5 µg (66.5 % and 76.1% cell viability respectively), 25 µg (74.1 and 95.4 % cell viability respectively), 50 µg (53.2 % and 89.2 % cell viability respectively) and 100 µg (47.6 % and 79.3% cell viability respectively). KT and VCO did not show significant cytotoxicity on SH-SY5Y cell lines up to 25 µg. But, at 50 µg and 100 µg, KT has shown more cytotoxicity than VCO (Figure 8).

Drosophila embryo study

The toxicological analysis at different concentrations of KT was performed. The screening was performed in the concentration range

from 0.005 % to 10 % KT. At 10 % of KT, the survival rate was found only 21.7 %, then it was found increasing in the order of 29.7 %, 56.50 %, 62.5 %, 65.70 %, 72 %, and 78.8 % at 5 %, 1 %, 0.1, 0.05, 0.01 %, and 0.005 % respectively as compared to control (80 %) i.e., without any treatment. The concentration of KT versus the survival rate of the insects was plotted (Figure 9).

When the concentration of KT increased the survival rate decreased. In the study, VCO was used as a base, and hence a toxicological analysis for VCO was carried out as well. The VCO had shown less toxicity [10 % (66 %), 5 % (68 %), 1 % (70 %), 0.1 % (71.4 %), 0.05 % (73.1 %), 0.01 % (74.8 %), and 0.005 % (76.8 %)] among all the treatments (Figure 9).

The result showed that as the concentration of KT in the medium increased, the rate of larvae transforming into pupae and pupa to adults was slowed. After being swallowed, KT caused toxic effects on the larva's body, slowing its growth and thus delaying the fly's development. Active locomotion of *Drosophila melanogaster* was observed in minor concentrations of KT, whereas activity and locomotion of flies were significantly reduced when the concentration of KT in the media was increased. The Pearson correlation coefficient showed a good correlation between the control and the VCO treated group. When compared to the control, KT treated group, has shown a significant correlation.

CONCLUSION

SH-SY5Y cell lines and *Drosophila melanogaster* were utilized in this work to assess the safety of KT or Rasayana. In this study, we demonstrated a simple method for investigating the effects of KT treatment in SH-SY5Y cell lines and *Drosophila melanogaster*. The results indicated that with the increased concentration of KT, the adverse effects on the viability of cell lines and the development of the fly. In the presence of relatively high concentrations of KT, developmental processes experienced severe imbalance during the transition from larval to adult stages. *Drosophila* developmental toxicity and survival rate were compared to an untreated control group. Developmental toxicity is defined as a structural and functional impairment in flies at any stage of their life cycle, including larvae, pupae, and adults. Our findings revealed that when the concentration of Rasayana in the medium increased, there was a noticeable adverse effect on SH-SY5Y cell lines and the number of offspring and larvae locomotor behaviour. The pupae's and larva's length and width were also observed to be altered. Long-term Rasayana exposure can have a major influence on the cell viability and flies' survival rate was in a dose and time-dependent way. But the same concentration of VCO has shown a protective effect on cell lines and flies. VCO was used as a vehicle. It can be concluded that the toxic effect could be the presence of protein in crab extract and not due to VCO. The toxicity has been shown only in higher concentrations of KT and the reasonable concentration is safe to use.

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CONFLICTS OF INTEREST

No

LIST OF ABBREVIATIONS

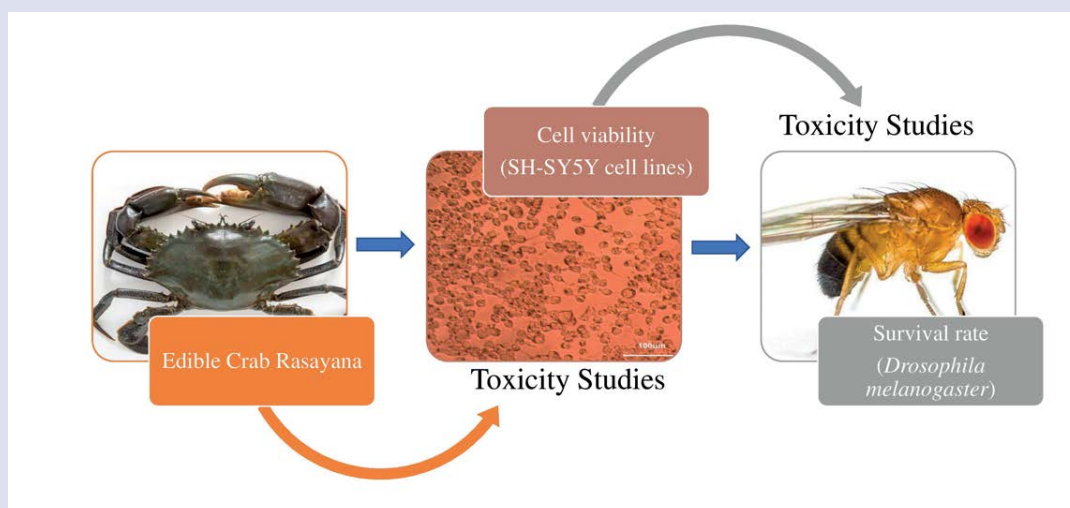
DMEM	Dulbecco's Modified Eagles medium
EDAX	Energy-dispersive X-ray spectroscopy
KT	Karkataka Taila
MTT	3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
SEM	Scanning Electron Microscopy
VCO	Virgin coconut oil

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GRAPHICAL ABSTRACT



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