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Research Article

ANTITUMOR ACTIVITY OF POTENT FRACTION OF *LAWSONIA INERMIS* ROOT EXTRACT BY *IN VITRO* AND *IN VIVO* MODELS

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ABSTRACT

In the present scenario, products derived from plants serve as a potential source of anticancer drugs with low toxicity and cost. *Lawsonia inermis* (LI) has been selected to investigate its antitumor activity due to the increased life span reported by alcoholic root extract in DLA bearing mice. The root of *Lawsonia inermis* was extracted with 70% alcohol and different fractions viz. *LIEA, LIBUT, LIALC* and *LIAQ* were prepared. The *LIEA* was discovered to have powerful antioxidant properties followed by *LIBUT, LIALC* and *LIAQ* among these distinct fractions of root extract prepared. Preliminary screening of cytotoxicity using SRB assay on different cell lines (HCT-116 and HeLa) showed *LIEA* and *LIBUT* more potent at 48 h of incubation. Detailed cytotoxicity study on *LIEA* and *LIBUT* fractions using various concentrations at different time points demonstrated the more potent activity of *LIEA* than *LIBUT* on all the cell lines at 24, 48 and 72 hours. Among the 3 cell lines, HCT-116 was found more sensitive to *LIEA* with an IC50 of 179 ± 15.5 µg/ml. *In vivo* study on the EAC mice model showed increased life span in *LIEA* treated mice at both selected doses (200 mg/kg and 300 mg/kg) and decreased percentage change in the body when compared to EAC bearing mice. *LIEA* at both the dose reversed the EAC induced alternation in the hematological parameters near to normal. However, 300 mg/kg was found more effective than 200 mg/kg. Thus, this study is an initial step in identification of a novel and selective herbal antitumor agent.

Keywords: *Lawsonia inermis* (LI), antitumor, Sulforhodamine B Assay (SRB), HCT-116, HeLa, Ehrlich Ascites Carcinoma (EAC) cells.

INTRODUCTION

Cancer is the second crucial cause of mortality, following cardiovascular disease and it is the cause of more than 20% of all deaths. It is progressively being noticed that lots of nowadays illnesses arise because of the disproportion among the formation and neutralization of pro-oxidants resulting in oxidative stress (OS). Apoptosis, a type of programmed cell death is important to maintain the integrity of multi cellular organisms[1]. Cancer cells avoid apoptosis by evolving various mechanisms. Modern cancer therapeutics involves apoptotic induction in cancer cells[2]. Free radicals like hydroxyl, peroxyl and superoxide radicals are starting off by OS[3], which becomes steady through electron pairing with biological macromolecules which includes proteins, lipids and DNA in healthy human cells and causing protein and DNA damage in conjunction with lipid per oxidation. OS causes damages that have been involved as a potential contributor to the pathogenesis of cancer, diabetes, atherosclerosis, cardiovascular diseases, aging, and inflammatory diseases[4,5]. Oxidative processes are hindered by antioxidants by reacting with free radicals, through metal chelation and by scavenging singlet oxygen[6].

Plants are rich sources of antioxidants and because of this reason, there is an increasing interest in isolating these antioxidants and utilizing them as natural antioxidants[7]. Plants are also well known as a major source of modern medicines. The plant chosen for the present study is *Lawsonia inermis* commonly called as Henna in English, Mehendi in Hindi and Madhurangi in Kannada. The ethanolic extract of *L. inermis* root increased the life span of DLA tumor bearing mice has already been reported[8]and in-housed studies reported its antitumor activity in EAC tumor bearing mice. Further, Gallic acid has been reported for its selective cytotoxicity to cancer cells through cell cycle arrest, apoptosis and DNA damage. The Gallic acid is also present in the *Lawsonia inermis's* root along with the presence of lawsaritol, structurally similar to stigmasterol, sitosterol and campesterol which also been reported to inhibit cancer. And it has been also reported that various fraction of *Lawsonia inermis* root extract possesses anti-clastogenic and chemo preventive activities. Therefore, this study has been carried out in order to explore various fractions of *Lawsonia inermis* root extract for a potent fraction with the antitumor property. Very high phenol content has been found in *LIEA* as per F-C assay performed and in-housed study has found that *Lawsonia inermis* roots extract contains Gallic acid. Many studies have been reported Gallic acid has anti-proliferative property on different cancers like cervical cancer[9], stomach cancer, prostate cancer, lung cancer, and many other cancer. Cisplatin has been reported to have caused sister chromatid exchange and chromosomal aberrations in mammalian cultured cells, mouse bone marrow cells, and patients peripheral blood lymphocytes[10,11].

MATERIALS AND METHODS

Plant Material and Extraction

The plant material (*Lawsonia inermis* roots) was gathered from Nellore, Andhra Pradesh and authenticated by Dr. K. Mruthunjaya, Professor, Department of Pharmacognosy, JSS Pharmacy College, Mysuru with Herbarium voucher specimen number LIPJ2015. The roots were cleaned, cut into tiny bits, dried for 20 days and then powdered coarsely. The coarse powder was introduced to maceration for extraction using 70% ethanol for 9 days with occasional stirring. The solvent was replaced with fresh ethanol every 3 days and the extracted solvent was concentrated using Rotary Flash Evaporator and dried under vacuum. Thus, 10 g of the obtained alcoholic extract (*LIALC*) was dissolved in 100 ml of distilled water and was fractionated in a separating funnel by vigorously shaking with petroleum ether till the upper layer becomes slightly clear. The upper petroleum ether (*LIPET)* layer was collected and the lower layer was again fractionated using ethyl acetate in the same way and the upper layer of ethyl acetate (*LIEA)* was collected. Again the lower aqueous layer was vigorously shaken with butanol until the upper layer of butanol (*LIBUT)* got saturated. Both the upper *LIBUT* and lower aqueous (*LIAQ*) were collected separately. All the fractions were concentrated and dried under vacuum using a Rotary flash evaporator.

Cell Lines

Cell lines used for *in vitro* study were procured from National Center for Cell Sciences, Pune. Cancer cell lines used in the present study were HCT-116 (human colorectal cancer) and HeLa (human cervical cancer). All cells were cultured in DMEM (Dulbecco modified eagles media) which is complemented with 10% v/v fetal bovine serum (FBS), 1% w/v Pen Strip (Penicillin and Streptomycin solution), 125 µl of ciprofloxacin (5 µg/ml) per 500 ml of DMEM and1% w/v L-glutamine (200 mM). DMEM and Pen Strip were procured from *In vitro* gen and remaining reagents from Sigma-Aldrich. EAC cells used for *in vivo* study were originally procured from Amala Cancer Research Center, Amala Nagar, Thrissur, Kerala and were maintained and produced in an aseptic setting through serial intra peritoneal implantation. The cells were used in Swiss albino mice to cause ascites tumor.

Animals

Each experiment was performed on Swiss albino mice of either sex procured from the animal house of JSS Medical College, JSS University, Mysuru. The weight of all the mice was about 25 ± 5 g along with that the age was nearly 10-12 weeks old. According to CPCSEA guidelines issued by the IAEC (Institutional Animal Ethics Committee), JSS College of Pharmacy, Mysuru, Karnataka, the animal care and handling was done. Animals were acclimatized before one week of performing the experiments in the experimental room. Furthermore, the temperature was maintained with (23 ± 2ºC) and also humidity (50 ± 5 %). The animals were stored in sterilized polypropylene cages containing sanitized paddy husk as bedding. The studies undertaken have been endorsed by the IAEC, JSS College of Pharmacy, Mysuru; Authorization No. (180/2015).

Chemicals and Drugs

Chemicals: Ethanol, petroleum ether, ethyl acetate, butanol were purchased from Merck Millipore. All the chemicals were procured from HiMedia and Sigma-Aldrich used for *in vitro* antioxidant assays.

