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PRECLINICAL EVALUATION OF SAGALA VAAIVU KUTTHALUKU KIYAZHAM-TOXICOLOGICAL AND PHARMACOLOGICAL STUDY ANTIOXIDANT AND ANTI CANCER ACTIVITY

Shasikala B. V.*[1] and Mohamed Musthafa M.[2]

[1]PG in Sirappu Maruthuvam, [2]Professor and Head of the Department of Sirappu Maruthuvam, Govt. Siddha Medical College, Chennai, Tamilnadu, India.

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***Corresponding Author**
Shasikala B. V.
PG in Sirappu Maruthuvam, Govt. Siddha Medical College, Chennai, Tamilnadu, India.

ABSTRACT

Siddha system of medicine is compiled by siddhars who embodiment of divine knowledge. Nowadays documented medical system which deals with physical, psychological, spiritual wellbeing of individual. Here the drug Sagala Vaaivu Kutthaluku Kiyazham taken from Siddha text. The primary purpose of this preclinical study is to evaluate the efficacy and safety of the drug which will help the medicine for widespread global acceptance. A study was performed as per OECD guidline-423 and no other significant changes were observed in animal models. The anticancer activity study was conducted in HT29 cell line and proven to be potent anticancer activity drug. The antioxidant study was determined by DPPH assay and hence proven to be a potent radical scavenging drug. The overall study results that Sagala Vaaivu Kutthaluku Kiyazham (SVKK) has high efficacy and safety in preclinical assessment and expect to give best result on management of colorectal cancer in future study.

KEYWORDS: Sagala Vaaivu Kutthaluku Kiyazham (SVKK), Anticancer activity, Antioxidant activity, HT29 Cell line.

INTRODUCTION

In Siddha system it has wide variety of medicine available which includes herbs, metals and minerals. Herbal medicine is an important complementary integrative cancer treatment with side of immune booster. Improving the quality of life of those living with chronic pain.

Siddha medicine sagalavaaivukutthallukukiyazham controls the multiplication of colorectal Cancer. Human colon adenocarcinoma cell line HT29, is not only used to study the biology of human colon cancer but it is receiving special interest in studies focused on food digestion and bioavailability due to the ability to express characteristics of mature intestinal cells. Herbal formulation drug SVKK taken from Thanvandhrivaithyam 1000 Siddha text. Many review articles show that the ingredients of the SVKK having antioxidant, anti inflammatory and anti cancer activity which is reason behind medicine to take up for the study on the management of colorectal cancer.

Antioxidant drug have the ability to demolish free radicals, protects the structural integrity of cells and tissues along with anti-inflammatory to manage the pain and inflammation. Preclinical study of pharmacology and toxicology profile of SVKK provides the efficacy safety of the drug and also plays an inevitable role in deciding and designing further clinical studies in future.

MATERIALS AND METHOD

Sagala Vaaivu Kutthaluku Kiyazham consist of Chukku (zingiberofficinale)**,** Velulli (Allium sativam), Karuvapattai (Cinnamomumverum), Kadugurohini (Picrorhizakurroa), Kazharchikai (Caesalpiniacrista)**,** Vasambu (Acorus calamus), Kadugu (Brassica nigra), Kodiveliverpattai (Plumbago zylanica).

Source of Raw Drug and Purification

The raw trial drugs are purchased from a famous traditional raw drug R.N. Rajan shop in Chennai. The raw drugs are authenticated by medicinal botanist in government Siddha medical college Chennai. Then raw drugs are purified separately in gunapadam department laboratory of government Siddha medical college Chennai as per classical text book.

Method of Preparation

All purified dry ingredients are powered coarsely by using stone mortar. Measured 5 gms and packed with butter paper. Stored in clean air tight glass container and dispensed to patients.

Dosage

50 ml per dose, two doses per day for 48 days.

1. Toxicological Study Method

After getting proper permission from the Institutional Animal Ethics Committee (IAEC NO:

XLVIII/27/CLBMCPM2016). Acute and Sub acute toxicity for the trial drug SVKK was carried out in wistar albino rats.

A. Acute Oral Toxicity Study of Sagala Vaaivu Kutthaluku Kiyazham (Oecd Guideline – 423)

Introduction

- The acute toxic class method is a stepwise procedure with the use of 3 animals of a single sex per step.
- Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance.
- This procedure is reproducible, uses very few animals and is able to rank substances in a similar manner to the other acute toxicity testing methods.
- The acute toxic class method is based on biometric evaluations with fixed doses, adequately separated to enable a substance to be ranked for classification purposes and hazard assessment.
- In principle, the method is not intended to allow the calculation of a precise LD50, but does allow for the determination of defined exposure ranges where lethality is expected since death of a proportion of the animals is still the major endpoint of this test.
- The method allows for the determination of an LD50 value only when at least two doses result in mortality higher than 0% and lower than 100%.
- The use of a selection of pre-defined doses, regardless of test substance, with classification explicitly tied to number of animals observed in different states improves the opportunity for laboratory to laboratory reporting consistency and repeatability.

