Toxicity studies of ZingiVir-H in in vitro and in vivo condition

Cytotoxicity studies of zingiVir-H in cell lines

Checking of cytotoxicity of ZingiVir-H against cancer cell lines

Study site

The *in-vitro* cytotoxicity of ZingiVir-H in cancer cell lines was carried out at Rajiv Gandhi Centre for Biotechnology (RGCB), Jagathy, Thiruvananthapuram, Kerala.

Cell culture and drug addition

Human breast cancer (MCF 7 & MDAMB-231) and human cervical cancer (SiHa) cells were grown in 96-well optical bottom plates in DMEM growth medium was used in the study. All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% Fetal Bovine Serum (FBS) and antibiotics in a humidified CO2 (5%) chamber at 37uC. For experiments, cells were seeded in 96 well plates at desired densities. After an overnight culture, medium was removed and 100 ml fresh phenol-red free DMEM containing 5% FBS with the compounds to be tested were added. Concentrated stocks of each drug was prepared in either DMSO or water according to solubility and stored at 28˚C.

After 48 h, the cells were exposed to ZingiVir-H in three dilutions, 1:50, 1:100 and 1:200 for 24 h, 48, and 72 h.

Breast cancer cell line expressing caspase sensor

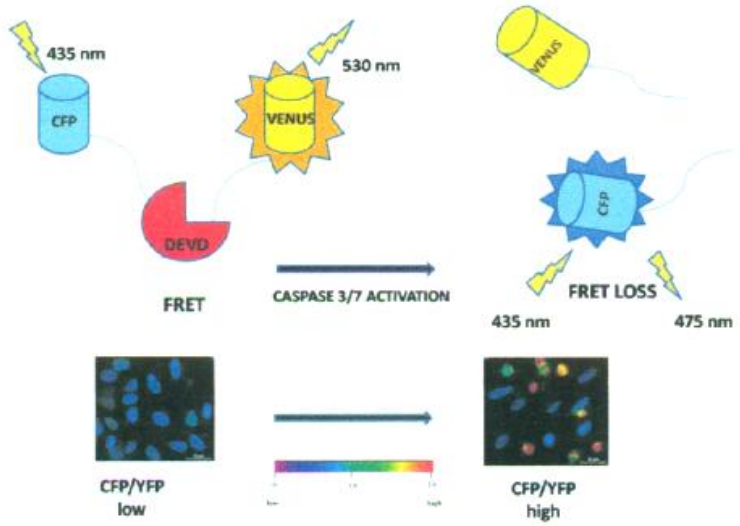
During cytotoxicity cells die by apoptosis through the activation of enzyme called caspase 3. The cell line used express a reporter sensor for monitoring caspase activation in live cells. Once caspase is activated by the test compound, the PROBE is cleaved with change in fluorescence colour under FRET microscopy.

FRET acceptor bleaching by confocal laser scanning microscopy

Cells seeded in chambered cover glass (Lab-TekTM, Nunc, Rochester, NY) were sequentially scanned using a laser scanning Leica TCS SP2 Confocal microscope (Leica Microsystems, Germany) for donor (ECFP) and acceptor (Venus) fluorescence signals under the 458 nm and 514 nm laser lines respectively to obtain pre-bleach donor intensity ($Donor_{pre}$) and pre-bleach images. Following this, the acceptor was bleached off completely with high intensity 514 nm laser pulses after which, the donor intensity was obtained ($Donor_{post}$) and FRET efficiency was calculated using the formula: % FRET

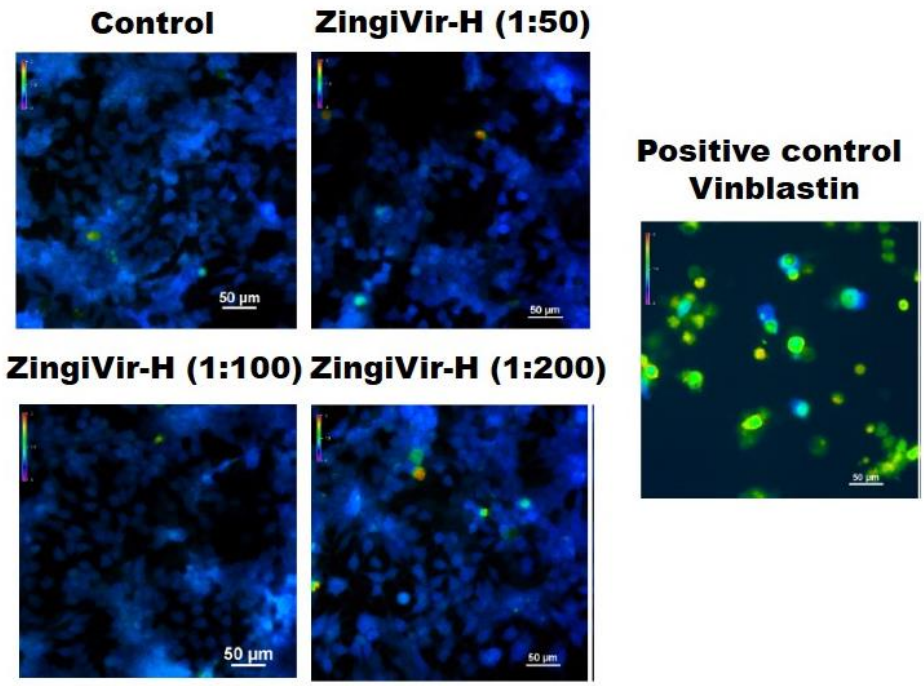
efficiency = ($Donor_{post}$ - $Donor_{pre}$)/$Donor_{post}$. Pre-bleach and post-bleach donor/acceptor intensity images were used to correlate the results visually. The increase in ratio scale reflects cell death and cytotoxicity (1).

Graphical method of FRET analysis



For analysing the cytotoxicity, the FRET imaging was carried out in ratio mode, collecting donor fluorescence and acceptor fluorescence. Vinblastine has been used as the positive control. From the results, it is clear that ZingiVir-H is non-toxic to human cells up to 72 h in all three dilutions tested. However, positive control showed significant cell death that is clearly evident from the increase in ratio.

