



***In Vivo* Antitumor Activity of Ethanolic Extract of *Lepidagathis Pungens* Nees Whole Plant**

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ABSTRACT

Cancer is the most dreadful of all the diseases and is the major contributors to the mortality in the world. Out of all the population, almost 1.2 million patients die due to cancer and related problems. The rate and spread of cancer are wide and even in both women and men. It is presently as high as 3.5 million people who have cancer in India alone. There are many ways of treatment of cancer like the surgery, radiation therapy, cryosurgery and chemotherapy. A part of chemotherapy is the usage of a lot of chemicals and synthetic drugs. Due to the side effects of chemotherapy, patients often get symptoms due to the usage of drugs, and so the cancer treatment often is toxic. Because of this, herbal drugs are seemed to have no side effects, and non-toxic effects, and so this project deals with identifying the plant sources of the drugs that treat cancers effectively. The present study focusses on the extraction of the chemical constituents from *Lepidagathis pungens* whole plant and using the same to estimate the anti-tumour potential in EAC induced tumour induction method. The activity was tested in two doses 200 and 400 mg/kg of the extract. They showed a dose-dependent activity when estimated for the tumour parameters and other haematological measures like RBC, WBC counts. They showed similar activity when estimated for the antioxidant enzymes like SGOT, SGPT, Catalases, LPO and ALP levels.



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INTRODUCTION

Cancer is the most dreadful of all the diseases and is the major contributors to the mortality in the world. Out of all the population, almost 1.2 million patients die due to cancer and related problems. The rate and spread of cancer are vast, and even in both women and men. It is presently as high as 3.5 million people who have cancer in India alone (Shrikhande *et al.*, 2007).

It is estimated that the growth of the cancer is caused by benign polyps and other growths in the organs of the body. An estimate is there up to 2/3 cases of the polyps turn into cancers which are malignant (World Health Organization, 2008).

There are often no symptoms of cancers in the initial stages, and they may spread to other parts of the body which causes malignancy. Symptoms start when the cancers grow to a more severe stage, usually till the patient is being diagnosed with the disease (Edwards *et al.*, 2010).

There are many ways of treatment of cancer like the surgery, radiation therapy, cryosurgery and chemotherapy. A part of chemotherapy is the usage of a lot of chemicals and synthetic drugs. Due to the side effects of chemotherapy, patients often get symptoms due to the usage of drugs, and so the cancer treatment often is toxic.

Because of this, herbal drugs are seemed to have no side effects, and non-toxic effects, and so this project deals with identifying the plant sources of the drugs that treat cancers effectively. About half of the drugs that are used to treat cancer are herbal in origin and their active principles too. Drugs like cardiac glycosides, alkaloids, terpenoid etc. have been the sources of anticancer drugs.

The present study focusses on the extraction of the chemical constituents from *Lepidagathis pungens* (*L.pungens*) whole plant and using the same to estimate the anti-tumour potential in EAC induced tumour induction method.

MATERIALS AND METHODS

Herbs and Extracts

Plant parts belonging to *Lepidagathis pungens* were collected from Tirunelveli district. A certified taxonomist duly authenticated the samples.

This dried powder is subjected to extraction with petroleum ether, chloroform, ethyl acetate, ethanol and aqueous based on polarity scale. Phytochemical screening was performed on the ethanol extract of the plant of *L.pungens* (LPE), and this extract was used in future studies.

Animals

Female Swiss mice of albino strain that weighed about 30-32 gms were selected for the work. Lab protocols were designed by following the institutional animal ethical committee (SVCP/IAEC/PhD/2/05/2015).

The mice were subjected to acclimatization in the lab where the temperature and humidity were controlled with light and dark cycles.

Feeding of animals was with the standard diet pellets that were bought from Hindustan suppliers in Bengaluru. This process of pre-experimental acclimatization was carried out for about 11 days.