Principle of the Test

It is the principle of the test that based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute toxicity of the test substance to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.

- No further testing is needed
- dosing of three additional animals, with the same dose
- dosing of three additional animals at the next higher or the next lower dose level. The

method will enable a judgment with respect to classifying the test substance to one of a series of toxicity classes.

METHODOLOGY

Selection of Animal Species

The preferred rodent species is the wistar albino rat, although other rodent species may be used. Healthy young adult animals are commonly used laboratory strains should be employed. Females should be nulliparous and non-pregnant. Each animal, at the commencement of its dosing, should be between 6 to 8 weeks old and the weight (150-200gm) should fall in an interval within±20% of the mean weight of any previously dosed animals.

Housing and Feeding Conditions

The temperature in the experimental animal room should be 22ºC + 3ºC. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. Animals may be group-caged by dose, but the number of animals per cage must not interfere with clear observations of each animal.

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

Test Animals and Test Conditions

Sexually mature Female Wistar albino rats (150-200gm) were obtained from TANUVAS, Madhavaram, Chennai. All the animals were kept under standard environmental condition (22±3°C). The animals had free access to water and standard pellet diet (Sai meera foods, Bangalore).

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 7 days prior to dosing to allow for acclimatization to the laboratory conditions.

Preparation for Acute Toxicity Studies

Rats were deprived of food overnight (but not water 16-18 h) prior to administration of the, ***Sagala Vaaivu Kutthaluku Kiyazham.***

The principles of laboratory animal care were followed and the Institutional Animal Ethical Committee approved the use of the animals and the study design.

IAEC approved Number: XLVIII/27/ CLBMCP/2016

Test Substance	: **Sagala Vaaivu Kutthaluku Kiyazham**
Animal Source	: TANUVAS, Madhavaram, Chennai.
Animals	: Wister Albino Rats (Female-3+3)
Age	**:** 6-8 weeks
Body Weight on Day 0	**:** 150-200gm.
Acclimatization	**:** Seven days prior to dosing.
Veterinary examination	**:** Prior and at the end of the acclimatization period.
Identification of animals	**:** By cage number, animal number and individual marking by using Picric acid.
Number of animals	: 3 Female/group,
Route of administration	: Oral
Diet	**:** Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore
Water	**:** Aqua guard portable water in polypropylene bottles.
Housing & Environment	**:** The animals were housed in Polypropylene cages provided with bedding of husk.
Housing temperature	**:** between 22ºC $\pm$ 3ºC.
Relative humidity	**:** between 30% and 70%,
Air changes	**:** 10 to 15 per hour and
Dark and light cycle	**:** 12**:**12 hours.
Duration of the study	**:** 14 Days

Administration of Doses

Sagala Vaaivu Kutthaluku Kiyazham was suspended in water and administered to the groups of wistar albino rats in a single oral dose by gavage using a feeding needle. The control group received an equal volume of the vehicle. Animals were fasted 12 hours prior to dosing. Following the period of fasting, the animals were weighed and then the test substance

was administered. Three Female animals are used for each group. The dose level of 5, 50, 300 and 2000 mg/kg body weight was administered stepwise. After the substance has been administered, food was withheld for a further 3-4 hours. The principle of laboratory animal care was followed. Observations were made and recorded systematically and continuously as per the guideline after substance administration. The visual observations included skin changes, mobility, aggressively, sensitivity to sound and pain, as well as respiratory movements. Finally, the number of survivors was noted after 24 hrs and these animals were then monitored for a further 14 days and observations made daily. The toxicological effect was assessed on the basis of mortality.

OBSERVATIONS

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary. The times at which signs of toxicity appear and disappear are important, especially if there is a tendency for toxic signs to be delayed. All observations are systematically recorded with individual records being maintained for each animal.

Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The principles and criteria summarized in the Humane Endpoints Guidance Document taken into consideration. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress was humanly killed. When animals are killed for human reasons or found dead, the time of death was recorded.

B.Sub Acute Toxicity Methodology

Repeated Dose 28-Day Oral Toxicity (407) Study of *Sagala Vaaivu Kutthaluku Kiyazham*

Test Substance	: **Sagala Vaaivu Kutthaluku Kiyazham**
Animal Source	: TANUVAS, Madhavaram, Chennai.
Animals	: Wister Albino Rats (Male -24, and Female-24)
Age	**:** 6-8 weeks

Body Weight : 150-300gm.

Acclimatization : Seven days prior to dose.

Veterinary examination : Prior and at the end of the acclimatization period.

Identification of animals : By cage number, animal number and individual marking by using Picric acid

Diet : Pellet feed supplied by Sai meera foods Pvt Ltd, Bangalore

Water : Aqua guard portable water in polypropylene bottles.

Housing & Environment : The animals were housed in Polypropylene cages provided with bedding of husk.

Housing temperature : between 22ºC ± 3ºC.

Relative humidity : between 30% and 70%,

Air changes : 10 to 15 per hour

Dark and light cycle : 12:12 hours.

