

# Evaluation of Cytotoxic and Anti-Tumor Activity of *Phyllanthus Acidus* (L.) Skeels Leaf Extracts

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**Abstract:** Cytotoxic and antitumor activities of ethyl acetate extract of leaves of *Phyllanthus acidus* were studied. The extract showed significant *in vitro* cytotoxic activity against Hep G2 and DLA cell lines. IC<sub>50</sub> value of Hep G2 cell line was 11µg/ml and for DLA cell line was 75µg/ml. The antitumor activity of the extract was determined by using DLA cell line induced solid tumor model in Swiss albino mice and its comparison with anticancer drug cyclophosphamide. There was significant reduction of tumor volume in *P. acidus* treated animals. Percentage reduction of in tumor volume after 30 days was 45.75% at lower concentration of 200mg/kg and 76.66% at 400mg/kg drug concentration.

**Keywords:** anti-tumor activity, cytotoxic activity, *Phyllanthus acidus*

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## I. INTRODUCTION

Cancer is the leading cause of mortality in developed countries and the second leading cause of death in developing countries. Now cancer is a common disease, and more than one in three people will develop some form of cancer in their lifetime. Cancer is associated with aging and has increased as life expectancy has increased. Chemotherapy is one of the methods for the treatment of cancer. A major advantage of chemotherapy is its ability to treat widespread or metastatic cancer whereas surgery or radiation therapies are limited to treating cancers that are confined to specific areas. Most of the synthetic chemicals now used for chemotherapy affect normal healthy cells at genomic level. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. Plants have a long history of use in the treatment of cancer. More than 3000 plant species that have reportedly been used in the treatment of cancer. Some plant products are less likely to cause side effects<sup>1</sup>. *Phyllanthus acidus* is a monoecious and semi evergreen and known as the Otaheite gooseberry or star gooseberry, or simply gooseberry tree, is one of the trees with edible small yellow berries fruit in the *Phyllanthaceae* family<sup>2,5</sup>. The species is named acidus because the taste of the fruit is acidic (highly sour). It is native of Madagascar and cultivated in gardens and in villages throughout India for fruit. The plant is used medicinally. The peppered leaves are used to make a poultice to treat sciatica, lumbago and rheumatism, while the seeds are used as a cathartic and the root as a purgative. The syrup is used to medicate the stomach, and in India the fruit is eaten as a blood-enhancer for the liver<sup>2</sup>. Sesquiterpenes, phyllanthusol A and B, aglycon saccharide has been isolated from the methanolic extract of the roots of *P. acidus*. Phyllanthusol A and B has been isolated from *P. acidus* has been proposed as possible antitumor agent<sup>2</sup>. From the root extracts of *P. acidus*, phyllanthusols A and B were isolated and were analysed by capillary electrophoresis<sup>16</sup>. Novel cytotoxic water-soluble norbisabolane glycosides, named phyllanthusol A and phyllanthusol B isolated from the methanolic extract of the roots of *P. acidus*<sup>3</sup>. Phyllanthusol A and B glycosides, isolated from the root of *P. acidus* have been reported to exhibit cytotoxic activity on KB nasopharyngeal cell lines<sup>14</sup>. Exhaustive literature survey showed that the plant is a good source of emetic and purgative<sup>2</sup>, hypertension<sup>4</sup>, hepatoprotective<sup>12</sup>, anti-diabetics<sup>6</sup>, antipyretic, analgesic, anti-inflammatory<sup>20</sup>, antiviral, antioxidant<sup>8,20,21</sup> and antibacterial activities<sup>21</sup>. This present study, ethyl acetate extract of *P. acidus* leaves have been analysed for cytotoxic and antitumor properties.

## II. MATERIALS AND METHODS

### Collection of plant sample:

*P. acidus* was collected from Ernakulam, Kerala and authenticated the plant by Rogimon. P. Thomas, Asst. Professor, Dept. of Botany, CMS College, Kottayam, Kerala. The fresh leaves were cleaned thoroughly with water and dried by shade for three weeks and powdered using grinder.

### Preparation of extract:

Approximately 150 g of the leaf powder was taken and was successively extracted using 350 ml volumes of solvents of increasing polarity (petroleum ether, ethyl acetate, ethanol and water) for 3 days period using percolator. Extracts were evaporated, residue was collected. Total residue was weighed and stored in an air tight container.

### Phytochemical screening:

The extracts of *P. acidus* was subjected to qualitative analysis for the various phyto-constituents like alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids and flavonoids. Phytochemical examinations were carried out as per the standard methods<sup>10,25</sup>.