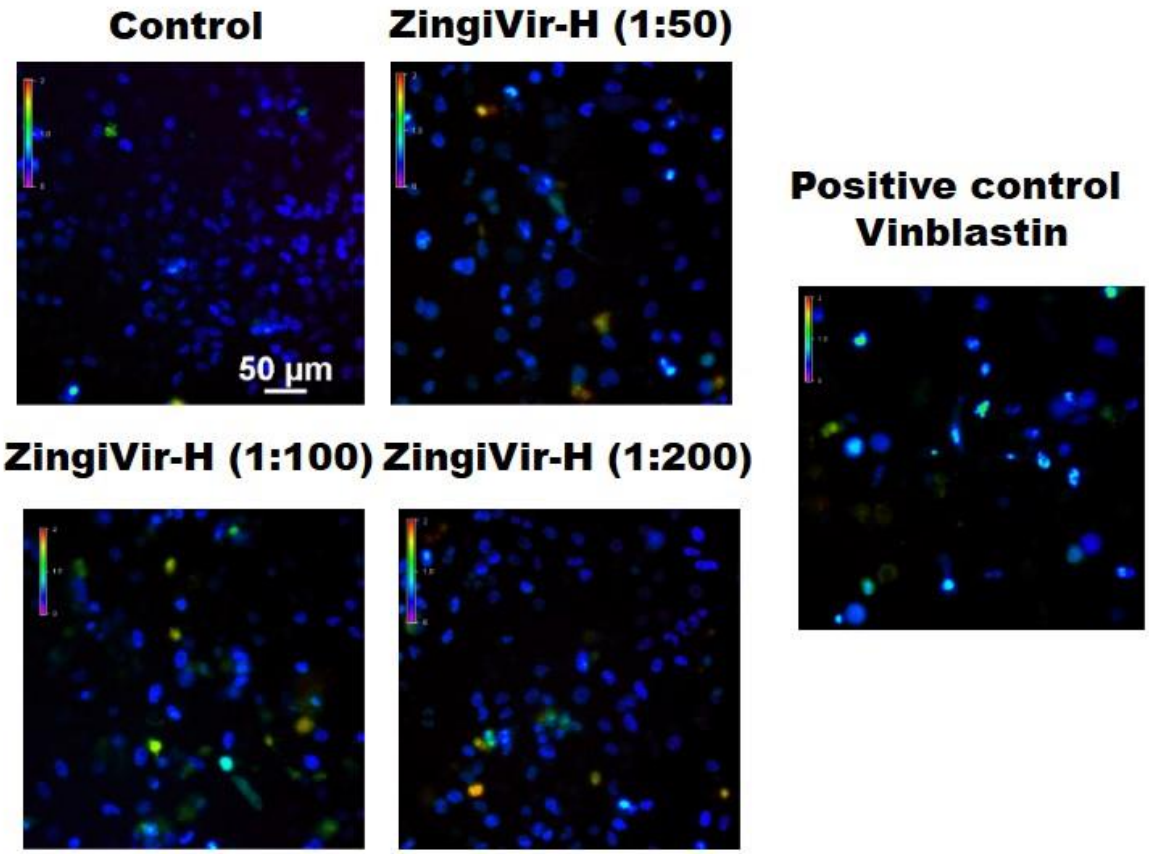
FRET image of the cell after ZingiVir-H treatment against MCF-7 in 72 h



MDAMB-231- A different breast cancer cell line expressing the FRET probe at the nucleus

The cells were grown in the 96-well plate and exposed to the drug at various concentrations as mentioned above.

FRET image of the cell after ZingiVir-H treatment against MDAMB-231 in 72 h



As shown in the figure 2, both 1:100 and 1:200 failed to induce any significant cell death. The cell showed almost similar ratio as that of the control. Even in 1:50 dilution, only few cell recorded mild toxicity at 72 hours. The positive control recorded significant increase in ratio confirming caspase dependent cell death. The morphological image of the Phase contrast microscope substantiate that the compound is non cytotoxic to the cells at the indicated concentrations.

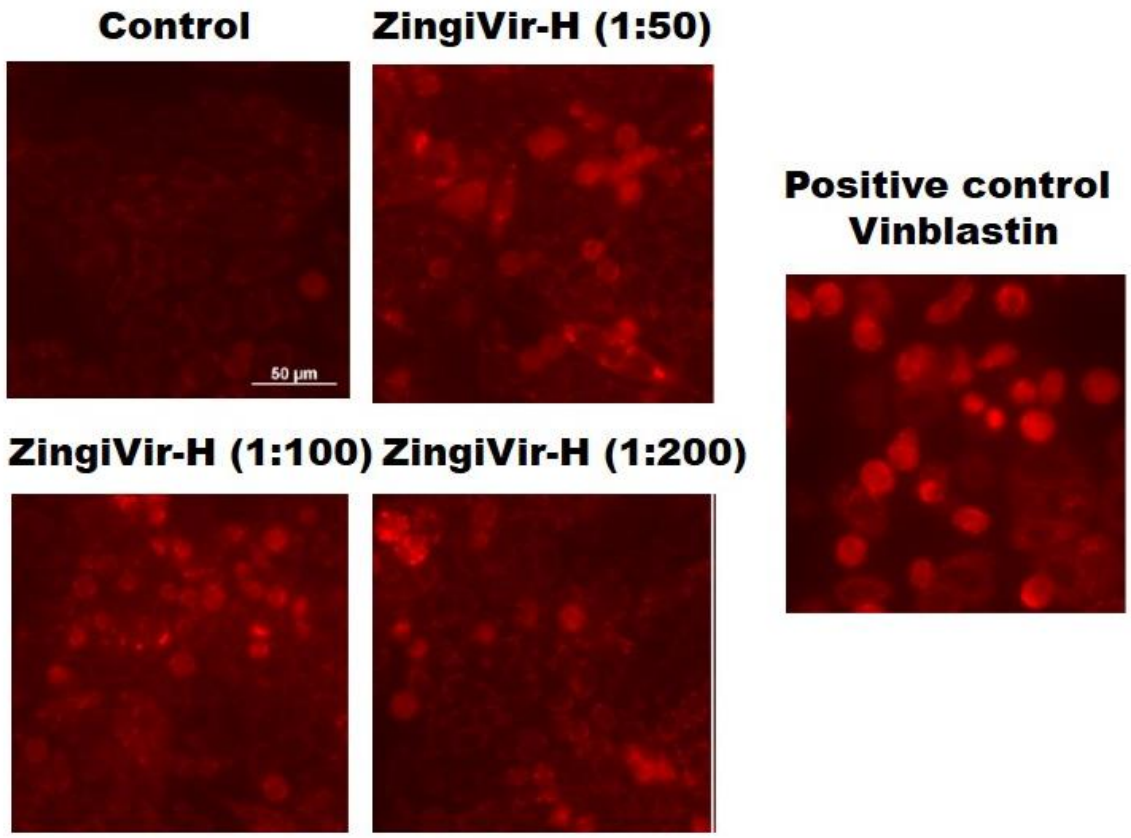
Determination of mitochondrial toxicity using

In order to evaluate whether the compound is having any effect on the mitochondria, cell expressing a genetically encoded mitochondrial permeabilization sensor has been employed.

Validation of cells using microscopy

The stable cells expressing SMAC-RFP were exposed to ZingiVir-H. Single time point imaging at 72 h of treatment was carried out to see the SMAC release by confocal microscopy. Most of the cells also showed mitochondrial granular red fluorescence indicating lack of mitochondrial permeabilization suggesting the absence of apoptosis in ZingiVir-H treated cells (2).