Duration of the study : **28 Days.**

Table 1: Svkk-Sagala Vaaivu Kutthaluku Kiyazham.

Groups	No of Rats
Group I Vehicle control (Water)	12(6 male,6 female)
Group II- SVKK low dose X (30mg)	12 (6 male,6 female)
Group III - SVKK Mid dose 5X (150mg)	12 (6 male,6 female)
Group IV - SVKK High dose 10X (300 mg)	12(6 male,6 female)

Methodology

Randomization, Numbering and Grouping of Animals

48 Wistar Albino Rats (24M + 24F) were selected and divided into 4 groups. Each group consists of 12 animals (Male -6, and Female-6). First group treated as a control and other three groups were treated with test drug (low, mid, high) for 28 days. Animals were allowed acclimatization period of 7 days to laboratory conditions prior to the initiation of treatment. Each animal was marked with picric acid. The females were nulliparous and non- pregnant.

Justification for Dose Selection

As per OECD guideline three dose levels were selected for the study. They are low dose (X), mid dose (5X), high dose (10X). i.e. X dose is (30mg), 5X dose is 150mg/animal, 10X dose is 300mg/animal.

Preparation and Administration of Dose

Sagala Vaaivu Kutthaluku Kiyazham suspended in with water, It was administered to animals at the dose levels of X, 10X, 20X. The test substance suspensions were freshly prepared every two days once for 28 days. The control animals were administered vehicle only. The drug was administered orally by using oral gavage once daily for 28 consecutive days.

Observations

Experimental animals were kept under observation throughout the course of study for the following

Body Weight

Weight of each rat was recorded on day 0, at weekly intervals throughout the course of study.

Food and water Consumption

Food and water consumed per animal was calculated for control and the treated dose groups.

Clinical signs

All animals were observed daily for clinical signs. The time of onset, intensity and duration of these symptoms, if any, were recorded.

Mortality

All animals were observed twice daily for mortality during entire course of study.

Necropsy

All the animals were sacrificed by excessive anaesthesia on day 29. Necropsy of all animals was carried out.

Laboratory Investigations

Following laboratory investigations were carried out on day 29 in animals fasted over-night. Blood samples were collected from orbital sinus using sodium heparin (200IU/ml) for Bio chemistry and potassium EDTA (1.5 mg/ml) for Hematology as anticoagulant. Blood samples were centrifuged at 3000 r.p.m. for 10 minutes.

Haematological Investigations

Haematological parameters were determined using Haematology analyzer.

Biochemical Investigations

Biochemical parameters were determined using auto-analyzer.

Histopathology

Control and highest dose group animals will be initially subjected to histopathological investigations. If any abnormality found in the highest dose group than the low, then the mid dose group will also be examined. Organs will be collected from all animals and preserved in 10% buffered neutral formalin for 24 h and washed in running water for 24 h. The organ sliced 5 or 6µ m sections and were dehydrated in an auto technicon and then cleared in benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50°C and then in a cubical block of paraffin made by the "L" moulds. It was followed by microtome and the slides were stained with Haematoxylin-eosin red.

Statistical analysis

Findings such as body weight changes, water and food consumption, hematology and blood chemistry were subjected to One-way ANOVA followed by Dunnet t test using computer software programmed – Graph pad version 7. All data were summarized in tabular form.

2. Pharmacological Study

A. Anticancer Activity-Cell Line Study

The evaluation of the anticancer activity of SVKK was carried out in cultured HT 29 Cell line in Biogenix Research Center.

Culture collection and media

HT-29(Human colorectal adenocarcinoma) cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Anti-cancer Assay by MTT Method

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µ l of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of control group}}$$

Anti-cancer Assay by Direct Microscopic observation

Entire plate was observed after 24 hours of incubation in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

B. Antioxidant Activity

Dpph Radical Scavenging Assay

The radical scavenging activity of different extracts was determined by using DPPH assay according to Chang et al [2001]. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517 nm. Ascorbic acid (10mg/ml DMSO) was used as reference.

Principle

1, 1-diphenyl-2-picryl hydrazyl is a stable free radical with pink colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as,

DPPH + [H-A] →DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability.

Reagent Preparation

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Procedure

Different volumes of extracts 1.25µl - 20µl (12.5 - 200µg/ml) from a stock concentration 10mg/ml were made up to a final volume of 20µ l with DMSO and 1.48ml DPPH (0.1mM) solution was added. A control without the test compound, but an equivalent amount of distilled water was taken. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm. 3ml of DPPH was taken as control.