Acute Oral Toxicity Study

Healthy mice were picked randomly and selected for the study. They are kept in the polypropylene cages for about five days before the start of the study. The mice were fasted overnight and then given the plant extract via the oral route that is suspended in 0.3% CMC dissolved in water. A single dose of the extract was given at a dose of 2gm/kg of the mice via the oral route. Oral intubation cannula was used to ingest the drugs into the mice. The extracts were administered, and the mice were let to set for the first four hours. They were observed for the signs and symptoms of toxicity and mortality in these 4 hours. Total mice which lived and healthy-looking were noted and a day after administration were observed and 14 days were issued as the observation period.

EAC Induced Ascitic Anti-tumor Studies

Cells of tumours

Ehrlich Ascites Carcinoma (EAC) cells are given as gift samples supplied by the Amala Cancer Research Centre, Thrissur, Kerala of India. These cells that are collected from the company are injected into the body of albino mice via the intraperitoneal cavities. The cells were subjected to aspiration, and the peritoneal cavity of the mice are washed with the saline solution and administered in the IP route and observed to develop the ascetic tumour in the cavity.

EAC cell line and induction of cancer

The donor mouse was injected intraperitoneally with 0.2ml of 0.9% normal saline using 1ml syringe. Immediately after injecting, 0.9 % saline ascites fluid is collected from the peritoneal cavity of the donor mouse (1ml). Collected ascites fluid is mixed with 3 ml of simple saline solution and was centrifuged for about 15mins at a speed of 1500 rpm inside a cooling centrifuge. The supernatant liquid is discarded, and the pellet is resuspended with the same quantity of simple saline and centrifuged, the process is redone three times to remove the unwanted matters. Finally, these cells were resuspended with a known amount of (10 ml) of simple saline. From this 10 μ l of fluid placed on Neubauer's chamber and the total number of cells appears on the 64 small squares were counted. From this 10 μ l of fluid placed on the Neubauer chamber and the total number of cells appears on the 64 small squares were counted. Calculations were made as 0.5 ml of solution contains 2 \times 10⁶ cells.

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ties. The cells were subjected to aspiration, and the peritoneal cavity of the mice are washed with the saline solution and administered in the IP route and observed to develop the ascetic tumour in the cavity.

The cells were counted, and the serial dilutions were prepared to the concentration should be maintained as the 2×10^6 cells/ ml/ mouse. The amount of the cells were given via the IP route were allowed to regenerate and divide inside the mice body for about 3 days before the start of the study by Pedro Figueiredo, 2008. The Transplantation was done in the mice using a sterile syringe that is disposed of under the aseptic conditions.

Procedure

60 Swiss albino mice are separated into five groups, with 12 animals in each Group. All groups apart from Group I receive 0.5 ml of EAC cells suspension (2×10^6 cells/mouse i.p.), and this is taken as the '0' day. Group, I served as normal control (0.3 % sodium CMC suspension, p.o). Group II served as EAC control. Twenty-four hours after the EAC administration, Group III received reference drug 5-Fluorouracil (5-FU, 20 mg/kg b.w, i.p) Groups IV and V received Test drug (200 and 400 mg/kg b.w, orally) and daily for 14 days.

Group-1: Normal group control and administered with 0.3% of S-CMC suspension

Group-2: Tumor group control and administered with 0.3% of S-CMC suspension

Group-3: Positive group control and administered with 20mg/kg of 5-Fluorouracil

Group-4: Drug group that is administered with LPE (200 mg/kg, p.o) suspended in sodium CMC

Group-5: Drug group that is administered with LPE (400 mg/kg, p.o) suspended in sodium CMC

The drug therapy was continued till 14 days at the start of the study. On day 15, i.e. after the previous dose and 24 hrs fasting, the blood samples were drawn out of the retro-orbital plexus. They were subjected for estimation of the haematological parameters such as RBC, WBC, DC, Hb counts etc. were estimated by an auto-analyzer. The counts of the WBC were performed out of the smear taking of the blood.