Drugs: 5-FU and cisplatin were procured from Sigma-Aldrich and Bio chem pharmaceutical industries Ltd.

Antioxidant Assays

Dpph Free Radical Scavenging Assay

The free radical scavenging activity of all the fractions of *Lawsonia inermis* was assessed by calibrating the decrease in absorbance of their methanolic solutions. A solution of 0.1 mM DPPH was prepared in methanol and 2.4 ml of this solution was mixed with 1.6 ml of all the fractions of *Lawsonia inermis* at different concentrations (20, 40, 60, 80 and 100 µg/ml) the reaction mixtures were incubated in dark for 30 minutes and their absorbance were recorded at 517 nm wavelength against methanol as blank[12]. Ascorbic acid was used as the standard in the concentration range of 2-10 µg/ml. By adding 2.4 ml of DPPH in 1.6 ml of methanol, a control reading was taken. All triplicate experiments were done, and values were reported as Mean ± SEM. The formula calculated the percentage scavenging activity:

$$\% \text{ scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Total Antioxidant Capacity

Antioxidant capacity was carried out by taking 100 µg of each *Lawsonia inermis* fraction in 0.1 ml of alcohol and blended individually with the addition of 1.9 ml of the reagent in Eppendorf pipes (4 mM ammonium molybdate and 28 mM sodium phosphate dissolved in 0.6 M of sulfuric acid). This was followed by incubation with capping at 95°C in thermal water baths for 90 min. The samples were then brought down to room temperature and subsequent analysis of absorbance was done at 695 nm against a blank (1.9 ml reagent, appropriate volume of same solvent, incubation). The standard graph of ascorbic acid was utilized to determine the ascorbic acid equivalents. Triplicate experimentation was done, and values were expressed in terms of ascorbic acid equivalents in mg per gram of the extract (Mean ± SEM).

Superoxide Anion Radical Scavenging Assay

According to a study carried out by Hongtao Bi, *et al*. 2013, the superoxide anion radical scavenging assay was performed as follows: The first step involved the generation in about 5 ml of Tris–HCl buffer (16 mM, pH 8.0) which possessed 1 ml of NBT (300 µM) solution, and equal amounts of NADH (936 µM) solution and all the fractions solutions followed by 2 ml of Tris HCl. The reaction was initiated by the addition of 1 ml of PMS solution (120 µM) incubation at 25ºC for 5 min and the absorbance was determined using spectrophotometer was measured against Tris-HCl buffer as blank. This mixture was incubated at 25ºC for 5 min. A lower absorbance signified the consumption of superoxide in the mixture at 560 nm. The standard and control readings were established using L-Ascorbic acid which contained most of the reagent compounds except the fraction. Mean± SEM was used to report the results and they were carried out in triplicate.[13] Percentage scavenging activity was calculated by the formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control} - \text{Absorbance of test}}{\text{Absorbance of Control}} x\ 100$$

Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was measured by means of studying the opposition between test extract and 2-deoxy-D-ribose for hydroxyl radical generated via Fenton's response[14]. The response combination containing 0.2 ml of 20 mm phosphate buffer (ph-7.4),0.2 ml fecl3 (10 mM), 0.1 ml ascorbic acid (0.1 mM), 0.1 ml EDTA (1 mM), 0.1 ml H_2O_2 (10 mM), 0.2 ml of 2-deoxy-D-ribose (10 mM) and various concentrations of *Lawsonia inermis* fractions (1 ml each) changed into incubated at room temperature for 60 min. To this 1 ml, each of 1% TBA and 2.8% TCA had been introduced. The very last mixture became stored in a boiling water bath for 30 min to get a crimson chromophore. The damage imposed because of unfastened radicals was determined colorimetrically by way of measuring the thiobarbituric acid reactive substances (TBARS) at 532 nm. A control sample becomes organized to comprise an equal volume without any extract. All experiments have been achieved in triplicate and values had been mentioned as Mean ± SEM. Hydroxyl radical scavenging pastime (%) was measured using a formula:

$$\% \text{ Scavenging} = \frac{\text{Absorbance of Control} - \text{Absorbance of test}}{\text{Absorbance of Control}} x\ 100$$

Reduction of Ferric ions

The reaction combination containing 0.5 mg of o-phenanthroline, ferric chloride (0.2 mM) and test samples (fractions/standard) become in a final quantity of 5 ml changed into incubated for 10 min at ambient temperature. The absorbance was measured at 510 nm in opposition to ethanol as blank. In another test, sodium dithionate (0.3 mm) became added in preference to the test compound and the absorbance acquired was taken as equal to a 100% reduction of all the ferric ions present.

Determination of Total Phenolic Content (TPC)

The entire phenolic content material of extracts determined approximately by the use of the Folin-ciocalteau phenol reagent approach[15]. The fractions, standard gallic acid and other reagents were prepared in DW. An aliquot (0.4 ml) of fractions or standard solution gallic acid (20, 40, 60, 80 and 100 µg/ml) was delivered to a 10 ml volumetric flask containing 3.6 ml of DW. 0.4 ml of Folin- ciocalteau phenol reagent was added to the mixture and shaken. After 5 min, 4 ml of 7% sodium carbonate solution was delivered to the mixture and the volume become made to 10 ml with DW and combined. After incubation for 30 minutes at room temperature, the absorbance against the reagent blank was determined at 750 nm with a UV-vis spectrophotometer (shimadzu). The entire phenolic content became expressed as gallic acid equivalents (GAE) in milligrams per gram of sample, the usage of a preferred curve generated with gallic acid. The experiment was conducted in triplicate and values are expressed in mean ± SEM.

Estimation of Total Flavonoids

Total flavonoid content material was determined via the aluminum chloride colorimetric assay[15]. An aliquot (0.5 ml) of fractions or standard solution of Quercetin become added to a test tube containing 2 ml of DW. To the test tube was added 0.15 ml of 5% sodium nitrite. After 5 min, 0.15 ml of 10% aluminum chloride solution become delivered. At 6th min, 2 ml of 1M sodium hydroxide was introduced and the entire quantity was made to 5 ml with DW. The solution was blended nicely, and the absorbance was measured in opposition to prepared reagent blank at 510 nm. The total flavonoid content of the extracts changed into expressed as mg of Quercetin equivalent to 1 g of the extract. All experiments had been finished in triplicate and entire flavonoid content was reported as mean ± SEM[16,17].

Preliminary I Cytotoxicity of Various Fractions of *Lawsonia inermis* by Sulforhodamine B Assay

Preliminary screening for the different fractions was assessed by SRB assay as per procedure with slight modification. The harvested cell suspension was checked for cell count using a hemocytometer and appropriate cell density was determined to get 5000 cells in 100 µL. 100 µL of cell suspensions (5000 cells) were seeded into 96-well plates and incubated for 48 hours. After cell suspension became 75% confluent, with the different concentrations (62.5- 500 µg/mL) of different fractions the cells were treated with different concentrations of different fractions and incubated for 48 hours. After complete incubation with drug samples, cells were removed from the CO_2 incubator and were kept for fixing with 10% TCA (50 µL per well) for 1 hour at 4ºC.

The media was eliminated after 1 hour and the streams were washed to unfasten TCA and serum-proteins with tap water. The plates were dried, 0.4% SRB incubated for 30 minutes with 100 µL to stain the cell proteins and washed quickly with 1% acetic acid to cast off unbound SRB. In 10 mM Tris base solution (100 µL / well), the bound SRB was solubilized and the absorbance measured in a Bio-Rad plate reader at 490 nm. The percent inhibition was calculated using the formula from the absorption obtained:

$$\%\ inhibition = \frac{Avg.O.D\ of\ Control - Avg.O.D\ of\ treated}{Avg.O.D\ of\ Control} \times 100$$

***In vitro* screening of Selected *Lawsonia inermis* Fractions by Five Dose Assay Using Sulforhodamine B Assay**

The promising fractions (*LIEA* and *LIBUT*) identified from the preliminary screening were then exposed to SRB assay using 5 different concentrations (62.5-1000 µg/ml) with a greater time points, i.e. 24, 48 and 72 hours on HCT-116, and HeLa cells to estimate the time dependent activity of fractions and to enhance the duration of action for addition assay.