Calculation

$$\% \text{ inhibition} = \frac{control - test}{control} X100$$

RESULT AND DISCUSSIONS

1. Toxicological Study

A. Acute Oral Toxicity Study of Sagala Vaaivu Kutthaluku Kiyazham

Table 2: Dose Finding Experiment And Its Behavioral Signs of Acute Oral Toxicity Observation done

SL	Group Control	Observation	SL	Group Test Group	Observation
1	Body weight	Normal	1	Body weight	Normally increased
2	Assessments of posture	Normal	2	Assessments of posture	Normal
3	Signs of Convulsion Limb paralysis	Normal	3	Signs of Convulsion Limb paralysis	Absence of sign (-)
4	Body tone	Normal	4	Body tone	Normal
5	Lacrimation	Normal	5	Lacrimation	Absence
6	Salivation	Normal	6	Salivation	Absence
7	Change in skin color	No significant color change	7	Change in skin color	No significant color change
8	Piloerection	Normal	8	Piloerection	Normal
9	Defecation	Normal	9	Defecation	Normal
10	Sensitivity response	Normal	10	Sensitivity response	Normal
11	Locomotion	Normal	11	Locomotion	Normal
12	Muscle gripness	Normal	12	Muscle gripness	Normal
13	Rearing	Mild	13	Rearing	Mild
14	Urination	Normal	14	Urination	Normal

Table 3: (Observational Study Results).

No	Dose mg/kg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1.	Control	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.	2000mg	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1. Alertness 2. Aggressiveness 3. Pile erection 4. Grooming 5. Gripping 6. Touch Response 7. Decreased Motor Activity 8. Tremors 9. Convulsions 10. Muscle Spasm 11. Catatonia 12. Muscle relaxant 13. Hypnosis 14. Analgesia 15. Lacrimation 16. Exophthalmos 17. Diarrhea 18. Writhing 19. Respiration 20. Mortality.(+ Present, - Absent).

Table 4: (Body Weight Observation).

DOSE	DAYS		
	1	7	14
CONTROL	280.2±42.30	281.4 ± 64.12	282.6 ±26.18
HIGH DOSE	280.4±21.24	281 ± 3.64	281.4 ± 2
P value (p)*	NS	NS	NS

Table 5: Water In Take (Ml/Day) of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

DOSE	DAYS		
	1	6	14
CONTROL	61 ± 1.12	62±2.22	63.9±1.14
HIGH DOSE	62.2±1.1	63±1.14	64.20±24
P value (p)*	NS	NS	NS

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 6: Food Intake (Gm/Day) of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

DOSE	DAYS		
	1	7	14
CONTROL	56.24±2.22	56.2±7.42	58.4±3.46
High DOSE	60.6±1.63	60.6±2.62	64.1±5.38

Inference

All data were summarized in tabular from (Table 2 -Table 6) showing for each test group the number of animals used, the number of animal displaying signs of toxicity, the number of found dead during the test, description of toxic symptoms, weight changes, food and water intake. Hence no significant changes are observed in body weight, water, food intake and no mortality are exhibited in rats.

B. Sub Acute Toxicity Result

REPEATED DOSE 28- DAY ORAL TOXIC STUDY OF SAGALA VAAIVU KUTTHALUKU KIYAZHAM.

Table 7: Body Weight of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

DOSE	DAYS				
	1	7	14	21	28
CONTROL	290.2±24.22	291.4 ± 14.24	291.5 ± 25.40	292.5±35.46	292.4 ± 45.15
LOW DOSE	265.2 ± 46.14	265.4 ± 27.20	267.6± 66.74	268 ± 62.18	268.8± 54.34
MID DOSE	270.4± 04.24	270.3 ± 46.54	271.2 ± 68.16	271.4 ± 54.26	272.4 ± 64.70
HIGH DOSE	250.6± 64.94	250.6 ± 50.53	251.4 ± 52.44	251 ± 24.68	252 ± 74.60
P value (p)*	NS	NS	NS	NS	NS

NS- Not Significant, **(p > 0.01),*(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 8: Water Intake (Ml/Day) of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

DOSE	DAYS				
	1	6	14	21	28
CONTROL	60.2 ± 1.21	60.6±6.12	62.2±4.10	62±4.12	64.6±1.32
LOW DOSE	62.1±1.10	62.6±2.42	62.9±1.72	63.2±6.86	64.4±1.54
MID DOSE	58.1±1.26	58.3±3.21	59.1±6.41	59.4±1.72	59.4±1.82
HIGH DOSE	54.1±1.41	54.2±1.42	54.4±1.44	54.6±1.52	55.8±2.82
P value (p)*	NS	NS	NS	NS	NS

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 9: Food Intake (Gm/Day) of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

DOSE	DAYS				
	2	7	23	22	28
CONTROL	36±4.12	36.2±3.12	37.3±2.84	37.2±1.41	38±2.43
LOW DOSE	38.2±1.41	38.3±1.13	38.1±1.21	39.5±1.23	39.5±1.26
MID DOSE	35.1±3.32	35.2±3.04	35.2±2.42	36.2±2.61	37.2±1.42
HIGH DOSE	37.1±1.32	37.1±1.41	37.6±2.62	38.2±1.10	39.6±3.42
P value (p)*	NS	NS	NS	NS	NS

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test.