The fluid that was collected from the cavity of the peritoneum of the mice into the centrifuging tube tumour volume was measured and divided into two parts. The first part is the centrifugation in the graduated tube with a centrifuge at the 1000rpm for amount 10min, and the PCV was also measured from the samples. The other cells part in the fluid were also separated using the centrifugation and

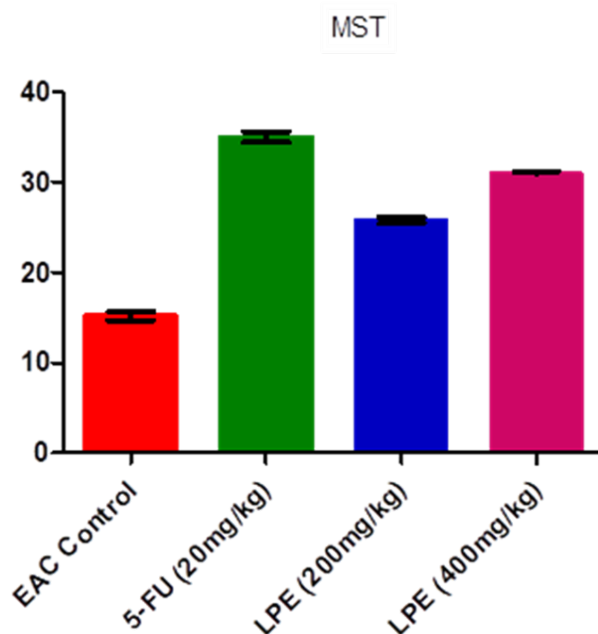


Figure 1: Effect of the ethanolic extract of L.pungens on MST

then stained using the trypan Blue solution that is suspended in 0.4% of the normal saline. The total number of viable cells and no viable cells were are measured.

Remaining animals (Mice) are grouped in to check the life span of the mice. The body weight changes and other parameters were checked for about weeks. % increase in the life span of the mice was calculated using standard procedures (Gaze, 2000). Similarly, the serum was analyzed for the following parameters: like the TP, SGOT, SGPT, ALP, GSH, LPO, CAT, SODs and other serum parameters. (Bel-lamakondi et al., 2014)

Anti-tumour Parameters

Percent of Increase in Life Span

The recording of the mortality of the mice was monitored with the effect of LPE on tumour growth, and per cent of raise in the life-span (ILS %) were measured as per procedures prescribed by standard procedures.

$$\text{ILS (\%)} = \left[\frac{(\text{Mean mice survived in the test group of A} \text{minal})}{(\text{Mean mice survived in the control group of A} \text{minal}) - 1} \right] \times 100$$

$$\text{Mean surviving time} = \frac{[\text{1st mortality} + \text{Last mortality}]}{2}$$

Body-weight analysis

The mean body weight of the animals was measured and noted in both groups. The measurements were taken in the starting day of the experiment and were again taken at 5th day and calculated on 15th day.