### Cell lines:

1. Hep G<sub>2</sub> or hepatic carcinoma cells were purchased from NCCS, Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO<sub>2</sub> in a humidified atmosphere in a CO<sub>2</sub> incubator.
2. Dalton Lymphoma Ascites (DLA) cells, maintained in intraperitoneal inoculation of 1x 10<sup>6</sup> viable cells in mice at Amala Cancer Research Centre, Amala Nagar, and Thrissur, India.

### Animals:

Swiss Albino female mice weighing 25-30 g were purchased from Sri Venkateshwara Enterprises No.4304, 13<sup>th</sup> day main, First cross, Subramanyanagar, Bangalore -560021. The animals were maintained under standardised environmental conditions (22-28°C, 60-70% relative humidity, 12 hr dark/ light cycle) and fed with standard rat feed (Sai Durga feeds and foods, Bangalore) and water in the animal house of Animal Cancer Research Centre. All the animals experiments were carried out in Amala Cancer Research Centre by the prior permission of Institutional Animal Ethics Committee (IAEC) and were strictly according to the guidelines of committee for the purpose of control and supervision of experiments of animals (CPCSEA) constituted by Animal Division Govt. of India.

### In-vitro cytotoxicity assays of *P. acidus* extracts:

#### 1. Cultured human (Hep G2) cancer cell line using MTT assay

Hep G<sub>2</sub> or hepatic carcinoma cells were purchased from NCCS, Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°C in 5 % CO<sub>2</sub> in a humidified atmosphere in a CO<sub>2</sub> incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia)) for 2 minutes and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25 µg, 12.5µg, 25µg, 50 µg and 100µg/ml from a stock of 1mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation.

The cells was washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37°C for 3 hours. MTT was removed by washing with 1x PBS and 200µl of DMSO was added to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micropate reader (ELISASCAN, ERBA). The cytotoxicity was determined by comparing the percentage death of treated cell population with the untreated control, indicated by their respective absorbance assessed with the MTT assay<sup>26</sup>. The growth inhibition was determined using:

**% Growth inhibition= [control O.D- sample O.D/ control O.D] x 100**

## 2. DLA cell line using trypan blue exclusion method

Cytotoxicity activity of plant extract was done by determining the percentage of viability of DLA using trypan blue exclusion technique. DLA cells were grown in peritoneal cavity of the mice weighing 25-30 g by injecting a suspension of cells ( $1 \times 10^6$  cells/ml). For this, tumor cells were aspirated from the peritoneal cavity of the mice on day 15 and washed with PBS (0.2 M, pH 7.4) and centrifuged for 15 min at 1500 rpm. The pellet was resuspended with PBS and the process was repeated 3 times. Finally, the cells were suspended in a known quantity of PBS and the cell count was adjusted to  $1 \times 10^7$  cells/ml. The cell suspension (0.1 ml) was dispensed in 0.8 ml of PBS and incubated with different concentrations of extracts (50-500  $\mu$ g/ml) at 37°C for 3 hrs. After incubation, 0.1 ml trypan blue dye was added and applied a drop of trypan blue- cell mixture to a hemocytometer and count the stained (non-viable) and unstained (viable) cells separately under a microscope. The number of stained and unstained cell was counted separately<sup>27</sup>. The trypan blue dye exclusion test was performed to determine the % viability and the IC<sub>50</sub> value. The % of dead cells is determined by using:

$$\% \text{ of dead cells} = \frac{\text{no: of dead cells}}{\text{no: of total cells}} \times 100$$

### Acute Toxicity:

Acute oral toxicity study was performed as per OECD guidelines. Female Swiss albino mice (n=9) selected by random sampling technique were used for the study. The animals were kept fasting for overnight, providing only water, after which the crude extract (200mg and 400mg/kg b.wt.) were orally supplied by intragastric tube to 2 groups of mice and propylene glycol alone was given to vehicle control group animals. They were noted individually after dosing, with special attention given during the first four hours and kept for 7 days of observation. Body weight, food and water intake and general behavioral changes were observed and thereafter for a total of 14 days (OECD 423, 2001).

### In-vivo study

#### Anti-tumor activity of *P. acidus* on DLA induced solid tumor model in mice

##### Tumor transplantation:

DLA were maintained by serial transplantation from tumor bearing Swiss albino mice at the log phase (day 78 of tumor bearing) of the tumor cells. The cell number was adjusted to  $1 \times 10^6$  cells/ml and showing more than 90% viability which was used for transplantation. Each animal received 0.2ml of tumor cell suspension containing  $1 \times 10^6$  cells/ml intraperitoneally.