Table 10: Haematological Parameters of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

Category	Control	Low dose	Mid dose	High dose	P value (p)*
Haemoglobin(g/d l)	15.8±0.68	15.60±0.84	15.8±0.26	15.92±0.65	N.S
Total WBC (×10^3 l)	8.71±0.32	8.75±0.26	8.68±0.27	8.60±1.22	N.S
Neutrophils (%)	29.22±0.01	30.02±0.10	31.11±1.12	32.02±1.02	N.S
lymphocyte (%)	58.12±1.32	58.12±1.12	58.10±2.33	58.20±2.62	N.S
Monocyte (%)	.06±0.02	.06±0.04	.06±0.01	.06±0.06	N.S
Eosinophil (%)	0.2±0.04	0.2±0.02	0.2±0.01	0.2±0.06	N.S
Platelets cells10^3/µl	543.14±3.43	543.41±4.12	544.13±4.0	545.12±2.54	N.S
Total RBC 10^6/µl	7.68±0.12	7.76±0.43	7.69±0.48	7.75±0.26	N.S
PCV%	49.42±0.2	49.42±1.12	49±1.22	49.60±2.21	N.S
MCHC g/dL	31.8±1.32	31.24±1.20	32.18±1.10	32.33±1.12	N.S
MCV fL(µm^3)	57.3±3.20	57.2±1.20	57.9±1.24	57.8±1.22	N.S

N.S- Not Significant, **(p > 0.01), *(p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 11: Biochemical Parameters of Wistar Albino Rats Group Exposed To *Sagala Vaaivu Kutthaluku Kiyazham*.

BIOCHEMICAL PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
GLUCOSE (R) (mg/dl)	105.14±8.2	105.16±4.10	106.02±11.10	106.12±6.2	N.S
T.CHOLESTEROL(mg/dl)	108.16±1.42	108.25±1.20	109.62±1.18	109.24±1.63	N.S
TRIGLY(mg/dl)	64.16±1.42	64.12±1.22	66.16±1.22	66.16±1.22*	N.S
LDL	69.6±2.13	69.12±2.34	69±1.32	69.24±12.12	NS
VLDL	13.4±1.32	13.42±4.24	13.24±2.84	13.54±14.16	NS
HDL	22.16±6.12	22.42±2.20	23.18±2.26	24.18±22.12	NS
Ratio 1(T.CHO/HDL)	4.61±1.12	4.62±1.24	4.64±1.14	4.64±2.30	NS
Ratio 2(LDL/HDL)	2.40±1.14	2.41±1.12	2.41±2.20	2.46±10.02	NS
Albumin (g/dL)	4.43±0.16	4.53±0.32	4.44±10.32	4.42±10.48	NS

NS- Not Significant, **(p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 12: Renal Function Test of Wistar Albino Rats Group Exposed To Sagala Vaaivu Kutthaluku Kiyazham.

PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
UREA (mg/dl)	21.30±0.99	21.20±0.36	21.16±1.18	21.48±1.21	N.S
CREATININE(mg/dl)	0.42±0.02	0.41±0.04	0.42±0.06	0.44±0.08	N.S
BUN(mg/dL)	14.1±0.11	14.10±0.60	14±0.32	14.46±1.12	NS
URIC ACID(mg/dl)	5.00±0.34	5.06±0.21	5.7±0.14*	5.62±0.26	N.S

NS- Not Significant, **(p > 0.01), * (p >0.05) , n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Table 13: Liver Function Test of Wistar Albino Rats Group Exposed To Pattaichoornam.

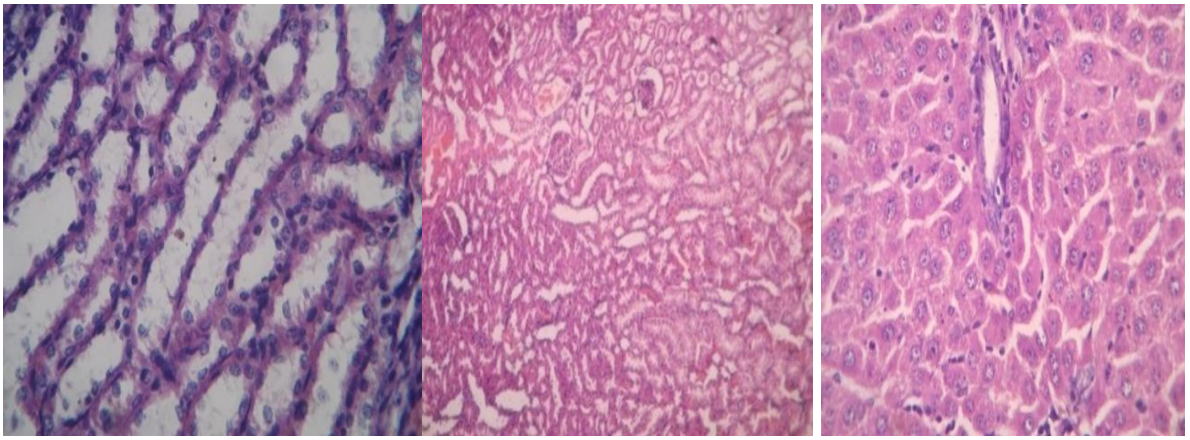
PARAMETERS	CONTROL	LOW DOSE	MID DOSE	HIGH DOSE	P Value (p)*
T BILIRUBIN (mg/dl).	0.03±0.03	0.03±0.02	0.04±0.02	0.04±0.04	N.S
SGOT/AST(U/L)	139.15±1.33	139.34±0.32	140.01±1.62	140.75±1.02	N.S
SGPT/ALT(U/L)	72.12±1.18	72.22±1.34	72.14±1.28	72.46±0.61	N.S
ALP(U/L)	129.22±3.16	129±12.14	130±14.04*	130.23±11.15*	N.S
T.PROTEIN(g/dL)	8.12±0.34	8.18±0.12	8.16±0.14	8.54±0.49	N.S