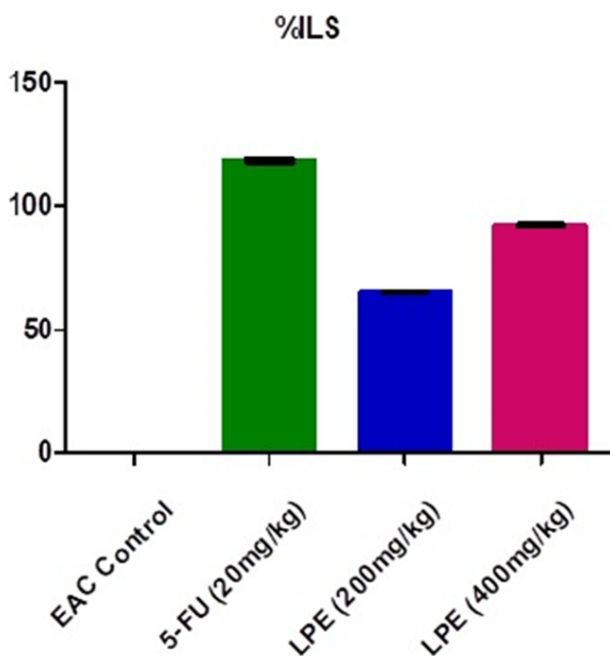


Figure 2: Effect of the ethanolic extract of L.pungens on %ILS

$$\% \text{ Increase in bodyweight} = \left(\frac{\text{Wt. of mice on day 0} - \text{Wt. of mice on day 15}}{\text{Wt. of mice on day 0}} \right) \times 100$$

Changes in the intake of food

Mice consume the animal feed in a cage in one week is obtained by subtracting the food that is leftover in the cage in the last day of the week from the total amount of food that is given to the animals in that week in gms.

Food that is consumed by a single mouse in one week equals food consumed by six animals divided by 6. Feed consumed by individual animal/week = Feed consumed by six animal per cage per week/6. Similarly, food consumed in a day equals food in a week divided by 7.

Estimation of tumour volume

The peritoneal lavage isolated the fluid that is extracted from the cavity of the tumour that was inside the mice by sacrificing the mice into the graduated tubes, and the measurements were taken (Bala et al., 2010)

Determination of tumour weight

The animals were all dissected, and the fluid was collected from the mice from the peritoneal cavity. This was then carefully stored in the LPE of 5 ml sterile syringe. This was transferred into the tubes that are already weighed (Kathiriya et al., 2010).

The fluid was then weighed and calculated using this formula.

Tumour Weight=Final Weight of a graduated tube –

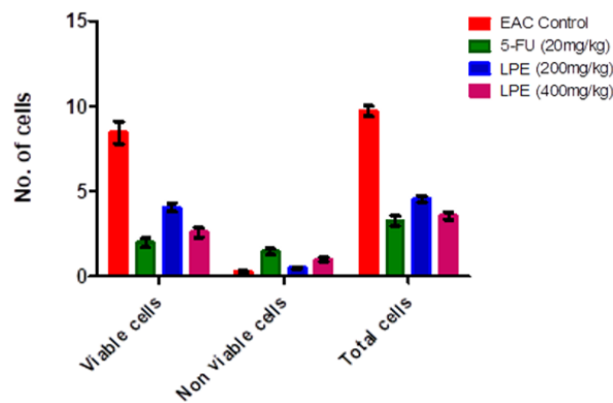


Figure 3: Effect of the ethanolic extract of L.pungens on PCV

Weight of pre-weighed graduated tube

Packed Cell Volume (PCV)

The animals were all dissected, and the fluid was collected from the mice from the peritoneal cavity. 1g of the fluid was then transplanted into the murine tumour and were very carefully collected into the LPE of 5 ml sterile syringe.

This fluid was transferred into the graduated glass tube and then centrifuged at a speed of 1000rpm-for about 5mins. The fluid was measured using the standard procedure (Dolai et al., 2012).

$$\text{Packed Cell volume (PCV) (\%)} = \frac{1 - \text{volume fluid}}{1} \times 100$$

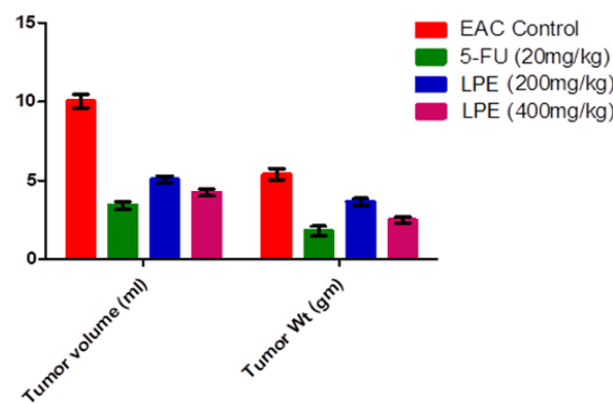


Figure 4: Effect of the ethanolic extract of L.pungens on Tumour parameters

Tumour cell (Viable/nonviable) count

The fluid was collected was pipetted into the tube and then diluted to 20times with the PBS solution. Then the drop was dropped in the cell suspensions was placed in the Neubauer’s chambers, and the numbers of cells were counted in 64 cells of the chambers (Karmakar et al., 2013).