##### Treatment schedule:

Swiss albino mice (female, 5-6 weeks old) weighing 25-30 g were divided into five groups comprising of six animals in each group. Tumor was induced by injecting DLA ( $1 \times 10^6$  cells/animal) onto the right hind limb of all the animals. Group I kept as DLA control without drug treatment and group II were treated with vehicle control propylene glycol. Similarly, standard drug cyclophosphamide (40 mg/kg b. wt.) and *P. acidus* EA extracts at different consecutive doses (200 and 400 mg/kg b. wt) were in groups III, IV and V respectively. Drug was administered orally from the first day of tumor induction and continued to next 10 days. Tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using a digital Vernier caliper starting from seventh day of tumor induction, up to 30<sup>th</sup> day. Tumor volume was calculated using the formula,  $V = \frac{4}{3} \pi r_1^2 r_2$  where  $r_1$  was the minor diameter and  $r_2$  was the major diameter<sup>27</sup>. The percentage of inhibition of tumor volume in animals on 30<sup>th</sup> day was calculated by,

$$\% \text{ of Inhibition} = \left[ \frac{\text{Tumor volume of control} - \text{tumor volume of treated}}{\text{tumor volume of control}} \right] \times 100$$

##### Statistical analysis:

All *in-vitro* assays were performed in duplicate and values were expressed in mean  $\pm$  SD. Data of animal experiments are mean  $\pm$  SD of 6 animals per group. Differences between group's means estimated using a one way analysis of variance followed by Dunnett's test, using GraphPad InStat Software. The results were considered statically when  $P < 0.05$ .

## III. RESULTS

### Preliminary phytochemical screening of plant extracts:

Evaluation of phytochemicals is various biochemical tests, PE extract revealed the presence of alkaloids, phytosterols and flavonoids. EA extract showed the presence of phytosterols and flavonoids. E extract showed the presence of saponins, phytosterols and tannins. Aqueous extract revealed the presence of saponins, phytosterols, phenols and tannins.

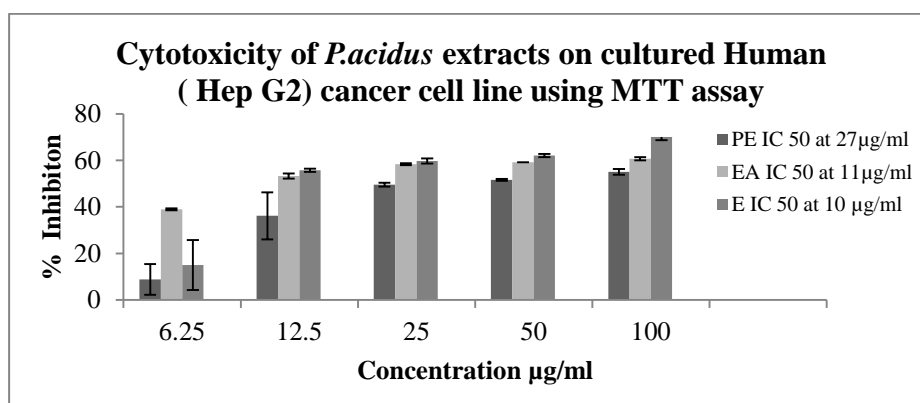
**In-vitro cytotoxicity assay of *P.acidus* extracts on cultured Human (Hep G2) cancer cell line using MTT assay**

The IC<sub>50</sub> value of EA extract showed significant anticancer activity by MTT assay. Various concentrations of *P.acidus* extracts produced significant cytotoxicity against Hep G2 cell line. IC<sub>50</sub> values were calculated for the plant extracts PE, EA and E indicated that the cytotoxicity of the extracts were as follows: E > EA > PE. (Tab.1; Graph.1).

**Table.1 Cytotoxicity of *P.acidus* extracts on cultured Human (Hep G2) cancer cell line using MTT assay**

Sl. no:	Concentration (µg/ml)	PE extract	EA extract	E extract
1.	6.25	49.36 ±1.24	79.93 ±0.52	54.79 ±0.39
2.	12.5	76.59 ±0.81	93.74 ±1.22	95.94±0.77
3.	25	89.82 ±0.81	98.88 ±0.33	100.03±0.89
4.	50	92.03±0.51	99.64 ±0.72	102.6 ±0.64
5.	100	95.33 ±1.03	101.14 ±0.73	110.53 ±1.27

Values are expressed as mean ± SD for triplicates.