The protocol was similar to the preliminary study described above. The % cytotoxicity of the fractions was determined for all the duration and IC_{50} was determined.

Mechanistic Study

The most potent fraction was subjected to DNA fragmentation and Fluorescent Dual staining

DNA Fragmentation Assay

This method can be used to recognize the impact of test compounds on apoptosis mechanisms[18]. To incubate 0.3 million cells 6 well-plates were used and after its 75% Confluency, treated with 3 concentrations of *LIEA* i.e. with the IC_{50} concentration, one below and one above IC_{50} and was incubated for 48 hours. After 48 hours, DNA isolation was carried out using an enzymatic method. The floating and adherent cells were collected in a 10 ml of the centrifuge tube, at 3000 rpm centrifuged for 10 minutes at 4ºC. The above acquired supernatant was discarded, and the pellets have been washed with 3 ml of PBS and centrifuged at 3000 rpm for 10 min at 4ºC. 20 µl of TES buffer was added to the pellets and mixed nicely using a

cut tip. 10 µl of proteinase K was delivered and incubated at 50°C for 90 minutes for digestion. All the samples were centrifuged at 3000 rpm for 3 minutes. The 21 µl of each supernatant was blended with 4 µl loading dye and fixed in the wells of 2% agarose gel and gel electrophoresis was run at 50V for 3 hours.

Fluorescent Imaging of Potent Fraction on the Most Effective Cell Lines Using Dual Staining

Double staining with Ethidium bromide (EB) and Acridine orange (AO) became accomplished to envision the viable cells (green nuclei), apoptotic cells (fragmented nuclei and colored in orange) and necrotic cells (red nuclei). From each treatment, treated cells were collected and pellets were suspended in PBS (25 µL) and were mixed with 10/10µL of AO/EB (100 µg/ml of AO in PBS; 100 µg/ml of EB in PBS) just before the microscopic evaluation for 15 min. On microscopic slides, a fine smeared stained cell was arranged and then images were taken using a fluorescent microscope.

***In Vivo* Activity of the Promising Fraction (*LIEA*) Against EAC Inoculated Ascites Tumor Model**

Among all of the *Lawsonia inermis* fractions, the potent one (*LIEA)* was taken to study its efficacy through the *in-vivo* model (EAC model). The ascitic fluid was aspirated from the peritoneal hollow space of tumor bearing mice, 10-12 days after the tumor cells inoculation. Trypan blue exclusion assay was employed to determine the viability of cells and the total number of cells per ml was calculated using hemocytometer. The ascitic fluid was properly diluted in PBS to acquire a concentration of $10x10^6$ cells/ml. From this stock suspension, 0.25 ml ($2.5x10^6$ cells) was administered intra peritoneal to each mouse to induce an ascetic tumor[19]. The tumor inoculated mice were randomized on the basis of body weight into five groups and the day was considered as day 0 when the tumor was inoculated.

The extract (*LIEA*) was administered p. o. on 1st, 3rd, 5th, 7th, 9th, 11th and 13th days of tumor inoculation. Cisplatin was used as a standard, 3.5 mg/kg i. p. at a single dose on day 1.

Treatment Groups and Parameters Evaluated

Group I: Normal - No Treatment
Group II: Control - CMC (0.25%) i.p
Group III: Standard - Cisplatin (3.5 mg/kg) i.p
Group IV: Test 1A - LIEA Dose 1 (200 mg/kg) p.o
Group V: Test 1B - LIEA Dose 2 (300 mg/kg) p.o

Parameters Evaluated

% Increase in Body Weight

Animals body weights were observed on every two days intervals and the % enhanced in the body weight of the animals on the respective days was calculated with respect to the weight of animals on 0 day using the formula[20]:

% increase in body weight = [(Bodyweight on respective day/body weight on day 0) -1] x100

Mean Survival Time (MST) and Increase in Life Span (%ILS)

The mortality of treated and untreated animals was noted and from the day when the tumor is inoculated, the total number of days an animal survived was counted. Subsequently % ILS was calculated using the formula:

% ILS = [(MST of treated group / MST of control group)-1] x100

Hematological Parameters

To determine the impact of treatment on hematological condition of EAC inoculated mice, blood was withdrawn (about 20 µl) from tail vein of animal into EDTA coated tube on 14th day and hemoglobin (Hb) content, total white blood cell (WBC), total red blood cell (RBC) count were determined using automatic hematological analyzer.

Statistical Analysis

The values of the specified number of animal experiments were expressed as Mean ± Standard Error of Mean (S.E.M.). Statistical analysis was conducted by one- way analysis of variance (ANOVA) followed by Multiple comparison test by turkey, values were significant if $p < 0.05$.

RESULTS

***In Vitro* Antioxidant Assays**

DPPH Free Radical Scavenging Assay

Among all the fractions, *LIEA* showed potent DPPH free radicals scavenging with IC_{50} of 27.72 ± 0.24 µg/ml whereas the standard ascorbic acid showed IC_{50} of 3.50 ± 0.04 µg/ml. The IC_{50} of other fractions are given in Table 1.

Total Antioxidant capacity

All the fractions were found to have complete antioxidant capacity however the ability turned into various in different fractions. It was found that 1 g of *LIEA* is equivalent to 703.74 ± 12.25 mg of ascorbic acid. *LIBUT* and *LIALC* were found to contain ascorbic acid equal to 562.70 ± 15.49 and 445.61 ± 10.43 mg per gram extract whereas the antioxidant capacity of aqueous was least. The total antioxidant capacity of various fractions is shown in Table 2.

Superoxide Scavenging Activity

The various fractions of *Lawsonia inermis* showed concentration dependent scavenging of superoxide ions. The % scavenging of all are shown in Table 3 and Figure 3. The most potent fraction in scavenging superoxide was *LIEA* with an IC_{50} of 71.16 ± 1.73 µg/ml and the least potent was *LIAQ* (116.84 ± 2.34 µg/ml) among all the fractions. The IC_{50} of ascorbic acid was found to be 17.88 ± 0.14 µg/ml.

Hydroxyl Radical Scavenging Activity

Among the fraction tested for hydroxyl radical scavenging activity, *LIEA* and *LIBUT* fractions were found more potent with IC50 values of 58.83 ± 1.29 µg/ml and 63.27 ± 2.92 µg/ml respectively. The IC50 of ascorbic acid was 21.11 ± 0.79 µg/ml. Data are shown in Table 4.

Reduction of Ferric Ions

The depletion of ferric ions by various fractions is shown in Table 5. *LIEA, LIBUT* and *LIALC* showed dose dependent reduction in ferric ion with the EC_{50} value of 26.23 ± 1.11, 31.49 ± 1.05 and 37.70 ± 2.05 µg/ml respectively. The EC_{50} of standard ascorbic acid, with 16.43 ± 0.30 µg/ml.