Determination of mitochondrial toxicity in SiHa SMAC mCherry after 72 hours



In order to evaluate whether the compound is having any effect on the mitochondria, cell expressing a genetically encoded mitochondrial permeabilization sensor has been employed. As shown in the control, the cells maintained the red fluorescence in granular pattern indicating they are retained within the mitochondria. However, in the positive control cells, the red fluorescence is diffused indicating the mitochondrial permeabilization. The test compound failed to show any indication for the release of inner mitochondrial membrane protein from the mitochondria. This result further substantiate that the drug is not toxic to mitochondria at the concentration tested up to 72 h.

The three different assay system used for the testing substantiate that the ZingiVir-H is not toxic to cells at the indicated concentrations.

Checking of cytotoxicity of ZingiVir-H, its raw materials and intermediates against normal cell lines

Site

The *in-vitro* cytotoxicity of ZingiVir-H, its raw materials and intermediates in normal cell lines was carried out at National Institute for Interdisciplinary Science & Technology (NIIST), Pappanamcode, Thiruvananthapuram, Kerala.

Samples tested

Sample No 1 - Raw Chayilyam (HgS)

Sample No 2 - Raw Thalakam (As_2S_3)

Sample No 3 - Chayilyam after 1st stage purification

Sample No 4 - Thalakam after 1st stage purification

Sample No 5 - Thalakam & Chayilyam mix - after final purification

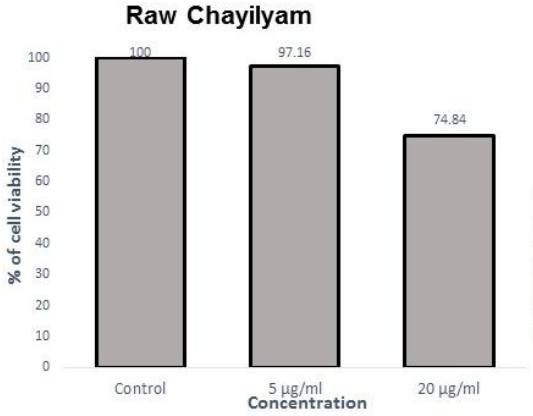
Sample No 6 - ZingiVir-H tablets (Containing mixture of Chayilyam, Thalakam, and other herbal ingredients).

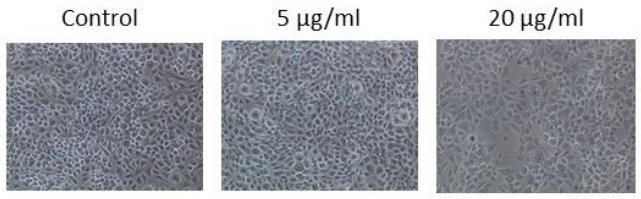
Determination of cell viability in normal cell lines using MTT assay

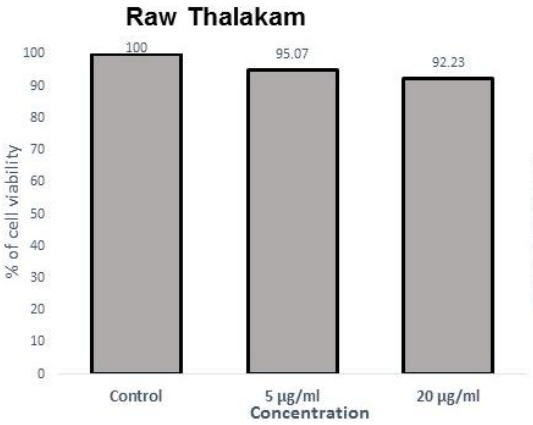
Principle

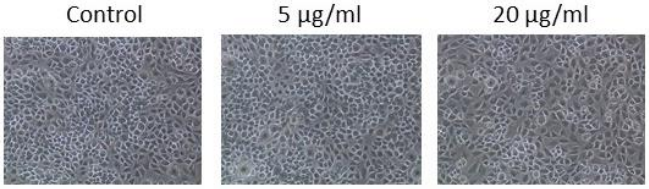
The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of chemicals on primary cells. This assay measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent and the released, solubilized formazan reagent is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of viability of the cells (3).

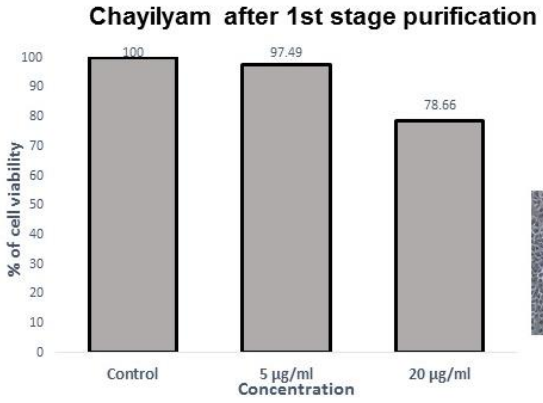
Figure showing the cell viability data after treatment with ZingiVir-H, its raw materials and intermediates.