**Graph.1 Cytotoxicity of *P.acidus* extracts on cultured Human (Hep G2) cancer cell line using MTT assay.**

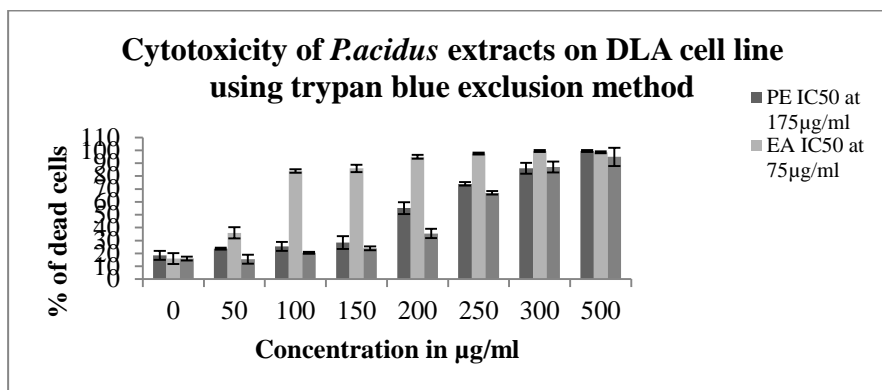
**In-vitro cytotoxicity assay of *P.acidus* extracts using DLA cell line using trypan blue exclusion method**

Addition of various concentrations (0-500µg/ml) of *P.acidus* extracts produced significant cytotoxicity against DLA cell line. IC<sub>50</sub> values were calculated for the plant extracts PE, EA and E indicated that the cytotoxicity of the extracts were as follows: EA > PE > E. The EA extract from the leaves of *P. acidus* showed good cytotoxicity against DLA cells with IC<sub>50</sub> value at 75µg/ml. The EA extract of *P. acidus* exhibited significant cytotoxicity, and it was therefore selected as candidate extract for more detailed antitumor study (Tab.2; Graph.2).

**Table.2 Cytotoxicity of *P.acidus* extracts on DLA cell line using trypan blue exclusion method**

Sl. No:	Concentration (µg/ml)	PE extract	EA extract	E extract
1.	0	18.5± 3.53	16 ±4.24	16±1.41
2.	50	23.75± 0.70	36±4.24	15.5± 3.53
3.	100	25.5 ±3.53	84±1.41	20.5± 0.70
4.	150	28.5±4.94	86±2.82	24±1.41
5.	200	55.25±4.59	95 ±1.41	35.5±3.53
6.	250	74 ±1.41	97.5 ±0.70	67±1.41
7.	300	86 ±4.24	99.5 ±0.70	87±4.24
8.	500	99.5 ±0.70	98.5± 0.70	95±7.07

Values are expressed as mean ± SD for duplicates.



**Graph.2** Cytotoxicity of *P. acidus* various leaf extract on DLA cell line using trypan blue exclusion method. Values are expressed as mean ± SD.

**Acute toxicity study**

Acute oral toxicity studies were carried out for *P. acidus* based on the OECD guidelines 423. The ethyl acetate extract was given by oral route. Toxicity study was done in animals for two weeks, no signs and symptoms of toxicity, mortality and intolerance were observed. Hence two doses i.e. 200 and 400 mg/kg b. wt. were selected for the present study.

**In-vivo antitumor studies**

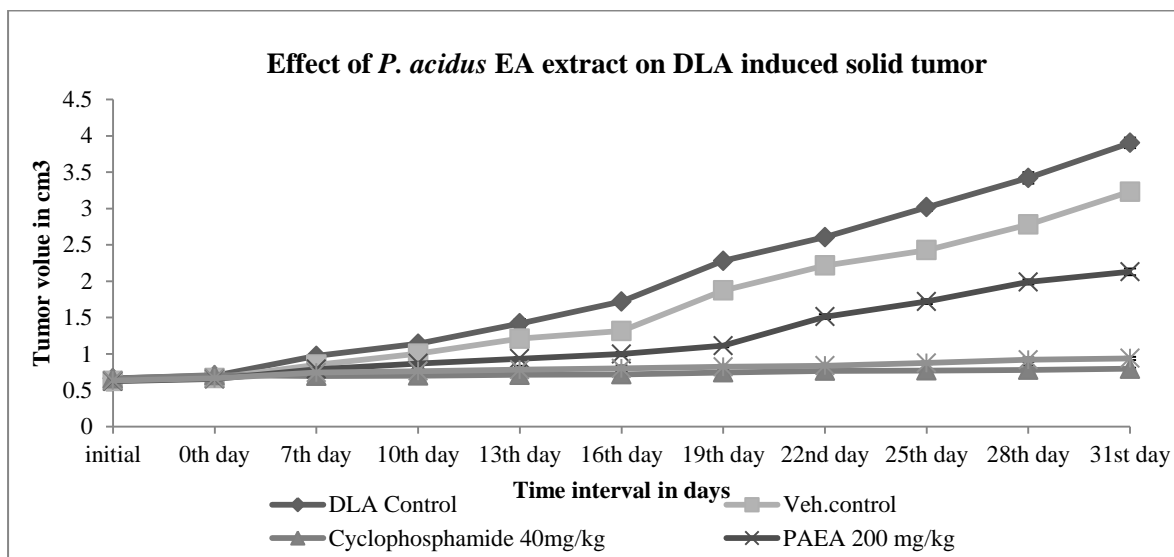
**Solid tumor activity of *P. acidus* EA leaf extract**