Total Phenolic Content

All the fractions were found to contain phenolics but with the different fractions amount of the content was varied. Phenolic content of *LIEA, LIBUT* and *LIALC* were 471.42 ± 4.06, 415.87 ± 4.19 and 375.39 ± 2.09 mg GAE/g (equivalent Gallic acid per gram) of each fraction, respectively and less amount present in *LIAQ* that is 135.71 ± 4.95 mg GAE/g *LIAQ*. The total phenolic contents of a different fraction are shown in Table 6.[21]

Total Flavonoid Content

The total flavonoid content of all the fractions is shown in Table 7. 1 gm of *LIEA* fraction was found to contain 165.75 ± 2.14 mg equivalent to quercetin. The *LIBUT* and *LIALC* were found to contain 142.46 ± 1.65 mg and 117.80 ± 0.96 mg equivalent quercetin per gram of both fractions respectively whereas *LIAQ* was found to contain 61.64 ± 3.02 mg.[16]

Preliminary *In-Vitro* Cytotoxicity of Various Fractions of *Lawsonia inermis* by Sulforhodamine B Assay

Sulforhodamine B assay at 48 hours on HCT-116 and HeLa cells determined preliminary screening of different fractions of *Lawsonia inermis* roots. Assessment of the percentage cytotoxicity on the cells for all the fractions was done. Among all the fractions, *LIEA* followed by *LIBUT* fraction showed a maximum % cytotoxicity compared to others. The dose dependent % cytotoxicity of all the fractions is shown in Figure 1 and 2.

***In Vitro* Screening of Selected Fractions by Five Dose Assay using Sulphorhodamine B Assay**

The selected fractions (*LIEA* and *LIBUT*), were further carried out for screening on cell lines at different time points. Both the fractions showed increased in % cytotoxicity with increasing dose but the increment in cytotoxicity was less in *LIBUT* when compared to that of the *LIEA.*

The IC_{50} value of the *LIEA* was found to be least at 48 hours when compared to 24 hours and 72 hours on HCT-116 and HeLa but the IC_{50} was 3 folds more on HeLa. IC_{50} of *LIBUT* was found very high and was independent of time points on both the cell lines. The IC_{50} of the *LIEA* at 48 hours was found least 179.0 ± 15.5 µg/ml whereas at 24 hours and 72 hours it was 230.7 ± 14.4 µg/ml and 184.30 ± 11.20 µg/ml on HCT-116 respectively. In case of HeLa, the IC_{50} of *LIEA* was found to be above 500 µg/ml at different time points on HCT-116. Upon treatment of both the cell lines with *LIBUT*, IC_{50} was found above 500 and 1000 on HCT-116 and HeLa respectively. But the potency of standard 5-FU was found to be higher than the *LIEA*. The IC_{50} of all the time point on all the cell lines are tabulated in Table 8.

Mechanistic Study

DNA Fragmentation Assay (Electrophoretic Determination of Apoptosis)

Gel electrophoresis assay of DNA from HCT-116 cells treated with ethyl acetate fraction of *LIEA* extract with different concentration (i.e. 80, 160 and 240 µg/ml) for 48 hours showed DNA fragmentation in the form of bands at 80 µg/ml (Figure 6, lane- 4) which indicates cell death by apoptosis whereas the higher concentration (160 and 240 µg/ml) didn't any show band but fine lines which are not prominent like bands was observed (Figure 6, lane 5 and 6).

Fluorescent Imaging of Potent Fraction (LIEA) on the Most Effective Cell Lines using Dual Staining

Dual staining of the cells collected after treatment of HCT-116 cells showed viable cells, necrotic and apoptotic cells. The viable cells are green coloured, necrotic are in red colour and the apoptotic cells had taken up the orange colour.

Evaluation of *In Vivo* Anti-tumor Activity of Potent Fraction of *Lawsonia inermis (LIEA*) by Ehrlich Ascites Carcinoma (EAC) Model

Effect of *LIEA* on Change in Body Weight of Ehrlich Ascites Carcinoma (EAC) Inoculated Mice

When compared to day 0, bodyweight of EAC inoculated mice were found to be increased with maximum gain of (44.94 ± 2.56 %) on day 15 and body weight was found to be reduced (13.34 ± 1.21%) by standard cisplatin at 3.5 mg/kg when compared to control. *LIEA* was treated at 200 mg/kg and 300 mg/kg significantly lowered the % increase in body weight (26.67 ± 1.64 and 22.06 ± 1.85) when compared to control.

Effect of LIEA on Mean Survival Time and % Increase in Life Span of EAC Inoculated Mice

Mean survival time was discovered to be decreased in EAC induced control mice (18.17 ± 0.40 days), MST (32.50 ± 0.61) was found to be increased by standard cisplatin at 3.5 mg/kg when compared to control, MST (27.34 ± 0.61) was found to be elevated after treatment with *LIEA* at 300 mg/kg when compared to control mice. It was found that MST (24.67 ± 0.49) was improved at 200 mg /kg of LIEA therapy relative to control. Whereas 300 mg/kg LIEA therapy showed a significant rise in lifespan (50.46%) when compared to cisplatin (78.89 %) Table 9.

Hematological Parameters

Effect on RBC Count

A significant decrease in total RBC counts was observed in EAC induced control mice (6.15 ± 0.36) when compared to normal mice (10.33 ± 0.30). Standard cisplatin at 3.5 mg/kg significantly reversed the decrease in the total RBC count (8.39 ± 0.4) compared to control; whereas the *LIEA* at 300 mg/kg and 200 mg/kg revealed a significant increase in RBC counts (8.99 ± 0.40) and (8.08 ± 0.21) when compared to control.

Effect on WBC Count

WBC count was found to be increased in EAC induced control mice (20.15 ± 0.73) when compared to normal mice (6.11 ± 0.24). Increased WBC count was significantly reduced (8.04 ± 0.16) by cisplatin at 3.5 mg/kg; whereas the *LIEA* at 300 mg/kg and 200 mg/kg showed a notable decrease in WBC counts (11.26 ± 0.25 and 13.10 ± 0.26) when compared to control.

Effect on Hemoglobin Count

A notable reduction in total hemoglobin counts was noticed in EAC induced control mice (10.23 ± 0.47) when compared to normal mice (15.70 ± 0.33). Standard cisplatin at 3.5 mg/kg significantly reversed the decrease in the total hemoglobin count (13.64 ± 0.16); whereas the *LIEA* at 300 mg/kg and 200 mg/kg exhibit a significant increase in hemoglobin counts (14.26 ± 0.44 and 13.32 ± 0.23) when compared to control.

Table 1: % scavenging of DPPH by various *Lawsonia inermis* fractions

Concentration (µg/ml)	% scavenging of DPPH by various *Lawsonia inermis* fractions				Ascorbic acid	
	LIEA	***LIBUT***	***LIALC***	***LIAQ***	**Conc. (µg/ml)**	**% scavenging of DPPH**
20	33.66 ± 0.29	30.75 ± 0.21	29.11 ± 0.50	11.12 ± 0.47	**2**	33.65 ± 0.50
40	66.18 ± 0.35	58.57 ± .431	53.24 ± 0.46	18.20 ± 0.21	**4**	51.97 ± 0.38
60	87.62 ± 0.52	77.95 ± 0.25	66.07 ± 0.23	39.14 ± 0.98	**6**	63.06 ± 0.50
80	91.792 ± 1.7	87.54 ± 1.14	84.37 ± 0.24	53.44 ± 0.22	**8**	79.85 ± 0.24
100	92.79 ± 0.13	89.54 ± .69	88.42 ± 0.32	73.36 ± 0.35	**10**	94.37 ± 0.41
IC_{50}	27.72 ± 0.24	31.44 ± 0.45	35.75 ± 0.36	71.47 ± 0.40	**IC_{50}**	3.50 ± 0.040

All values are expressed as Mean ± SEM, n = 3

Table 2: Total antioxidant capacity of different *Lawsonia inermis* fractions

Fraction	Total antioxidant capacity (mg ascorbic acid equivalent/g)
LIEA	703.74 ± 12.25
LIBUT	562.70 ± 15.49
LIALC	445.61 ± 10.43
LIAQ	102.32 ± 8.49

All values are expressed as Mean ± SEM, n = 3

Table 3: % scavenging of superoxide by various *Lawsonia inermis* fractions and their IC_{50}