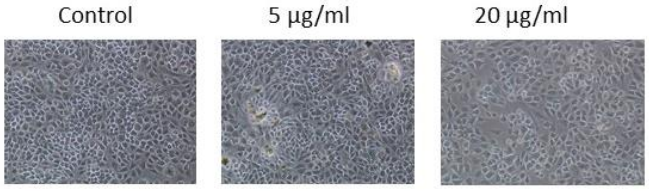


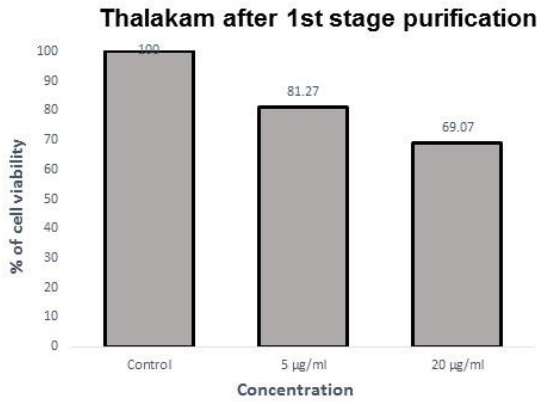


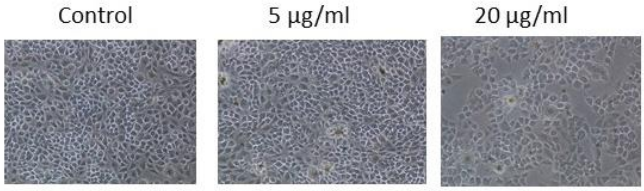


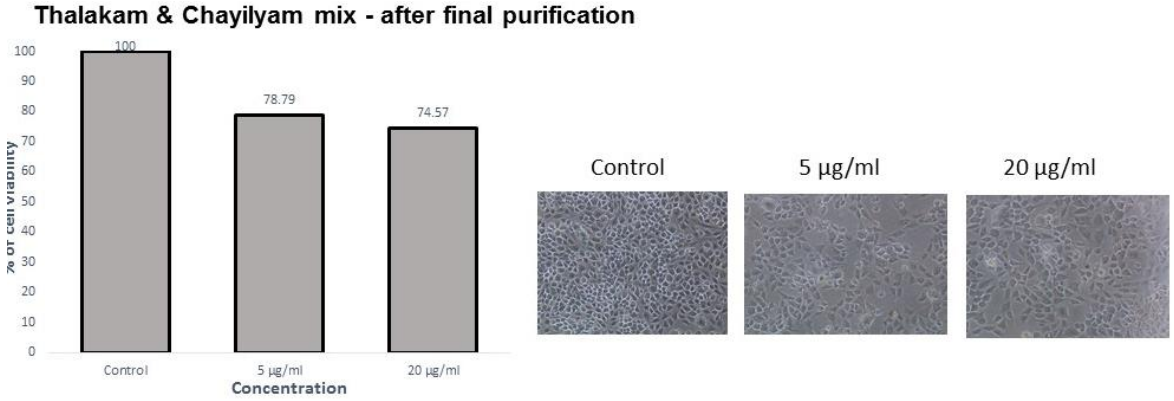


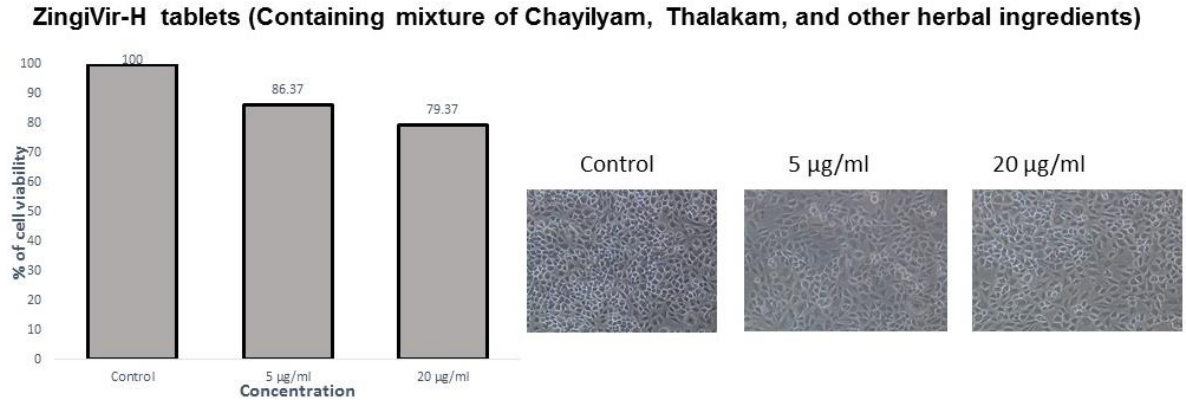












From the preliminary result it is clear that the raw material and the final product is non-toxic to the cell line tested.

Summary of cytotoxicity studies

The cytotoxicity test is one of the biological evaluation and screening tests that use tissue cells *in vitro* to observe the cell growth, reproduction and morphological effects by any agents. Cytotoxicity is preferred as a pilot project test and an important indicator for toxicity evaluation of any agents especially drug molecules as it is simple, fast and has a high sensitivity. The preliminary cytotoxicity testing of ZingiVir-H against cancer (Human breast cancer (MCF 7 & MDAMB-231) and human cervical cancer (SiHa) and normal cell lines (L6) was conducted at two national institute situated at Thiruvananthapuram (RGCB and NIIST). From the result it was very clear that the ZingiVir-H, its raw materials and intermediates are nontoxic to the cell lines tested as it does not inhibit the cell growth and morphology. So from the result it is clear that ZingiVir-H is safe for human cells, as it is nontoxic towards normal cell line tested.

References

1. Joseph J, Seervi M, Sobhan PK, Retnabai ST (2011) High Throughput Ratio Imaging to Profile Caspase Activity: Potential Application in Multiparameter

High Content Apoptosis Analysis and Drug Screening. PLoS ONE 6(5): e20114. doi:10.1371/journal.pone.0020114.

2. Santhik Subhasingh Lupitha1, Leena Chandrasekhar1, Shankara Narayanan Varadarajan, Aparna Geetha Jayaprasad, Aneesh Chandrasekharan, Sanoj Namdev Patil, Minsa Mini, Prakash Rajappan Pillai, T.R. Santhoshkumar. Reporter cell line for real-time imaging of autophagy and apoptosis. Toxicology letter. 326 (2020) 23-30.
3. Reshmitha, T. R., Thomas, S., Geethanjali, S., Arun, K. B., & Nisha, P. (2017). DNA and mitochondrial protective effect of lycopene rich tomato (Solanum lycopersicum L.) peel extract prepared by enzyme assisted extraction against H 2 O 2 induced oxidative damage in L6 myoblasts. *Journal of Functional Foods*, *28*, 147-156.

In vivo Toxicity Studies in Wistar rats

Repeated dose 28-day oral toxicity study of Pankajakasthuri ZingiVir-H in Wistar rats

Form B No: PKHRF/B1/125/RP/01-2020

Study site

Pankajakasthuri Herbal Research Foundation, Pankajakasthuri Ayurveda Medical College Campus, Killy, Kattakada P.O, Thiruvananthapuram, Kerala, Pin-695572.

CPCSEA No: 2093/PO/ReRcBi/S/20/CPCSEA

Test item information

Test item name	: ZingiVir- H
Name to be used in report	: ZingiVir- H
Test item code by Test facility	: ZH
Physical appearance	: Brown coloured tablet
Storage conditions	: Room temperature, avoid direct sun light
Name of the supplier	: Pankajakasthuri Herbals India (P) Ltd., Poovachal, Thiruvananthapuram, Kerala

The responsibility for the correct identity of the test item rests with the sponsor.

Test System and Management

Animal species	Rats
Strain	Wistar albino rats
Source of animal	SCTIMST, Trivandrum
No. of animals and sex	52 Wister rats (26 male and female each) are used for the experiment. Females will be nulliparous and non-pregnant
Body weight range	175-250 g
Age at treatment	8 to 12 weeks

Study Design

Considering the results obtained in the in-vitro cytotoxicity test, the present proposal limits to examine the subactue toxicity effect of the study drug in the rats as per OECD-407 guidelines. A dose range of 500, 1000, 1500 mg/ kg bw/day was fixed as per limit test guidance prescribed by OECD guidelines which shall explore the overall toxicity assessments.

Six groups consisting of equal male and female rats was maintained in the study. Group- I, I R, II, III, IV and IV R served as control, control recovery, low, mid, high dose and high dose recovery group respectively.