The viable and non-viable cells were counted and then determined by the trypan blue assay method.

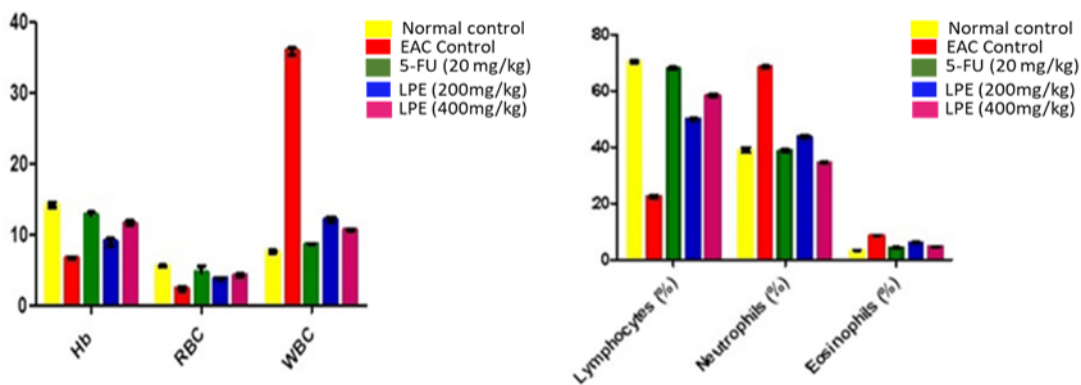


Figure 6: Effect of ethanolic extract of *L.pungens* on the blood profile of the tumour induced mice

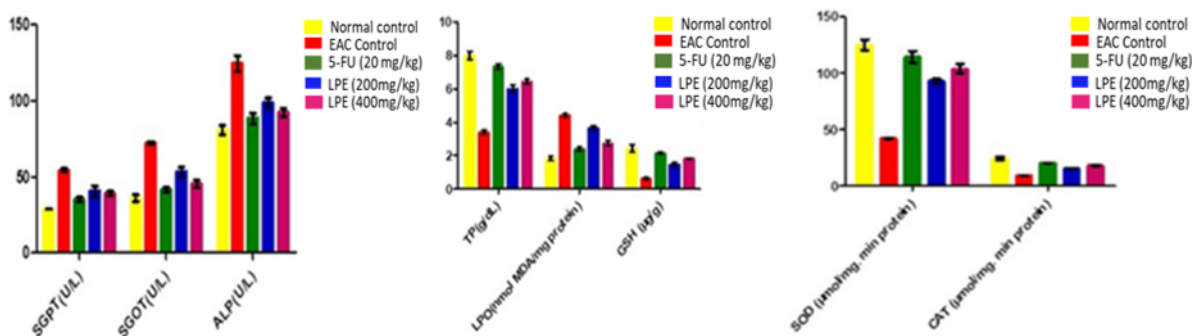


Figure 7: Effect of ethanolic extract of *L.pungens* on the biochemical parameters of the tumour induced mice