Concentration (µg/ml)	% scavenging of superoxide by various *Lawsonia inermis* fractions				Ascorbic acid	
	LIEA	***LIBUT***	***LIALC***	***LIAQ***	**Conc. (µg/ml)**	**% scavenging**
20	20.45 ± 0.15	15.25 ± 0.54	12.54 ± 0.27	5.45 ± 0.35	**10**	34.11 ± 0.24
40	27.89 ± 0.21	23.98 ± 0.12	25.43 ± 0.09	7.84 ± 0.12	**20**	49.83 ± 0.39
60	42.67 ± 0.11	39.55 ± 0.24	33.63 ± 0.16	18.91 ± 0.13	**30**	63.79 ± 0.15
80	52.60 ± 0.13	49.59 ± 0.16	44.56 ± 0.16	32.29 ± 0.24	**40**	76.61 ± 0.21
100	63.38 ± 0.21	60.74 ± 0.48	58.94 ± 0.06	40.83 ± 0.51	**50**	89.79 ± 0.11
IC_{50}	71.16 ± 1.73	77.31 ± 1.86	84.20 ± 1.77	116.84 ± 2.3*	**IC_{50}**	17.88 ± 0.14

All values are expressed as Mean ± SEM, n = 3

*IC_{50} of *LIAQ* is not in the range of tested concentration. It is a projected value.

Table 4: % scavenging of hydroxyl radical by various *Lawsonia inermis* fractions and their IC_{50}

Concentration (µg/ml)	% scavenging of hydroxyl radicals by various *Lawsonia inermis* fractions				Ascorbic acid	
	LIEA	***LIBUT***	***LIALC***	***LIAQ***	**Conc. (µg/ml)**	**% scavenging**
20	16.76 ± 0.17	14.38 ± 0.71	10.62 ± 0.30	8.96 ± 0.14	**10**	31.14 ± 0.32
40	28.59 ± 0.67	25.88 ± 1.21	22.06 ± 0.36	15.91 ± 0.89	**20**	44.41 ± 0.24
60	44.65 ± 1.19	39.36 ± 0.78	35.65 ± 0.23	28.86 ± 0.38	**30**	57.02 ± 0.49
80	63.55 ± 0.29	58.93 ± 1.09	53.43 ± 0.79	39.74 ± 0.58	**40**	72.68 ± 0.15
100	81.12 ± 1.79	76.82 ± 0.81	74.07 ± 0.41	55.02 ± 0.28	**50**	79.50 ± 3.21
IC_{50}	59.95 ± 0.95	65.81 ± 1.37	71.64 ± 0.39	94.96 ± 0.26	**IC_{50}**	21.11 ± 0.18

All values are expressed as mean ± SEM, n = 3

Table 5: % reduction of ferric ions by various *Lawsonia inermis* fractions and their EC_{50}

Conc. (µg/ml)	% reduction of ferric ions by various *Lawsonia inermis* fractions				Ascorbic acid	
	LIEA	***LIBUT***	***LIALC***	***LIAQ***	**Conc. (µg/ml)**	**% reduction**
20	38.32 ± 1.65	35.54 ± 0.54	29.54 ± 0.27	15.45 ± 0.35	**10**	30.32 ± 1.08
40	59.89 ± 1.21	55.98 ± 0.82	52.43 ± 0.39	27.84 ± 0.57	**20**	55.09 ± 0.91
60	79.67 ± 0.45	67.55 ± 0.24	63.63 ± 0.96	38.91 ± 0.13	**30**	71.76 ± 0.78
80	83.43 ± 0.13	74.59 ± 0.16	70.56 ± 0.16	48.29 ± 0.24	**40**	82.61 ± 0.89
100	88.38 ± 1.01	81.02 ± 0.48	76.94 ± 0.86	54.83 ± 0.51	**50**	92.80 ± 1.11
EC_{50}	26.23 ± 1.11	31.49 ± 1.05	37.70 ± 2.05	83.43 ± 0.11	**EC_{50}**	16.43 ± 0.30

All values are expressed as mean ± SEM, n = 3

Table 6: Total phenolic content of different fractions of *Lawsonia inermis*

Fraction	Total Phenolic Content (mg GAE/g)
LIEA	471.42 ± 4.06
LIBUT	415.87 ± 4.19
LIALC	375.39 ± 2.09
LIAQ	85.71 ± 4.95

All values are expressed as Mean ± SEM, n = 3

Table 7: Total Flavonoid content in different fractions of *Lawsonia inermis*

Fraction	Total Flavonoid Content (mg QE/g)
LIEA	165.75 ± 2.14
LIBUT	142.46 ± 1.65
LIALC	117.80 ± 0.96
LIAQ	61.64 ± 3.02

All values are expressed as Mean ± SEM, n = 3

Table 8: IC_{50} values of *LIEA* and *LIBUT* on various cell lines using SRB assay at different time points

Lawsonia inermis Fractions	IC_{50} (µg/ml)			IC_{50} (µg/ml)		
	HCT-116			HeLa		
	24 h	48 h	72 h	24 h	48 h	72 h
LIEA	230.7 ± 14.4	179.0 ± 15.5	184.30 ± 11.20	819.70 ± 56.70	649.40 ± 91.80	717.85 ± 78.75
LIBUT	589.90 ± 69.19	811.35 ± 53.35	660.600 ± 70.5	1476.0 ± 70.59	955.3 ± 47.29	1102.05 ± 158.95
5-FU	81.9 ± 2.31	52.26 ± 5.23	28.50 ± 2.35	99.57 ± 5.23	88.48 ± 3.67	34.23 ± 0.98

Table 9: Effect of *LIEA* on Mean survival time and % increase in life span

S. No.	Groups	Mean survival time (days)	% increase in life span
1	EAC Control	18.17 ± 0.401	--
2	Standard (cisplatin 3.5 mg/kg)	32.50 ± 0.619[a]	78.895
3	*LIEA* (200 mg/kg)	24.67 ± 0.494[a,b]	35.795
4	*LIEA* (300 mg/kg)	27.34 ± 0.615[a,b]	50.463

All the values are represented as Mean ± SEM of six mice. [a]$p < 0.05$ compared to Normal and [b]$p < 0.05$ compared to Control. The data was analyzed by one way ANOVA by followed post hoc Tukey's multiple comparison test.

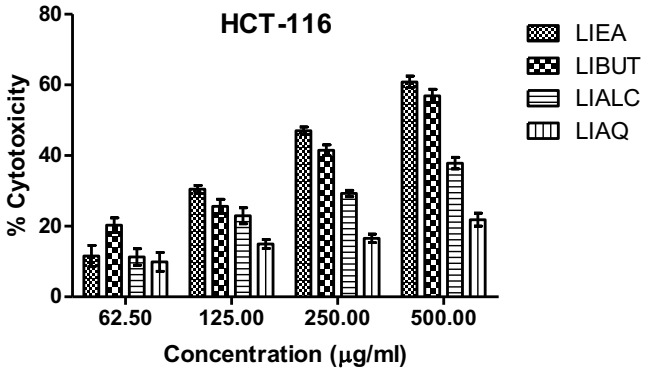


Figure 1: Preliminary screening dose dependent % cytotoxicity of various *Lawsonia inermis* fractions at 48 hours on HCT-116 (Colorectal Carcinoma) cell line by SRB assay

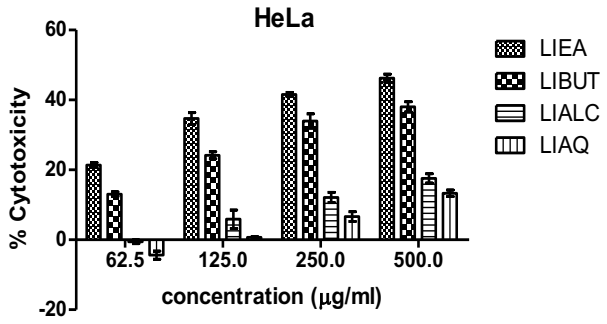


Figure 2: Preliminary screening- dose dependent % cytotoxicity of various *Lawsonia inermis* fractions at 48 hours on HeLa (Cervical Carcinoma) cell line using SRB assay