Group No.	Group	Dose	No. of rats/group	No of rats	
				male	female
Group- I	Control	Distilled water (1 ml/ 100 g body wt, orally)	6	3	3
Group- I R	Control Recovery	Distilled water (1 ml/ 100 g body wt, orally)	6	3	3
Group- II	Low dose	ZingiVir- H (500 mg/ kg bw/day, orally)	10	5	5
Group- III	Mid dose	ZingiVir- H (1000 mg/ kg bw/day, orally)	10	5	5
Group- IV	High dose	ZingiVir- H (1500 mg/kg bw/day, orally)	10	5	5
Group- IV R	High dose Recovery	ZingiVir- H (1500 mg/ kg bw/day, orally)	10	5	5

Dose Formulation

The weighed test item was suspended in Distilled water to get desired concentration as per the dose (mg/kg body weight) following OECD Guideline. Test item will be formulated shortly before dosing. The homogeneity of the test formulation will be maintained by continuous stirring with glass rod.

Administration of Test Item

The test item ZingiVir- H was administered through oral route by gavage to the animal after formulation preparation using rat gavaging needle fitted to graduated syringe. The administration of the ZingiVir- H will be done after calculating the dose for each

respective group and formulation will be made with the dose concentration as mentioned in the study design. This procedure of administration will follow for 28 consecutive days as per OECD Guideline.

Study compliance

The study was performed in accordance with the following:

- The standard operating procedures of Pankajakasthuri Herbal Research Foundation Animal House.
- The recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for laboratory animal facility published in the gazette of India, January 7th 2010 and the protocol approved by Institutional Animal Ethics Committee (IAEC).

Observations

Following observations was made during the course of the study.

Clinical Signs and pre-terminal deaths

All animals was observed daily in the morning and again in the afternoon for clinical signs and mortality during the study period.

Body weight (bw)

The body weight of each rat was recorded prior to treatment on Day 1, weekly thereafter and at terminal sacrifice of the study. Group mean body weights and body weight gained was calculated.

Food Consumption

The quantity of food consumed by rats in each cage was measured and recorded from the day of commencement of treatment and average weekly consumption was calculated. Food intake per rat was calculated using the amount of food offered to and left in each cage in each group. Food consumption of recovery group rats was recorded weekly during post-treatment period.

Water Consumption

The quantity of water consumed by rats in each cage was measured and recorded from the day of commencement of treatment and average weekly consumption was calculated. Water intake per rat was calculated using the amount of water offered to and left in each cage in each group. Water consumption of recovery group rats was record weekly during post-treatment period.

Haematology

At the end of treatment (on 28th day) all treatment group animals and on 42nd day all recovery group animals was fasted overnight. *Ad libitum* water shall be given during fasting. Blood samples was collected by cardiac puncture, under general anaesthesia, with EDTA anticoagulant for determining the following haematological parameters;

- Haemoglobin (Hb)
- Erythrocyte Count (Total RBC)
- Leukocyte Count (Total WBC)
- Platelet Count
- Neutrophil
- Basophil
- Eosinophil
- Lymphocytes
- Monocytes

Serum Biochemistry

At the end of treatment (on 28th day) all treatment group animals and on 42nd day all recovery group animals was fasted overnight. *Ad libitum* water shall be given during fasting. Blood samples was collected by cardiac puncture, under general anaesthesia. The serum was separated by centrifuging the blood samples at 3000 rpm for 10 mins for determining the following clinical chemistry parameters as per OECD Guideline;

- Total Protein
- Glucose
- Alanine aminotransferase (ALT)
- Aspartate aminotransferase (AST)
- Alkaline phosphatase (ALP)
- Creatinine (Creat)
- Total Cholesterol (TC)
- Triglycerides
- Bilirubin
- Urea
- Uric acid
- HDL
- LDL

- Serum albumin
- Calcium
- Potassium
- Sodium
- Chloride
- Phosphorous

Tissue collection

All animals was euthanized by injecting Sodium pentobarbital at 200 mg/kg body weight dissolved with saline intra peritoneal. The representative tissue samples of heart, liver, spleen, kidneys, lungs and brain was collected and preserved in 10% neutral buffered formalin. The tissues was embedded in paraffin wax, sectioned at five micrometres and stained with haematoxylin and eosin.

Necropsy experiments

At the end of the test (28^{th} day), inspection of the externum and body orifices was performed to assess abnormalities. Following, the surviving animals was injected by Sodium pentobarbital for humane euthanasia. The nasal, cranial, oral, thoracic, and abdominal cavities shall be opened and the visceral organs examined by dissection. After taking the wet weight of the internal organs, the histopathological examination of the tissue samples was performed. The adverse changes in the tissue pattern was photographed and the results discussed. Necropsy procedure are performed within 4 hours after the death of the animal to minimize autolysis (Ghosh, 1984).

Histopathology experiments

Processing of sample: The methods employed were adapted from (Culling et al., 1985). The processes include: fixation, infiltration/impregnation, embedding, sectioning, staining and mounting.

Fixation: The organs are fixed by putting them in a large volume of 10% formalin (ten volumes of fixative for one volume of tissue).

Dehydration: After a few brief washes in distilled water, the tissues are dehydrated through a series of alcohol as shown in the schedule; (i) 50% concentrated alcohol for 5 min and changed twice (a total of 10 min); (ii) 70% concentrated alcohol for 5 min and changed 3 times (a total of 15 min); (iii) 95% concentrated alcohol for 5 min and changed 3 times (total of 15 min); (iv) absolute alcohol (100%), for 10 min and then changed 3 times (a total of 30 min).

Cleaning: The tissues are placed in xylene solution and allowed for a period of 30 min.

Infiltration or impregnation: The tissues are infiltrated or impregnated by using molten paraffin wax at little above the melting point of the wax on a hot plate for a period ranging from 1 - 2 h.

Embedding: This was done by using fresh molten paraffin wax, embedding mould, embedding pot and hot plate. The molten wax shall be poured into the mould and left for few seconds before the specimen is placed or embedded into the mould. This is kept for at least 1 h to solidify, before microtomy or sectioning.

Sectioning: The embedded tissue in the paraffin block is sliced into thin section using microtome with a thickness of 10µm for microscopic examination. Sliced tissues are then placed on a clean microscopic slide, and then stained.

Staining: The following method is employed for staining the tissues.

Deparaffinization of tissue: the processes followed include leaving the slide in a solution of; i. Xylene for 5 min; ii. Absolute alcohol for 5 min; iii. 95% alcohol for 5 min. iv. 75% alcohol for 5 min. v. 50% alcohol for 5 min; vi. Finally wash the slide for 5 minutes using distilled water.

Staining: the processes followed include the following, i. the slides are stained with haematoxylin for 30 min. ii. Excess stain is then washed off with water. iii. Tissues are then differentiated with acid alcohol for a few seconds Removal of excess stain: excess stain is removed using the following processes. Slides are placed in 50% alcohol for 5 min. i) Slides are placed in 70% alcohol for 5 min. ii) Slides are placed in 90% alcohol for 5 min. iii) Slides are placed in 100% alcohol for 5 min. iv) Slides are placed in pure xylene for 5 min.