Table 1: Anti-Tumor properties of the ethanolic extract of *L.pungens*

| Parameters | EAC Control | 5-FU (20mg/kg) | Ethanol Extract | |
|-------------------|--------------|--------------------|---------------------------|---------------------------|
| | | | 200mg/kg | 400mg/kg |
| MST | 15.17 ± 0.48 | 34.98 ± 0.59 a*** | 25.77 ± 0.36 a*** b*** | 30.85 ± 0.35 a*** b*** |
| % ILS | 0.00 | 118.28 ± 0.72 a*** | 65.18 ± 0.20 a*** b*** | 91.83 ± 0.45 a*** b*** |
| Tumor volume (ml) | 10.03 ± 0.42 | 3.43 ± 0.24 a*** | 5.06 ± 0.23 a*** b*** | 4.24 ± 0.22 a*** |
| Tumor wt(gm) | 5.39 ± 0.35 | 1.79 ± 0.31 a*** | 3.64 ± 0.22 a*** b*** | 2.49 ± 0.19 a*** |
| PCV | 7.14 ± 0.20 | 2.35 ± 0.21 a*** | 5.99 ± 0.33 a** b*** | 3.99 ± 0.21 a*** b*** |
| Viable cells | 8.43 ± 0.65 | 1.99 ± 0.27 a*** | 4.06 ± 0.23 a*** b** | 2.59 ± 0.28 a*** |
| Non-viable cells | 0.28 ± 0.06 | 1.45 ± 0.18 a*** | 0.50 ± 0.05 b*** | 0.99 ± 0.12 a*** b** |
| Total cells | 9.70 ± 0.31 | 3.27 ± 0.28 a*** | 4.57 ± 0.19 a*** b** | 3.57 ± 0.21 a*** |
| Viable % | 95.79 ± 0.74 | 65.55 ± 3.57 a*** | 88.56 ± 1.65 a* b*** | 76.17 ± 1.33 a*** b** |
| Non-viable % | 3.34 ± 0.35 | 37.46 ± 3.06 a*** | 12.09 ± 1.57 a** b*** | 25.15 ± 1.41 a*** b*** |

The values were given as mean ± SEM, n=6; A=Group 1vs 2, 3, and 4; b=Group 2 vs 3 and 4 ***P<0.001, **P <0.01, *P<0.05

Table 2: Effect of the ethanolic extract of *L.pungens* on haematological parameters

| Parameters | Normal | EAC Control | 5-FU (20mg/kg) | Ethanolic Extract | |
|--|--------------|----------------------|--------------------------|---------------------------|---------------------------|
| | | | | 200mg/kg | 400mg/kg |
| Hb (g %) | 14.21 ± 0.33 | 6.60 ± 0.14 a*** | 12.78 ± 0.37 a** b*** | 9.02 ± 0.47 a*** b*** | 11.69 ± 0.23 a*** b*** |
| RBC (million/mm ³) | 5.57 ± 0.08 | 2.36 ± 0.27 a*** | 4.69 ± 0.29 a** b*** | 3.78 ± 0.06 a*** b*** | 4.26 ± 0.26 a*** b*** |
| WBC (10 ³ cells/mm ³) | 7.62 ± 0.13 | 35.90 ± 0.51 a*** | 8.67 ± 0.08 a* b*** | 12.12 ± 0.38 a*** b*** | 10.66 ± 0.06 a*** b*** |
| Lymphocytes (%) | 70.32 ± 0.54 | 22.45 ± 0.45 a*** | 67.91 ± 0.43 a** b*** | 49.94 ± 0.42 a*** b*** | 58.37 ± 0.36 a*** b*** |
| Neutrophils (%) | 38.97 ± 0.28 | 68.45 ± 0.38 a*** | 38.67 ± 0.47 b*** | 43.64 ± 0.48 a*** b*** | 34.59 ± 0.36 a*** b*** |
| Eosinophils (%) | 3.46 ± 0.06 | 8.68 ± 0.06 a*** | 4.34 ± 0.07 a*** b*** | 6.16 ± 0.17 a*** b*** | 4.75 ± 0.07 a*** b*** |

The values were given as mean ± SEM, n=6; A=Group 1 vs 2, 3, and 4; b=Group 2 vs 3 and 4, ***P<0.001, **P<0.01, *P<0.05