Administration of *P. acidus* (200 and 400 mg/kg b. wt.) inhibited DLA induced solid tumor in a dose when compared to a control group. Cyclophosphamide received at 40mg/kg b.wt orally, simultaneously offered significant protection. The tumor volume in untreated control groups, there was a steady increase in tumor volume from initial value  $0.665 \pm 0.027$  to  $3.75 \pm 0.074$  following tumor injections over a period of 30 days (Graph.3). In cyclophosphamide groups, there was a decrease in tumor volume from the initial  $0.662 \pm 0.01$  to  $0.797 \pm 0.047$  on 30<sup>th</sup> day (Graph. 3) and percentage reduction was 90.99%. In *P. acidus* EA 200mg/kg b.wt. there was a steady increase from initial volume of  $0.624 \pm 0.042$  to  $2.13 \pm 0.021$  with (45.27%) reduction in tumor volume in 200mg/kg b.wt. extract treated animals. Simultaneous oral administration of *P. acidus* at 400mg/kg b.wt. extract administration the tumor volume was reached to maximum of  $0.939 \pm 0.015$  with the percentage reduction of 76.66% which significantly prevented the increase in tumor burden when compared to standard drug cyclophosphamide 40mg/kg (Graph.3).

**Table.3** Antitumor effect of PAEA leaf extract in reducing solid tumor volume induced by DLA cell in Swiss albino mice.

Days	DLA Control	Veh. control	Cyclophosphamide 40mg/kg	<i>P. acidus</i> EA 200mg/kg	<i>P. acidus</i> EA 400mg/kg
Initial day	$0.66 \pm 0.027$	$0.62 \pm 0.054$	$0.66 \pm 0.038$	$0.62 \pm 0.059$	$0.63 \pm 0.049$
0 <sup>th</sup> day	$0.701 \pm 0.033$	$0.66 \pm 0.068$	$0.709 \pm 0.046$	$0.65 \pm 0.0648$	$0.66 \pm 0.056$
7 <sup>th</sup> day	$0.97 \pm 0.0059$	$0.85 \pm 0.014$	$0.69 \pm 0.024^{**}$	$0.79 \pm 0.04^*$	$0.74 \pm 0.031^{**}$
10 <sup>th</sup> day	$1.14 \pm 0.039$	$1.00 \pm 0.047$	$0.70 \pm 0.04^{**}$	$0.86 \pm 0.003^{**}$	$0.76 \pm 0.031^*$
13 <sup>th</sup> day	$1.42 \pm 0.058$	$1.21 \pm 0.042$	$0.71 \pm 0.023^{**}$	$0.93 \pm 0.051^*$	$0.78 \pm 0.024^{**}$
16 <sup>th</sup> day	$1.721 \pm 0.009$	$1.31 \pm 0.031$	$0.72 \pm 0.028^{**}$	$0.99 \pm 0.041^{**}$	$0.801 \pm 0.035^*$
19 <sup>th</sup> day	$2.2 \pm 0.04$	$1.87 \pm 0.052$	$0.74 \pm 0.023^{**}$	$1.11 \pm 0.027^*$	$0.82 \pm 0.035^{**}$
22 <sup>nd</sup> day	$2.605 \pm 0.044$	$2.21 \pm 0.041$	$0.76 \pm 0.031^{**}$	$1.51 \pm 0.02^{**}$	$0.83 \pm 0.031^*$
25 <sup>th</sup> day	$3.01 \pm 0.049$	$2.42 \pm 0.039$	$0.77 \pm 0.031^{**}$	$1.72 \pm 0.017^*$	$0.87 \pm 0.021^*$
28 <sup>th</sup> day	$3.42 \pm 0.08$	$2.77 \pm 0.017$	$0.78 \pm 0.035^{**}$	$1.99 \pm 0.03^*$	$0.92 \pm 0.061^{**}$
31 <sup>st</sup> day	$3.90 \pm 0.074$	$3.23 \pm 0.085$	$0.79 \pm 0.047^{**}$	$2.13 \pm 0.021^{**}$	$0.93 \pm 0.015^{**}$