Mounting: Permanent mounts are made by using Canada balsam dissolved in xylene (mountant). The stained slides are removed from the xylene and excess xylene is wiped off from the slide. One drop of the mountant is placed at the middle of the section. A clear cover-slip is lowered onto the section by resting the cover slip against a finger and leaving it down gently with a mounting needle. Excess mountant is then wiped off, from the edge of the cover slip with care and the slide is left to dry up in an oven at 37°C for 60 minutes. These slides are then examined under 100 X objective microscopically.

Data collection

All the observations made during the study period such as clinical signs of intoxication, body weight changes and gross pathological observations will be recorded and tabulated.

Statistical Analysis

All values are expressed as the mean ± SD (standard deviation) and the results are analysed statistically by one-way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Clinical signs and pre -terminal deaths

No clinical signs and pre-terminal deaths were recorded in any of the doses tested.

Body weight and Body weight changes

None of the animal in treatment group showed any statically significant variation in body weight compared to respective control group. This clearly indicate that the **ZingiVir-H** did not had any effect on body weights.

Food Consumption

There observed slight reduction in the food consumption of male and female during treatment period. However there was no statically significant difference noted in the average weekly food consumption of animals in treatment group, negligible variation noted in recovery (male rats) group compared to the respective control group.

Water Intake

The average water intake of male and female animals in treatment group and recovery group had slight reduction in water intake compared to the respective control group.

Haematology

Treatment did not affect on haematological parameters estimated in both the sexes of treatment and recovery groups when compared with control.

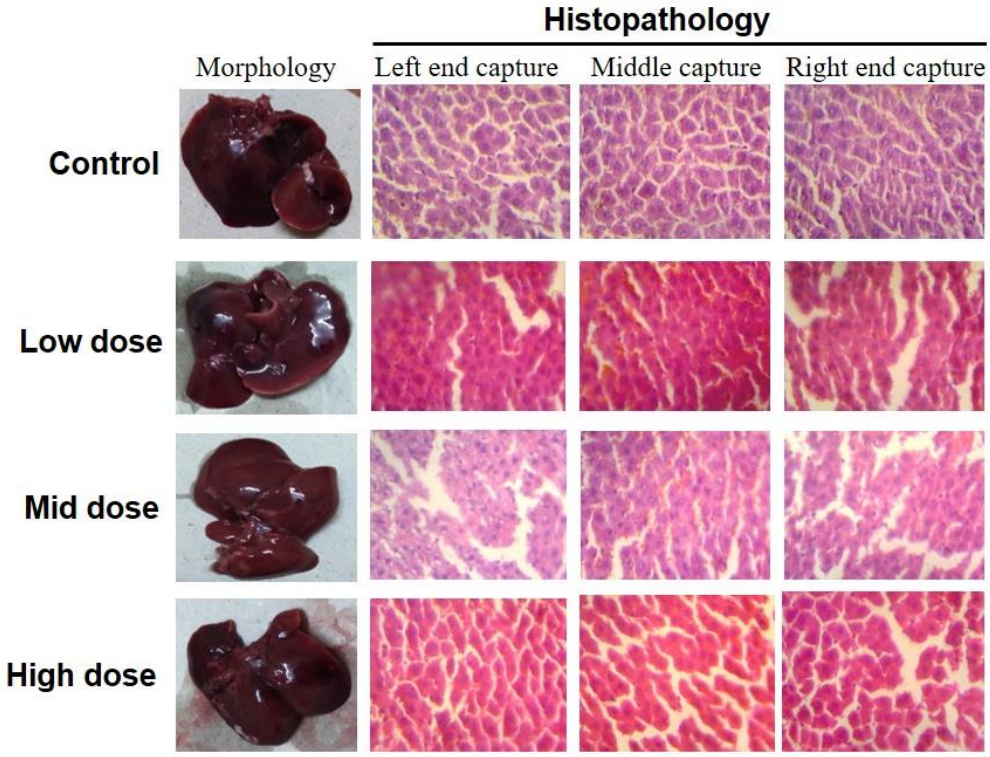
Serum Biochemistry

Clinical chemistry parameters of the treatment and recovery groups were compared with the control groups. There were no treatment related significant differences in biochemical values of study animals. These results indicates that the **ZingiVir-H** does not had an adverse effect on blood and serum parameters at a dose up to 1500 mg/kg.

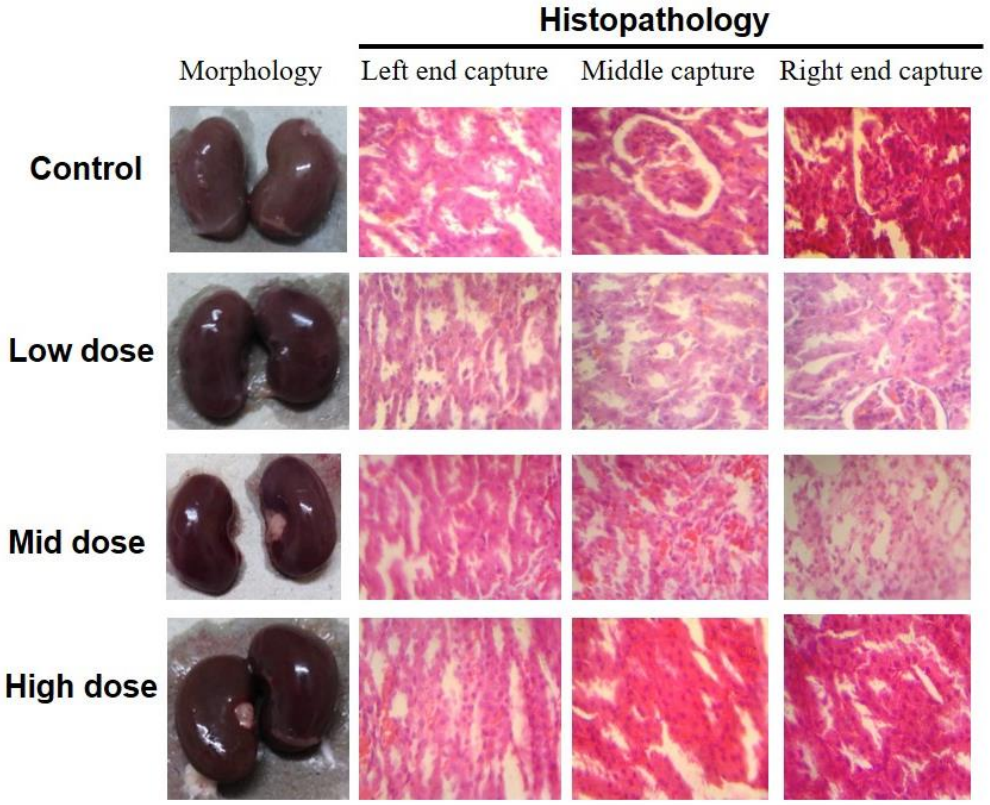
Histopathology experiments

On gross examination any pathologically relevant lesions were not detected in any of the organs. None of the animals in treatment and recovery groups showed any histological changes during evaluation, indicating that the **ZingiVir-H** did not cause any histological changes in the tissues.