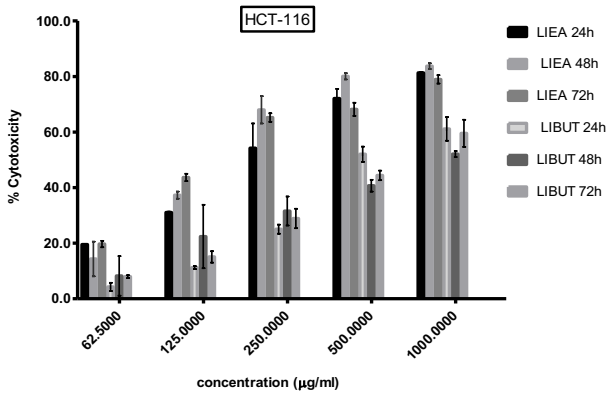


Figure 3: Time- and dose-dependent % cytotoxicity on HCT-116 (Colorectal Carcinoma) cell line during treatment with *LIEA* and *LIBUT* using SRB assay

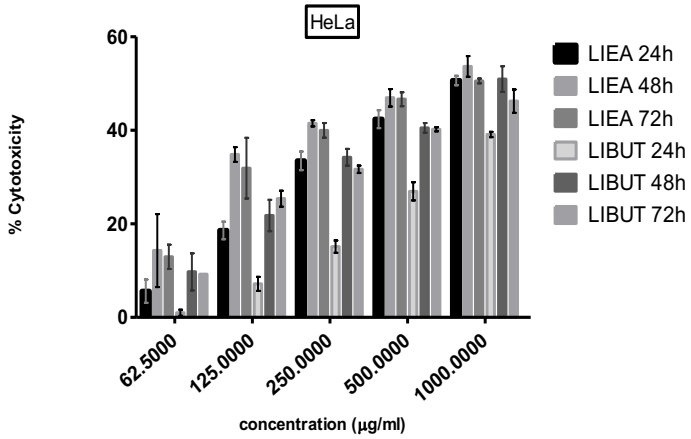
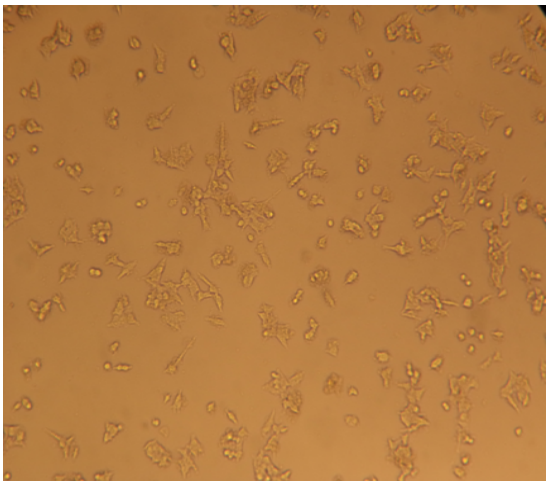
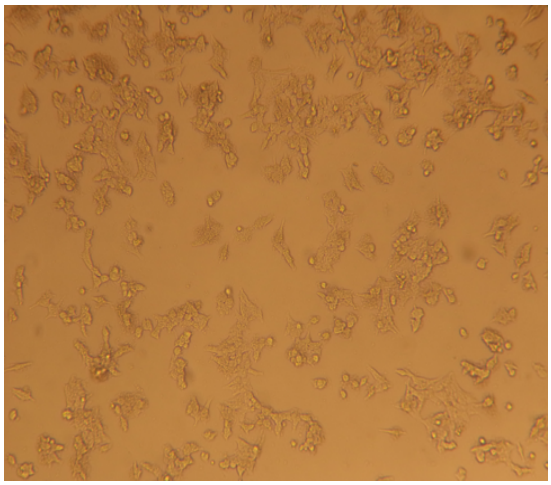


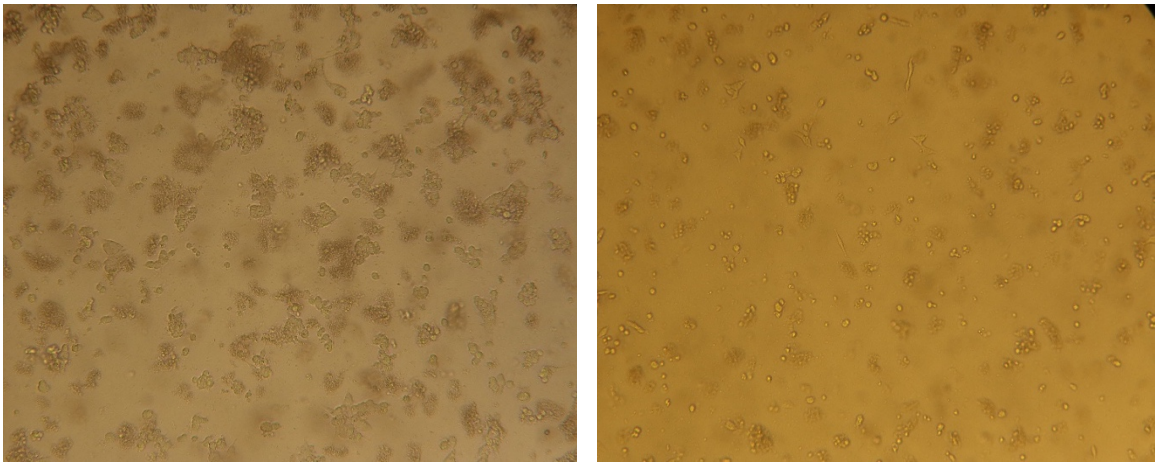
Figure 4: Time- and dose-dependent % cytotoxicity on HeLa (Cervical Carcinoma) cell line during treatment with *LIEA* and *LIBUT* using SRB assay



Control



Vehicle control (DMSO)



***5-FU* (65 µg/ml)** ***LIEA* (250 µg/ml)**

Figure 5: Images of Cytotoxic effect of *LIEA* and 5-FU at 48 h on HCT-116 cell line