Values are expressed as mean ± SD for 6 animals. \*P< 0.05, \*\* P< 0.01 significantly compared with control groups.



Graph.3 Antitumor effect of *P. acidus* EA leaf extract on DLA induced solid tumor volume. Values are expressed as mean ± SD for 6 animals

Tab.12: Inhibition of solid tumor induced tumor volume in mice by *P. acidus* EA extract treatment on 30<sup>th</sup> day.

Groups	Doses	Tumor volume in cm <sup>3</sup> ( 30 days after tumor inoculation )	% of inhibition of tumor volume after 30 days
DLA Control	---	3.90 ± 0.074	---
Veh. Control	Propylene glycol	3.23 ± 0.085	---
Cyclophosphamide	40mg/kg b.wt	0.797 ± 0.047**	80.34
<i>P. acidus</i> EA low dose	200mg/kg b.wt	2.134 ± 0.021**	45.75
<i>P. acidus</i> EA high dose	400mg/kgb.wt	0.939 ± 0.015**	76.66

Values are expressed as mean ± SD for 6 animals.\*P< 0.05, \*\* P< 0.01 significantly compared with control groups.

#### IV. DISCUSSION

The result of present investigation reveals that *P.acidus* has cytotoxicity against DLA and Human cancer cell line. Cytotoxicity is one of the chemotherapeutic targets of antitumor activity. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. *P.acidus* ethyl acetate extract exhibited significant cytotoxic effect to DLA in trypan blue exclusion method, and human cancer cell line in MTT assay.

Lymphoma is a disease of the lymphocytes (a type of white blood cell involved in immune responses) and the lymphatic system, which includes the spleen, thymus and liver, as well as other lymphatic tissues. Lymphoma is defined as malignant tumors of lymphoreticular origin i.e. form lymphocytes and histocytes and their precursor cells. Many studies have reported the useful effects of plant products against DLA. DLA is transplantable, poorly differentiated malignant tumor which appeared originally as lymphocytes in mouse. It grows in both solid and ascites. When DLA is induced in animals, the cancer cell count in the peritoneal fluid has been used as the marker to confirm the proliferation of cell. Transplantable tumors in animal models are effective methods to investigate antineoplastic effects of drugs. DLA is poorly differentiated transplantable malignant tumors, which grows as both ascites and solid tumor mice. The antitumor activity was evaluated in DLA induced solid tumor model. Results of the present study demonstrated that the oral administration of ethyl acetate leaves extract of *P.acidus* exerts significant antitumor activity in DLA induced solid tumor models in a dose dependent manner. Ethyl acetate leaves extract of *P.acidus* at 400mg/kg reduced the tumor burden effectively by decreased DLA induced solid tumor volume in mice model.



The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. The IC<sub>50</sub> value of EA extract showed significant anticancer activity by MTT assay against human cell line (Hep G2) at 11 µg/ml.

In trypan blue exclusion method live cells exclude the dye trypan blue and dead cells take up the dye and appear in blue colour. The ethyl acetate extract of *P. acidus* showed good cytotoxicity against DLA cells with IC<sub>50</sub> value at 75 µg/ml. The ethyl acetate extract of *P. acidus* exhibited significant cytotoxicity against DLA cell line.

Phytochemical screening of ethyl acetate extract shows the presence of phytosterols, flavonoids and saponins. Acute toxicity studies have revealed that the ethyl acetate extract of the plant were safe up to 2000 mg/kg. There was no mortality observed during the acute toxicity study period. There was no change in body weight and water consumption.