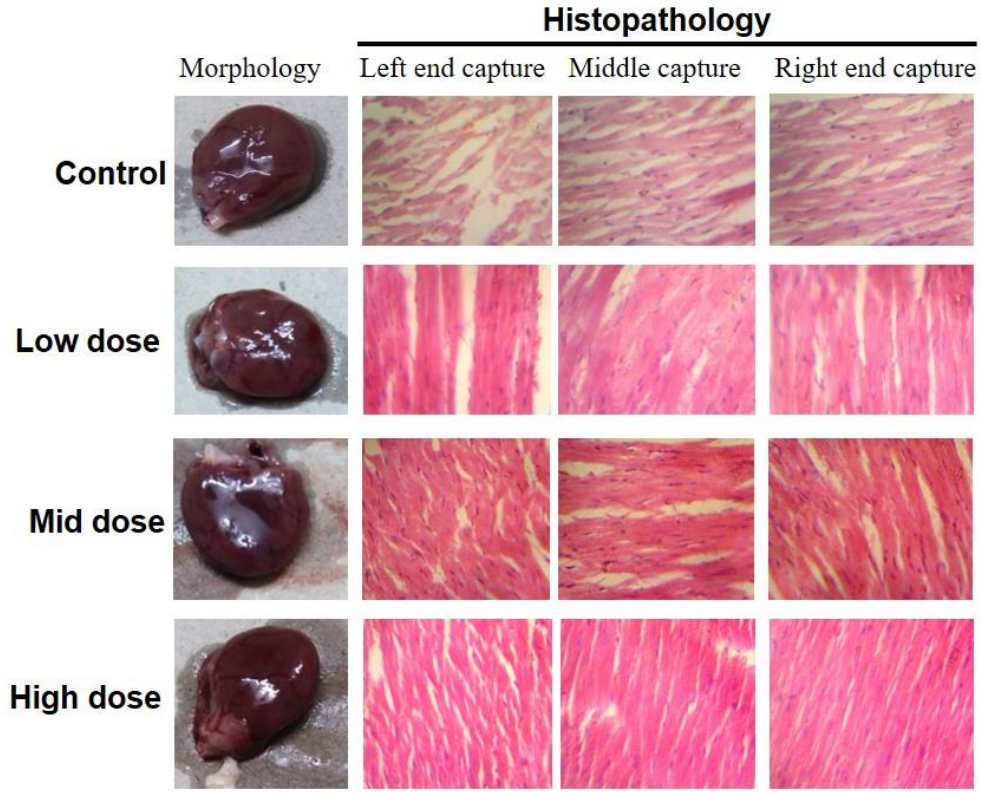
Morphology and Histopathology data of liver



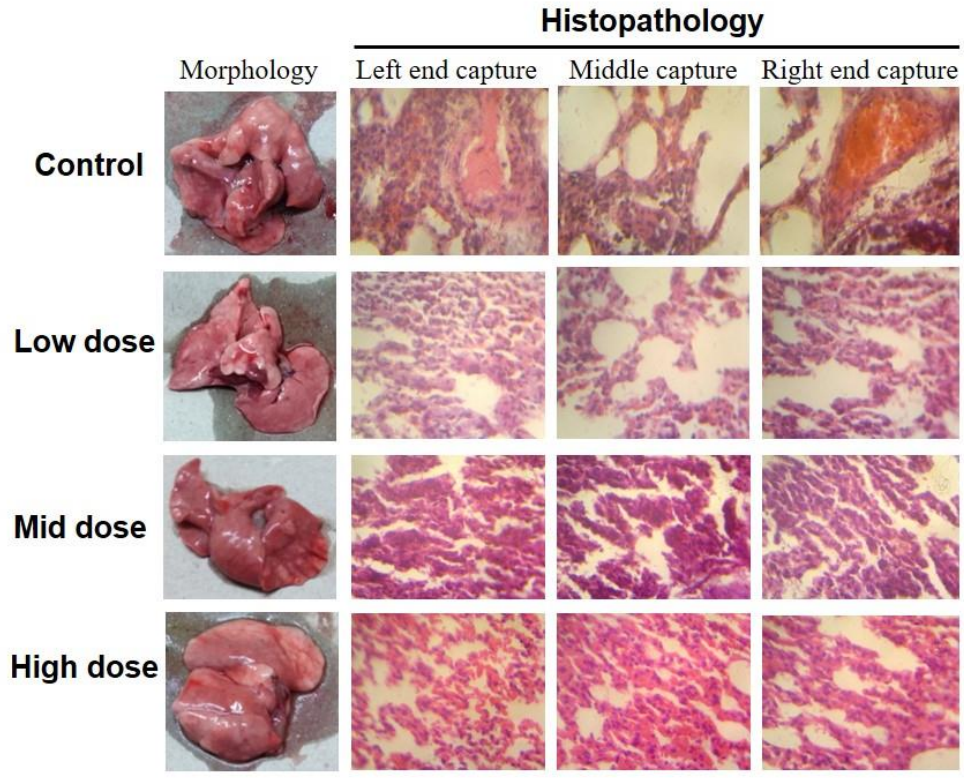
Morphology and Histopathology data of kidney



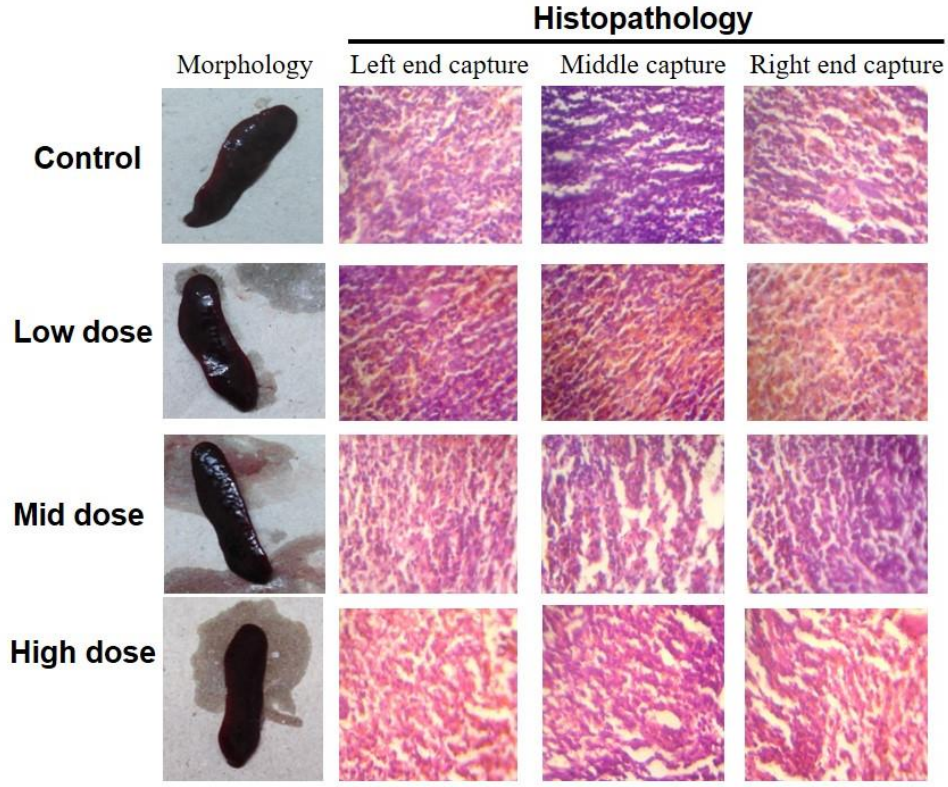
Morphology and Histopathology data of heart