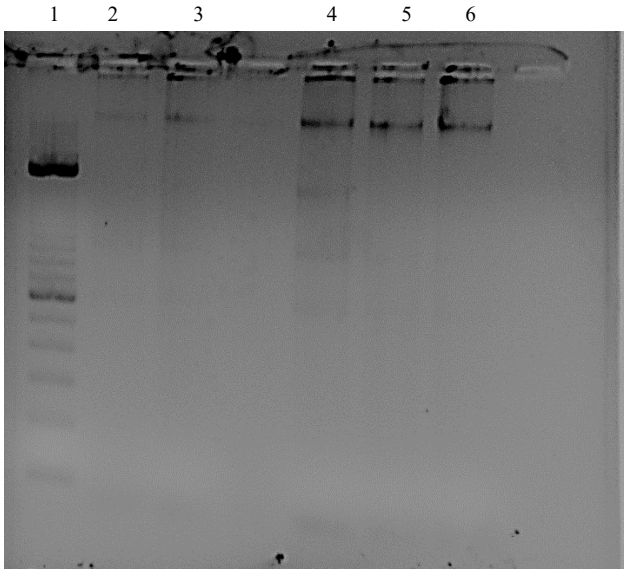
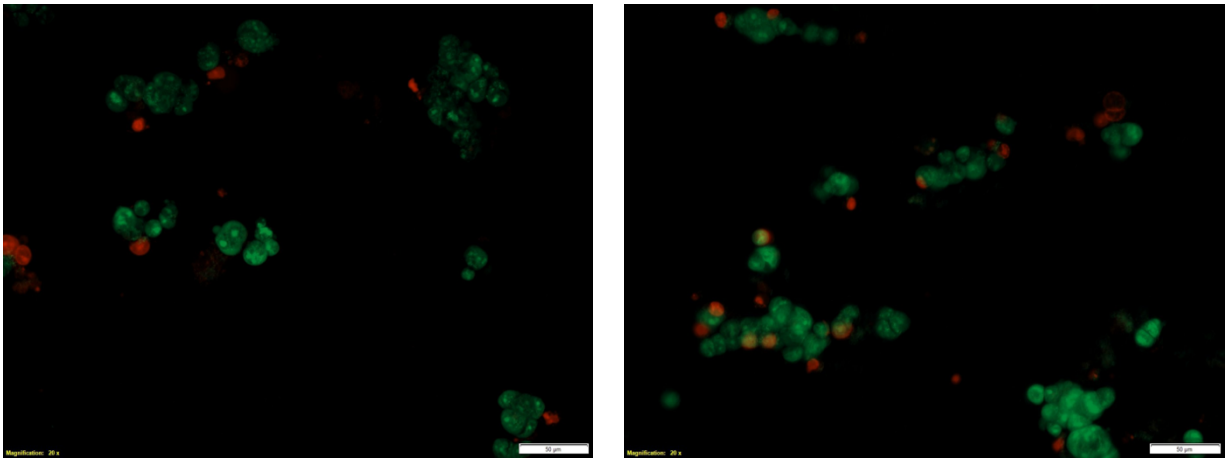
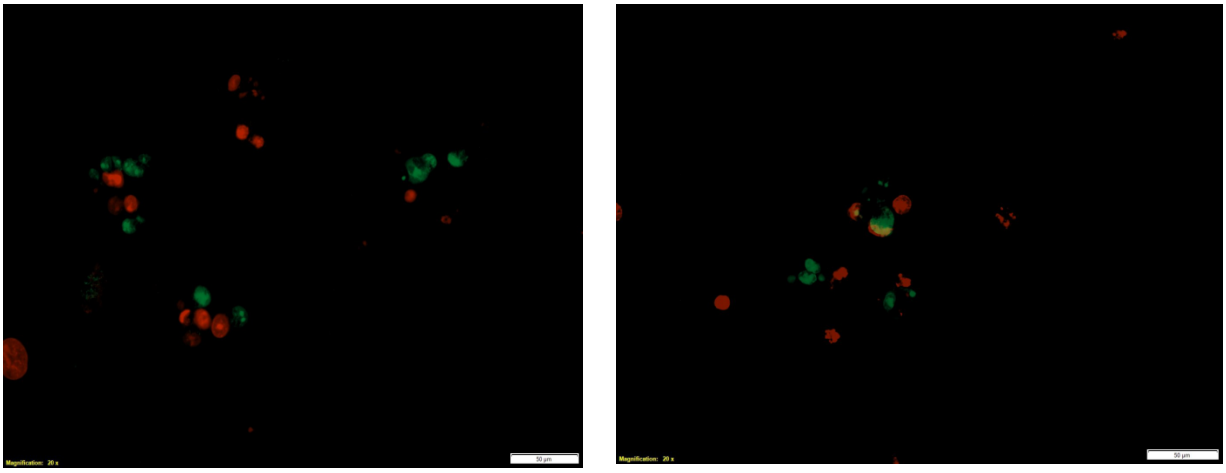


Figure 6: Agarose gel electrophoresis showing nucleosomal DNA fragmentation of HCT-116 cells induced by *LIEA*

[Lane 1- 100 bp DNA marker, Lane 2- control, Lane 3- vehicle control, Lane 4, 5 and 6 represents- cells treated with *LIEA* at 80, 160 and 240 µg/ml.]

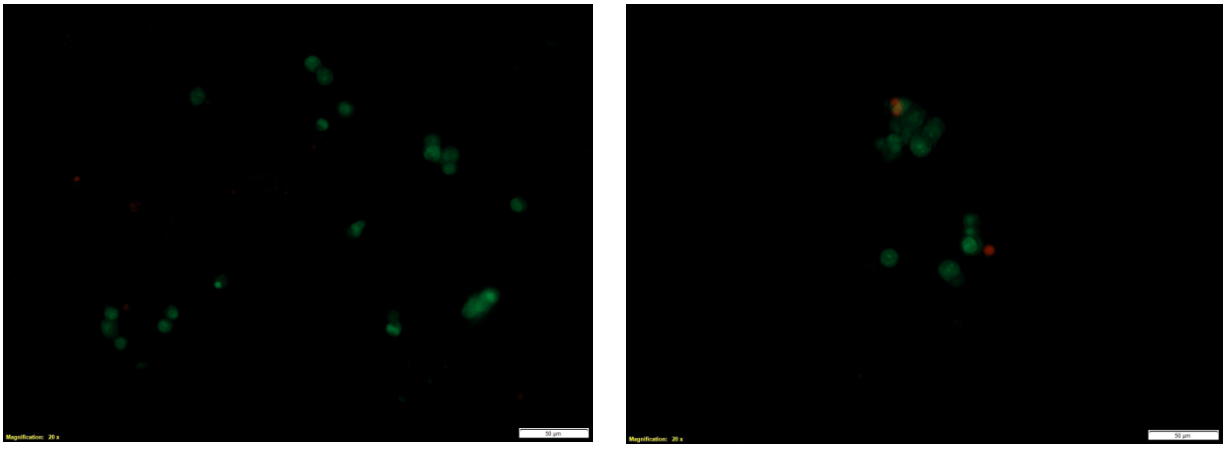


5-FU 65 µg/ml ***LIEA* 80 µg/ml**



***LIEA* 160 µg/ml** **LIEA 240 µg/ml**

Figure 7: **Fluorescent images of treated cells after double staining with AO/EB**



Control **Vehicle control (1% DMSO)**

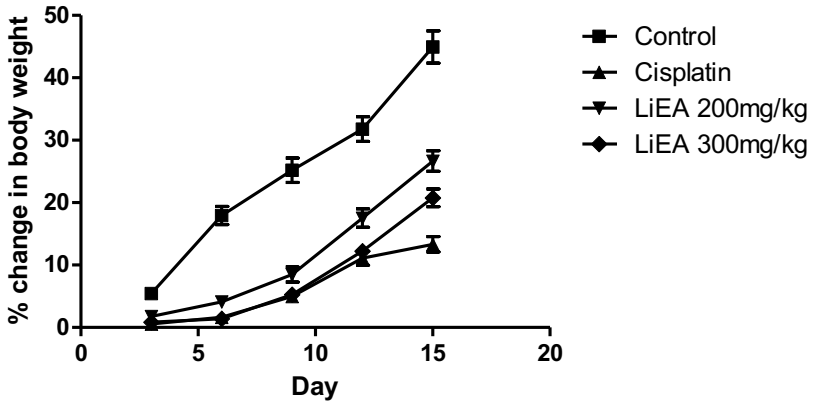


Figure 8: Effect of *LIEA* on body weight change in EAC inoculated Mice

All the values are represented as Mean ± SEM of six mice. The data was analyzed by one way ANOVA followed by post hoc Tukey's multiple comparison test