NS- Not Significant, **(p > 0.01), * (p >0.05), n = 10 values are mean ± S.D (One way ANOVA followed by Dunnett's test).

Histo Pathology

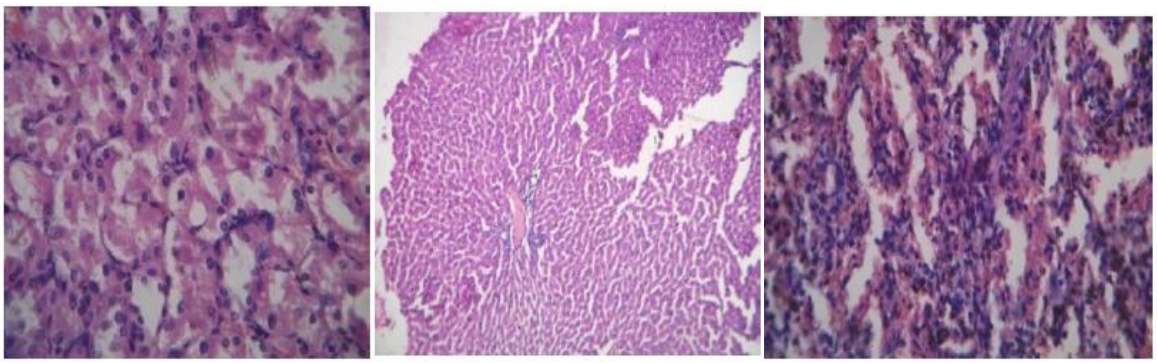
Control Group

Kidney **Liver** **Spleen**



High dose

Kidney **Liver** **Spleen**



2. Pharmacological Study

In vitro cytotoxicity assay of Sagalavaivakuthazhakkukiyazham decoction on HT-29 (human colorectal adenocarcinoma cell line)

As shown in Graph-1, the cytotoxicity effects of the Sagalavaivakuthazhakkukiyazham decoction were investigated HT-29 (human colorectal adenocarcinoma cell line) cell lines as normal and cancerous cell lines, respectively. The cells were treated with the Sagalavaivakuthazhakkukiyazham decoction at various concentrations (6.25, 12.5, 25, 50, and 100 µg/ml) incubated at 37°C for 72 h. The prepared Sagalavaivakuthazhakkukiyazham decoction also demonstrated no significant toxicity even in concentrations up to 100 µg/ml on normal cell lines in the resazurin reduction assay, meaning that the Sagalavaivakuthazhakkukiyazham decoction are well tolerated by 3T3 cells. Hence, the IC_{50} was 67.489µg/mL. These results are demonstrated the possibility of these Sagalavaivakuthazhakkukiyazham decoction for different biomedical applications.