## V. CONCLUSION

The present investigation was mainly centered to evaluate the cytotoxic and anti-tumor activity of *Phyllanthus acidus* using cell lines. The present study demonstrated that ethyl acetate extract of *Phyllanthus acidus* presented anti-tumor activity, comparable with the standard drug. The *in-vitro* cytotoxic activity of ethyl acetate extract of *Phyllanthus acidus* done by using membrane integrity and cell proliferation assay methods on cancer cell lines, revealed a significant dose dependent increase in cytotoxic activity. This might be correlated with the presence of phytosterols, saponins, and flavonoids constituents in the extract. As this drug is unexplored further studies on its active constituents, its isolation, purification and characterization of the phytochemicals along with investigations is needed to provide some additional insight into the mechanism of action by which it exhibit anti-tumor activity with a view to obtain useful chemotherapeutic agent.

## REFERENCES

- [1] Mohammad Shoeb. Anticancer agents from medicinal plants. Bangladesh journal pharmacology-2006; 1: P (35-41).
- [2] Saraju Devi.S and Satya Paul. An overview on *Cicca acida* (*Phyllanthus acidus*). Assam University Journal of Science & Technology: Biological and Environmental Sciences-2011; 7(1): P (156-160).
- [3] Namphung Vongvanich, Prasat Kittakooop, Jarin Kramyu, Morakot Tainticharoen and Yodhathai Thebtaranonth. Phyllanthusols A and B, Cytotoxic Norbisabolane Glycosides from *Phyllanthus acidus* Skeels- 2003: P (1-4).
- [4] Yuttapong Leeya, Michael Mulvany. J, Emerson.F.Queiroz, Andrew Marston, Kurt Hostettmann, Chaweewan Jansakul. Hypotensive activity of an n-butanol extract and their purified compounds from leaves of *Phyllanthus acidus* (L.) Skeels in rats. European Journal of Pharmacology-2010; 649: P (301–313).
- [5] Orwa.C, Mutua. A, Kindt. R, Jamnadas.R, Anthony. S. *Phyllanthus acidus*. Agroforestry Database 4- 2009: P (1-5).
- [6] Yanadaiah. J.P, Mohana lakshmi. S, Jayaveera.K.N, Sudhakar.Y, Ravindra Reddy.K. Assessment of antidiabetic activity of ethanol extracts of *Phyllanthus acidus* Linn. and *Basella rubra* Linn. leaves against streptozotocin induced diabetes in rats. International Journal of Universal Pharmacy and Bio Sciences- 2012: P (2319-8141).
- [7] Diding.H.P, Andri Putranto.R.P, Sarsono. Effect of *Phyllanthus acidus* (L).Skeels leaves extract on Bronchial CD4<sup>+</sup> expression in mice model of allergic asthma. Journal of Indonesia-2010; 7(ISSN 1412-2855): P (2).
- [8] Shilali. K, Ramachandra.Y .L, Rajesh.K.P, Kumara Swamy.B.E. Assessing the antioxidant potential of *Phyllanthus acidus* bark extracts. International Journal of Pharmacy and Pharmaceutical Sciences- 2014; 6(6): P (522-531).
- [9] Santosh Kumar .C, Chiranjib Bhattacharjee, Subal Debnath, Atul.N.Chandu. *In-vitro* antimicrobial synergistic and anti-TB activities of *Phyllanthus acidus* methanolic extract. Indo American Journal of Pharmaceutical Research- 2011; 1(1): P (18-25).
- [10] Jagajoithi Angamuthu, Manimekalai Ganapathy, Vasthi Kennedy Evanjelene, Nirmala Ayyavuv and Vasanthi Padamanabhan. Antimicrobial Activity and Phytochemical Analysis of *Phyllanthus acidus*. International Journal of Innovation in Science and Mathematics-2014; 2(1): P (2347–9051).