Table 3: Effect of ethanolic extract of *L.pungens* on the biochemical parameters of the tumour induced mice

| Parameters | Normal | EAC Control | 5-FU (20mg/kg) | Ethanolic Extract | |
|----------------------------|---------------|-----------------------|----------------------------|---------------------------|----------------------------|
| | | | | 200mg/kg | 400mg/kg |
| SGPT(U/L) | 28.95 ± 0.29 | 54.46 ± 1.12 a*** | 34.98 ± 1.59 a*** b*** | 40.63 ± 3.47 a*** b*** | 38.97 ± 1.52 a*** b*** |
| SGOT (U/L) | 36.07 ± 2.13 | 72.17 ± 1.16 a*** | 41.77 ± 1.40 a*** b*** | 53.16 ± 3.10 a*** b*** | 45.35 ± 2.55 a*** b*** |
| ALP (U/L) | 80.79 ± 3.25 | 124.41 ± 5.29 a*** | 88.45 ± 3.63 a*** b*** | 98.55 ± 3.24 a*** b*** | 92.28 ± 2.75 a*** b*** |
| Total Protein (g/dL) | 8.01 ± 0.25 | 3.41 ± 0.14 a*** | 7.34 ± 0.17 a* b*** | 6.01 ± 0.23 a*** b*** | 6.44 ± 0.15 a*** b*** |
| LPO (nmol MDA/mg protein) | 1.84 ± 0.14 | 4.42 ± 0.10 a*** | 2.4 ± 0.14 a* b*** | 3.67 ± 0.14 a*** b** | 2.75 ± 0.18 a*** b*** |
| GSH (μg/g) | 2.44 ± 0.22 | 0.63 ± 0.07 a*** | 2.16 ± 0.06 b*** | 1.49 ± 0.12 a*** b*** | 1.83 ± 0.04 a*** b*** |
| SOD (μmol/mg. min protein) | 125.00 ± 4.64 | 42.24 ± 0.18 a*** | 114.46 ± 5.21 a*** b*** | 92.88 ± 2.16 a*** b*** | 103.92 ± 4.15 a*** b*** |
| CAT (μmol/mg. min protein) | 24.69 ± 1.25 | 9.44 ± 0.12 a*** | 20.52 ± 0.26 a*** b*** | 15.61 ± 0.18 a*** b*** | 18.18 ± 0.14 a*** b*** |

The values were given as mean ± SEM, n=6; A=Group 1 vs 2, 3, and 4; b=Group 2 vs 3 and 4, ***P<0.001, **P<0.01, *P<0.05

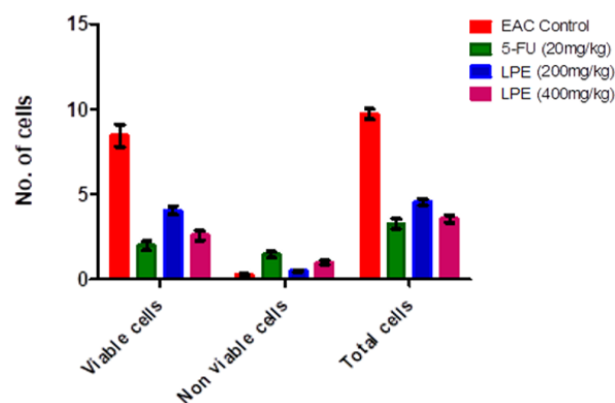


Figure 5: Effect of the ethanolic extract of *L.pungens* on the viability of cells

The cells were stained using the blue die that is suspended in the normal saline. The cells were then stained, and they were named as non-viable cells, and those which are stained are named as viable cells. They are counted based on the below formula.

$$\text{Cells count} = \frac{(\text{number of total cells} \times \text{dilution factors})}{(\text{area} \times \text{thickness of the film})}$$

Histopathology studies

Histopathological study was conducted using standard sectioning and staining techniques that were mentioned. The sections were viewed under the microscope, and the pictures were taken.