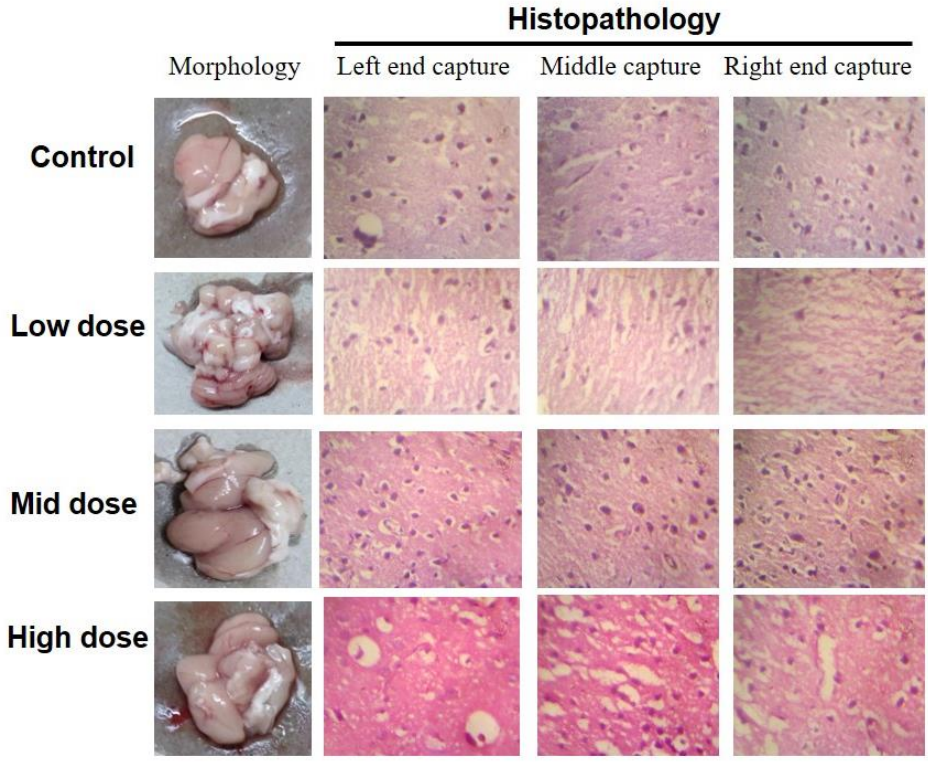
Morphology and Histopathology data of lung



Morphology and Histopathology data of spleen



Morphology and Histopathology data of brain



CONCLUSION

No clinical signs and mortality were noticed up to the high dose of 1500 mg/ kg bw/day, orally.

- There were no significant treatment related changes in average weekly water intake of males and females during treatment and recovery period.
- No significant effect on average daily food consumption was noted in both the sexes up to the high dose.
- No significant treatment related differences were noted in body weight and body weight gains of animals across different groups.
- No effect on haematological parameters of male and female rats treated up to the dose of 1500 mg/ kg bw/day was noted.
- There were no treatment related significant differences in serum biochemical values of study animals treated at and up to the level of 1500 mg/ kg bw/day, orally.

No gross and microscopic pathological changes were noted in the tissues of male and female rats treated at and up to the level of 1500 mg/kg bw/day. Based on the above findings of the study, the No-Observed-Adverse -Effect –Level (NOAEL) of **ZingiVir-H** in Wistar rats following oral route administration for 28 Days was found to be 1500 mg/ kg bw/day, orally.

SUMMARY

Six groups consisting of equal male and female rats will be maintained in the study. Group- I, I R, II, III, IV and IV R served as control, control recovery, low, mid, high dose and high dose recovery group respectively. A dose range of 500, 1000, 1500 mg/ kg bw/day is fixed as per limit test guidance prescribed by OECD guidelines which shall explore the overall toxicity assessments. The test item ZingiVir- H will be administered through oral route by gavage to the animal after formulation preparation using rat gavaging needle fitted to graduated syringe for 28 days. The animals control group and control recovery group were administered with distilled water. The vehicle and test item administration to control recovery and high dose recovery was stopped after 28 days of administration and observed for another 14 days to evaluate the effects during recovery period.

The animals were observed for health status, clinical signs of toxicity and mortality, Body weights and food consumption were recorded at weekly intervals .On

completion of 28 day for treatment group or 42 days for recovery groups respectively, the blood samples were collected from all the animals and subjected to haematological and clinical chemistry evaluation. At termination the animals were humanely sacrificed and subjected to necropsy. Histopathological examination was conducted on the specified list of tissues from the control and the treatment groups.

Food consumption was comparable across the groups. The haematological data did not show any statistically significant differences. Serum biochemical data also showed no statistically significant differences in enzyme levels of dose treated groups. On necropsy, no gross pathological changes were noted in the treatment groups compared to the control groups. From the results of the study, the No-Observed-Adverse -Effect–Level (NOAEL) of **ZingiVir-H** in Wistar rats, following oral route administration for 28 days was found to be 1500 mg/kg Body weight.

References

1. Canadian Council on Animal Care (CCAC) guidelines (www.ccac.ca).
2. Culling CFA, Allison RT, Barr WT (1985). Cellular Pathology Techniques 4th edition London.
3. Ghosh, M. N (1984). Toxicity studies. In fundamentals of experimental pharmacology; scientific book agency, Kolkata, pp. 153–158.
4. OECD Guideline for Testing Chemicals, Section 4. Test No. 407: Repeated Dose 28- Day Oral Toxicity Study in Rodents.
5. OECD Guideline for Testing Chemicals, Section 4. Test No. 407: Repeated Dose 28- Day Oral Toxicity Study in Rodents.

Final conclusion on in vitro and in vivo studies

The in vitro and in vivo studies on ZingiVir-H was duly and scientifically demonstrated. Based on the results, ZingiVir-H was found to be safe both cellular and organ system level at a maximum dose of 1500 mg/kg/day body weight. There is no physical, behavarial, vital-functional, physiological, anatomical, haematological, cytological, biochemical, histopathological toxicities or changes were observed during in vivo and in vitro studies. Thus the safety of ZingiVir-H for the use of humans at a maximum dose of 1500 mg/ kg/day is scientifically established.

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