- [11] Watchara Chongsa, Nisaudaih Radenahimad ,Chaweewan Jansakul. Six weeks oral gavages of *Phyllanthus acidus* leaf water extract decreased visceral fat, the serum lipid profile and liver lipid accumulation in middle-aged male rats. *Journal of Ethanopharmacology*-2014; 155(1): P (396-404).
- [12] Nilesh Kumar Jain, Abhay.K.Singha. Protective effects of *Phyllanthus acidus* (L.) Skeels leaf extracts on acetaminophen and thioacetamide induced hepatic injuries in Wistar rats. *Asian Pacific Journal of Tropical Medicine*-2011: P (470-474).
- [13] Talubmook, Chusri, Buddhakala, Nopparat. Hypoglycemic and hypolipidemic properties of leaf extract from *Phyllanthus acidus* (L.) Skeels. *Leucaena leucocephala* (Lam.) de Wit. and *Psidium guajava* (L.) in streptozotocin-induced diabetic rats. *Journal of BioSciences*-2013: P (30).
- [14] Chulabhorn Mahidol, Hunsu Prawat, Vilailak Prachyawarakorn, Somsak Ruchirawat. Investigation of some bioactive Thai medicinal plants-2012: P (287-297).
- [15] Jun-Jiang, Shan Yu, Feng Wang, Dong Wang, Hong-Tao Zhu, Ying-Jun Zhang. Anti-Hepatitis B Virus Norbisabolane Sesquiterpenoids from *Phyllanthus acidus* and the Establishment of Their Absolute Configurations Using Theoretical Calculations. *Journal of Organic Chemistry*-2014; 79. P (5432- 5447).
- [16] Durham.D.G, Reid. R.G, Wangboonskul.J, Dadoee. S. Extraction of phyllanthusols A and B from *Phyllanthus acidus* and analysis by capillary electrophoresis. *Phytochemical Analysis*-2002; 13(6). P (358 – 362).
- [17] Clara Elizabeth Quijano, David Linares, Jorge A. Pino. Changes in volatile compounds of fermented *Cereza agria* [*Phyllanthus acidus* (L.) Skeels] fruit. *Flavour and Fragrance Journal*-2007; 22(5): P (392–394).
- [18] Jorge A. Pino, Luis F. Cuevas-Glory, Rolando Marbot and Víctor Fuentes. Volatile compounds of grosella (*Phyllanthus acidus* [L.] Skeels) fruit. *Revista CENIC Ciencias Químicas*-2008; 39(1): P (3-4).
- [19] Marisa Sousa, Jiraporn Ousingsawat, Roswitha Seitz, Supaporn Puntheeranurak, Ana Regalado, Andre Schmidt. An Extract from the Medicinal Plant *Phyllanthus acidus* and Its Isolated Compounds Induce Airway Chloride Secretion: A Potential Treatment for Cystic Fibrosis. *Molecular Pharmacology*-2007; 71(1): P (366–376).
- [20] Raja Chakraborty, Biplab De, Nayakanti Devanna, Saikat Sen. Anti-inflammatory, antinociceptive and antioxidant activities of *Phyllanthus acidus* L. extracts. *Asian Pacific Journal of Tropical Biomedicine*-2012: P (S953-S961).
- [21] Md. Mominur Rahman, Md. Razibul Habib, Raquibul Hasan.S. M. , Mohammed Aktar Sayeed, Md. Sohel Ran. Antibacterial, cytotoxic and antioxidant potential of methanolic extract of *Phyllanthus acidus*. *International Journal of Drug Development & Research*-2011;3(2): P (1-8).
- [22] Jagessar.R.C, Marsa.A, Gomes.A. Selective Antimicrobial properties of *Phyllanthus acidus* leaf extract against *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* using Stokes Disc diffusion, Well diffusion, Streak plate and a dilution method. *Nature and Science*-2008; 6(2): P (1-15).
- [23] Md. Razibul Habib, Md. Mominur Rahman, Adnan Mannan Abu Hasanat Md. Zulfiker, Muhammad Erfan Uddin and Mohammed Aktar Sayeed. Evaluation of antioxidant, cytotoxic, antibacterial potential and phytochemical screening of chloroform extract of *Phyllanthus acidus*. *International Journal of Applied Biology and Pharmaceutical Technology*-2011; 2(1): P (ISSN: 0976-4550).
- [24] Sujatha Ramasamy, Norhanom Abdul Wahab , Nurhayati Zainal Abidin and Sugumaran Manickam .Cytotoxicity evaluation of five selected Malaysian *Phyllanthaceae* species on various human cancer cell lines. *Journal of Medicinal Plants Research*-2011; 5(11): P (2267-2273).
- [25] Prashant Tiwari, Bimlesh Kumar, Mandeep Kaur, Gurpreet Kaur, Harleen Kaur. Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Scientia*-2011; 1(1): P (98-106).
- [26] Purushoth Prabhu.T, Panneerselvam. P, Selvakumari.S, Sivaraman.D. *In-vitro* and *in-vivo* anticancer activity of ethanolic extract of *Canthium parviflorum* Lam on DLA and HeLa cell lines. *International Journal of Drug Development & Research*-2011; 3(4): P (280-285).
- [27] Jayaseelan.R.S, Fijesh.P.Vijayan, Mathesvaran.M, Suresh .V, Jose Padikkala. Cytotoxic and antitumor activity of methanolic extracts of *Desmodium triangulare* (Retz) Merr. root. *International Journal of Pharmacy and Pharmaceutical Sciences*-2012;4(3): P (540-542).