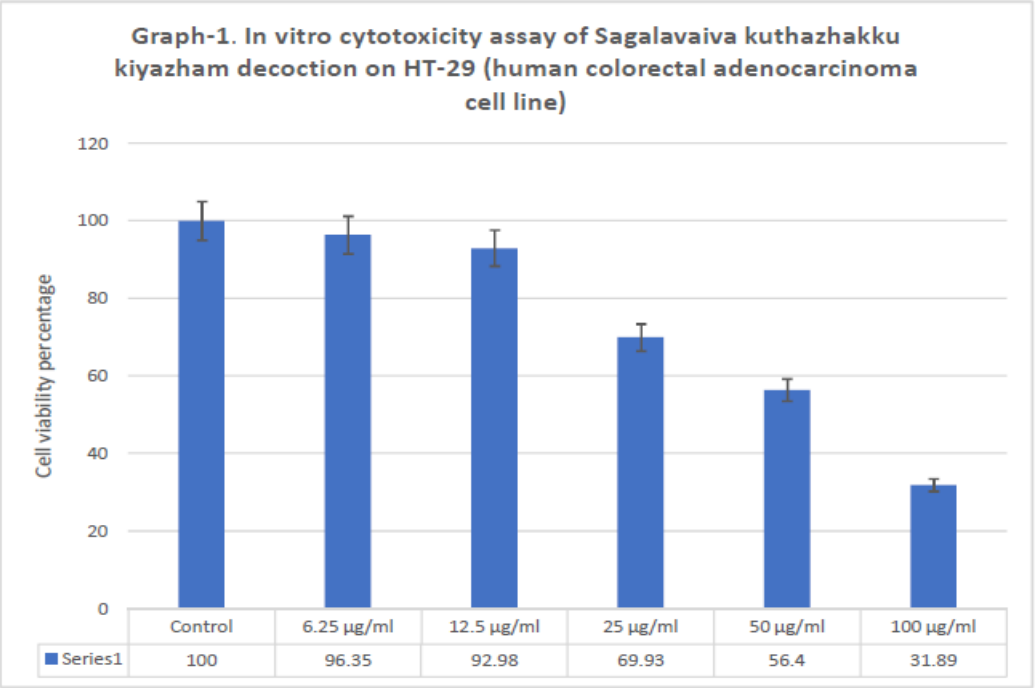
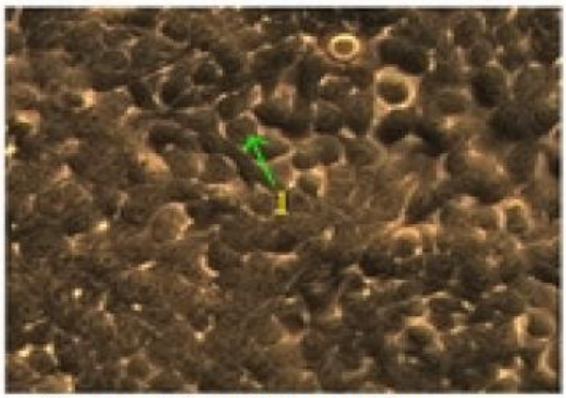
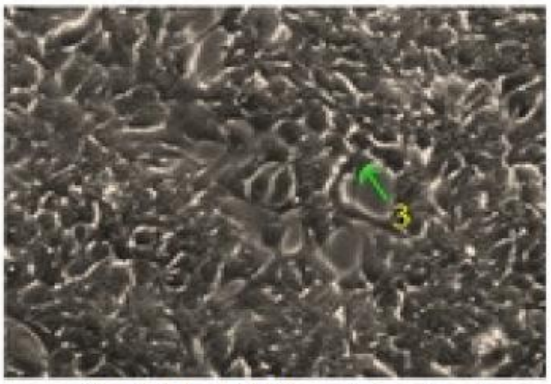


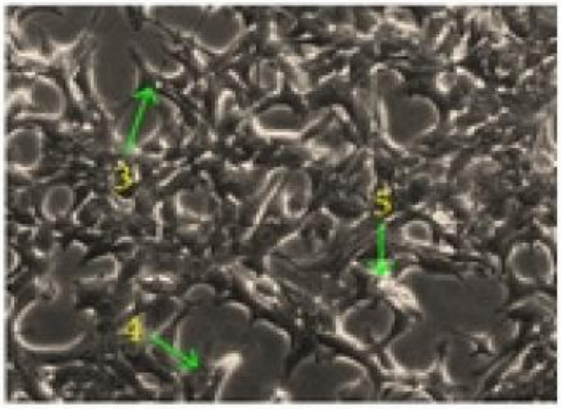
Fig. 1: (Human colorectal adenocarcinoma (HT-29) cell lines after 72 h of treatment with Sagalavaivakuthazhakkukiyazham decoction.



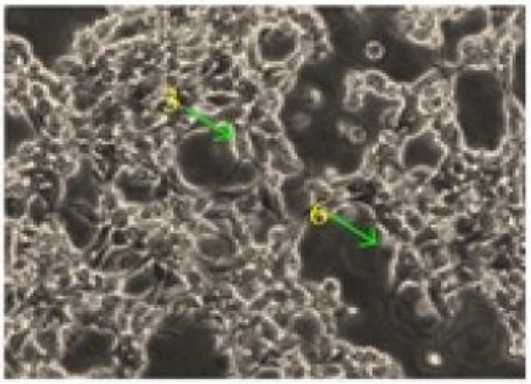
Control (HT-29) cell lines



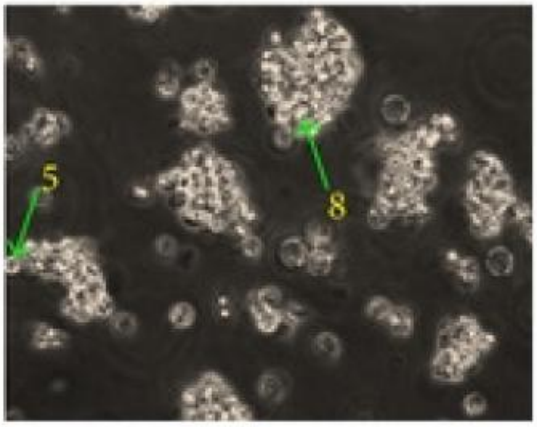
(HT-29) cell lines Treated with 6.25 µg/ml