Statistical Analysis

All the values were expressed as mean \pm SEM ($n = 6$). Statistical analysis was carried out by using one-way ANOVA followed by Dunnett's multiple comparison test with GraphPad Prism 5.0 (San Diego, CA, USA) and values of $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

The whole plant of *L.pungens* was extracted with various solvents and ethanolic extract tested for acute toxicity, and the plant is proven to be safe. No signs of toxicity were observed in this study. The ED_{50} was fixed, and the tests were continued for anti tumour activity.

The results of the anti-tumour activity were given in Table 1. The MST of the ethanol extract at two doses of 200 and 400mg/kg was found to be 25 and 30, respectively, which was lower than that of the standard drug that is 5-FU. The control group showed a very low MST at 15. The percentage ILS of the standard drug is found to be more than 100, and extract at 400 showed a nearer value at 91%, which is significant when compared to the standard. There was a significant change in the tumour volume. The control group had a tumour of 10ml, which is very much

higher than the extract-treated Group of 5 and 4.2 at two doses and of the standard drug of 3.4. a similar result was shown in terms of the tumour weight also. The extract group showed a lesser size of the tumour compared to the control group, and the standard drug was effective in this case (Figures 1 and 2).

In terms of cellular values, the packed cell volume was higher in the control group which indicates that the growth of tumour cells was rapid and the extract group showed similar values with that of the standard drug. In this value, the amount of viable and active cells is higher in the control group compared to the other three groups, and the non-viable cells in the standard Group are lesser. This indicated that the standard Group of animals has stronger cells that are not actively dividing which were similar to that of the extracts. The percentage of the viable cells in the control group is near to 100 % which indicates that the cancer was successfully induced to the mice and the extract group remained at 76% at the higher dose indicating that the extract controlled the spread of the tumour and the viability of cells.

The effect of ethanolic extract of *L.pungens* on the blood parameters is shown in Table 2. There was a significant lowering of the blood cells like RBC, Lymphocytes, and there is a greater increase in the WBC, Neutrophils and eosinophils in the control group. This indicates that the response of inflammation and the tumour is apparent in the blood cells. The values are significant compared to the normal limits of the cells in the body. The extract showed an elevation in the RBC and haemoglobin levels, but the values are not equal to the normal values. The extract showed a dose-dependent activity in suppressing the tumours. 400mg extract showed a better and significant activity compared to the same at a lesser dose of 200mg. The standard drug also showed similar results compared to the extracts by lowering the WBC counts, neutrophils and rising the haemoglobin and RBC counts (Figures 3, 4 and 5) (Kaplow, 1955; Natt and Herrick, 1952).

The effect of ethanolic extract of *L.pungens* on the biochemical parameters was shown in Table 3. The parameters like SGOT, SGPT, ALP, total protein, lipid peroxidases and catalases were estimated after treating the tumour induced mice with extracts and standard drug. The following table shows the results of the activity. There was a significant lowering of the values of total protein, GSH, SOD and catalases in the tumour induced groups. This was raised to almost normal with the treatment of standard drug, and the values were competent with the extract treatment at 400mg dose. In this activity, also the extracts showed a dose-dependent activity.

Other parameters like SGOT, SGPT, ALP and LPO's are significantly elevated with the induction of the tumour indicating that there are a mutation mechanism and oxidation that is involved in the causation of the tumour (Shiau and Chang, 1983). These values were normalized with the treatment if the mice with standard drug and the extracts at two doses (Figures 6 and 7).

CONCLUSIONS

In the present investigation, *Lepidagathis pungens* was extracted with various solvents and ethanolic extract was used to study the anti-tumor activity in the ascites induced method. The activity was tested in two doses 200 and 400mg of the extract. They showed a dose-dependent activity when estimated for the tumour parameters and other haematological measures like RBC, WBC counts. They showed similar activity when estimated for the antioxidant enzymes like SGOT, SGPT, Catalases, LPO and ALP levels. This research concerns for further research in estimating the chemical constituents that are responsible for the activity and determining the molecular mechanisms.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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