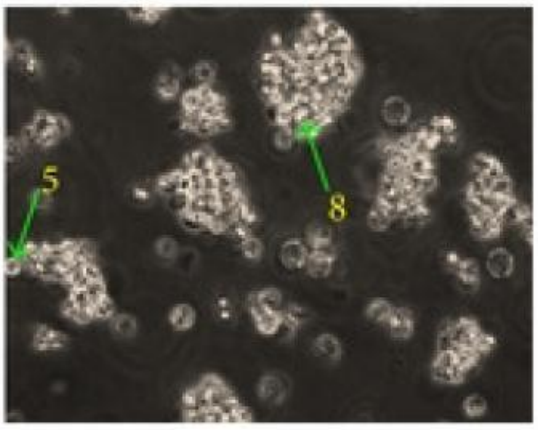
(HT-29) cell lines Treated with 12.5 µg/ml



(HT-29) cell lines Treated with 25 µg/ml



(HT-29) cell lines Treated with 50 µg/ml



(HT-29) cell lines Treated with 100 µg/ml

Arrows indicates, (1) Control cell, (2) Condensed nuclei, (3) Cell shrinkage, (4) Membrane blebbing, (5) Apoptotic bodies, (6) Bubbling and (7) echinoid spikes.

Fig 1 shows images of control cell lines in the absence of Sagalavaivakuthazhakkukiyazham decoction. As shown, the cells are neatly connected with each other with a high concentration of cells in the cell lines. Fig 1 b represents images in the presence of Sagalavaivakuthazhakkukiyazham decoction interact with membrane proteins and disrupt the signaling process with the result that some of the cells were dying and also cell concentration is less than before.

C. Anti Oxidant Activity

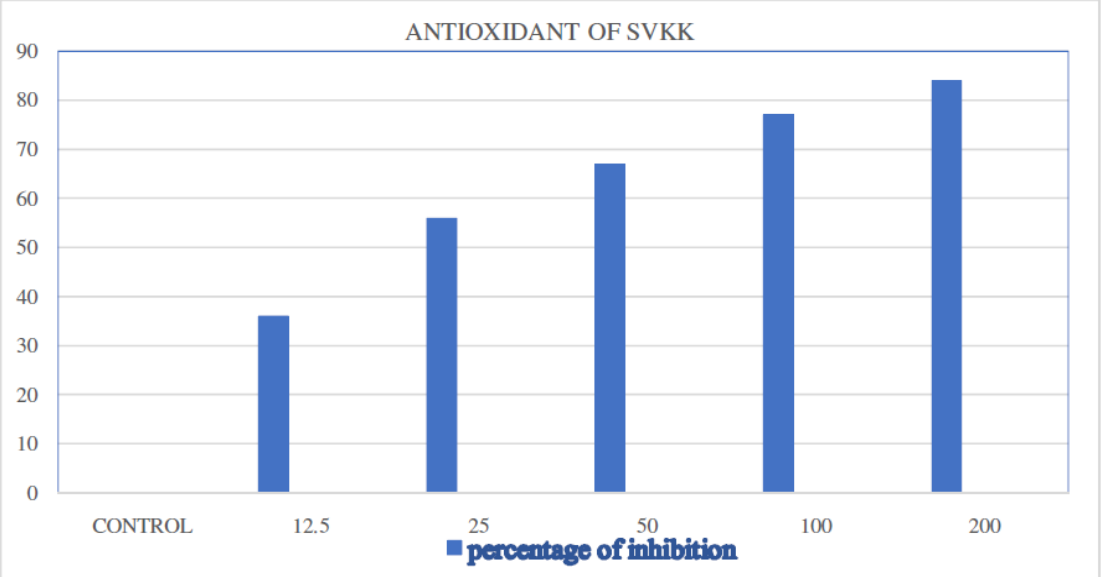
Table 14: Ascorbic acid standard.

Concentrations (µg/ml)	Absorbance	Percentage of inhibition
Control	1.7983	
12.5	1.4044	21.90
25	1.0782	40.04
50	0.7121	60.40
100	0.2921	83.75
200	0.0692	96.15

Table 15: Anti Oxidant Activity of Svkk.

Concentrations (µg/ml)	Absorbance	Percentage of inhibition
Control	0.8085	
SAMPLE CODE		
12.5	0.5170	36.05
25	0.3554	56.04
50	0.2674	66.93
100	0.1844	77.19
200	0.1294	84.00

Chart 11 Anti Oxidant Study.



Inference

200µ/ml level of ski has 84% of inhibition the free radical production and oxidative degeneration and proven to be a rich antioxidant drug.

CONCLUTION

Acute and sub acute oral toxicity study was done in wistar Albino Rats for the sample Sagala Vaaivu Kutthaluku Kiyazham and hence no other significant changes and mortality were observed in their behavior, bodyweight, waterintake, food in take, LFT, RFT. It proves the trial drug SVKK is safe. In pharmacological study, while 84% of inhibition of free radicals, thus proven to be a rich Antioxidant drug.

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