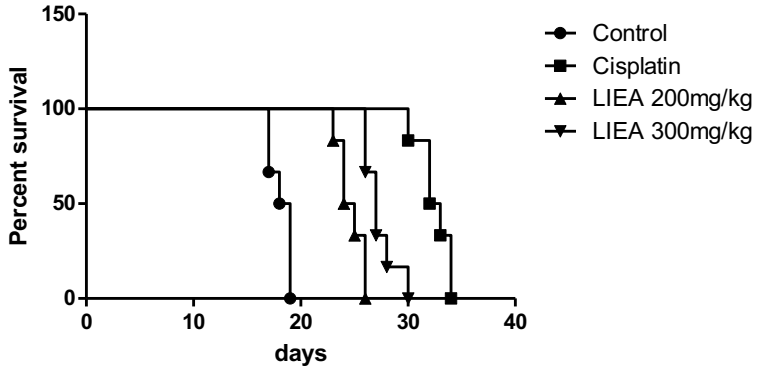


Figure 9: Kaplan Meier's Graph showing the effect of *LIEA* on % MST of tumor bearing mice

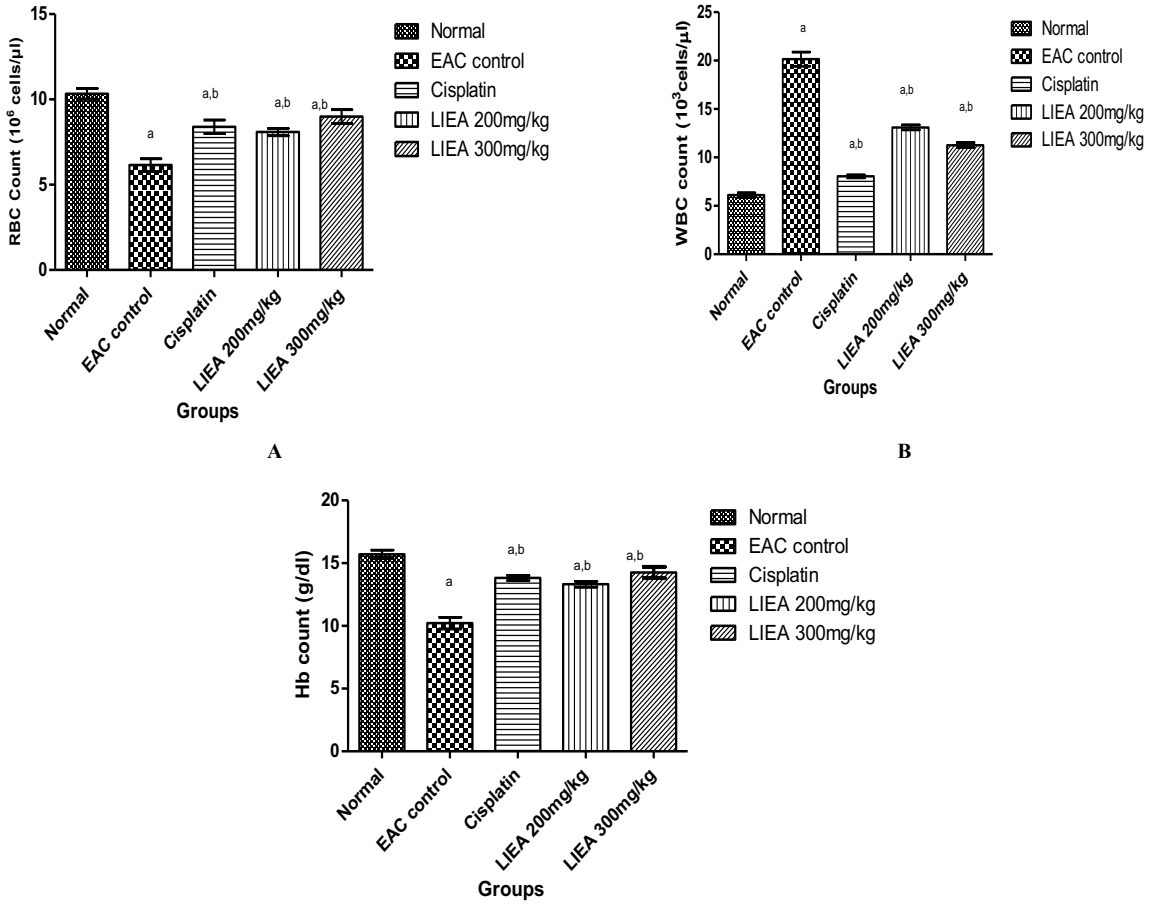


Figure 10: (A) Effect of *LIEA* on RBC Count; (B) Effect of *LIEA* on WBC Count and (C) Effect of *LIEA* on Hemoglobin count in EAC inoculated mice on day 14

All the values are represented as Mean ± SEM of six mice. $^{a}p < 0.05$ compared to Normal and $^{b}p < 0.05$ compared to Control. The data was analyzed by one way ANOVA post hoc Tukey's multiple comparison test.

DISCUSSION

Many cancer cells have a pro-oxidant status and promote oxidative stress. The oxidative process is inhibited by reacting with free radicals, through metal chelation and by looking singlet oxygen. We have found in the preliminary *in vitro* antioxidant assay that *LIEA* has very potent antioxidant property among other *Lawsonia inermis* fractions, has significantly scavenged DPPH, hydroxyl radicals, hydrogen peroxide and superoxide and was able to reduce ferric ions. The herbs with natural antioxidants including phenolics reduce cellular damages of oxidative stress and commonly used for cancer treatment and prevention. As well as, they have been identified as anti-proliferative agents due to their capability to cell cycle arrest, apoptotic induction, destruction mitotic spindle formation and inhibit angiogenesis. Plants with excessive phenol content are reported to possess efficacious antioxidant and antitumor properties.

Cell based assay provides mechanistic data pertaining to various investigations on cancer studies. In the present study, SRB assay

was used which provides preliminary information about the level of protein content and damage to cellular DNA. In the cytotoxicity screening, *LIEA* showed potent activity on HCT-116 when compared to that on HeLa cell line. Cell death plays an important role in maintaining embryonic development, tissue homeostasis, immune function, tumor suppression and resistance to infection. The anticancer agents exert their cytotoxic effects by negatively regulating the cell cycle checkpoints and triggering apoptosis in cancer cells thereby, disturbing their proliferation. *LIEA* produced DNA damage with subsequent cleavage of chromatin DNA into inter nucleosomal fragments and presence of apoptotic cell (orange color), necrotic cells (red fragmented nuclei) assessed by fluorescent images against control cells.

In the ascites tumor model, substantial growth in body weight of the animals was noted in EAC bearing control mice owing to rapid and increasing accumulation of ascites tumor cells. LIEA is treated at both doses (200 mg/kg and 300 mg/kg) on alternate days up to 14 days showed a reduction in the body weight when compared to the control indicating that tumor cell growth progression has been inhibited.

Mean survival time (MST) has been increased at both the doses of LIEA in EAC bearing mice. Increased MST indicates an enhancement of % life span which was evident when compared to control. To determine the anti-tumor property of any compound the improvement of life span is a well-founded criterion. Usually, 100% of mortality is caused by EAC inoculation within 18 days, and the current data support this fact. If the life span is elevated by more than 25% compared to control is considered as an efficacious antitumor response. The % increase in life span by the *LIEA* at 300 mg/kg and 200 mg/kg was 50.46 and 35.79 respectively which is comparable to cisplatin (78.89%).

Tumors caused a marked increase in WBC count in control animals, which is a well-known phenomenon seen in tumor-bearing animals and humans while declining in RBC count and content of hemoglobin on the 14th day. Anemia in tumor-bearing mice is primarily due to decreased RBC or Hb output, which can happen either owing to iron deficiency or hemolytic or other myelopathic diseases. Treatment with *LIEA* at dose 300 mg/kg was more effective in reversing the elevated WBC count on day 14 along with significant elevation in level of RBC count and Hb counts near to normal when compared to 200 mg/kg dose. Whereas, cisplatin treatment at single dose of 3.5 mg/kg reversed the WBC count significantly. Cisplatin treatment did affect RBC count and Hb count, but the values were found less than the normal value indicating myelo suppression. The findings showed that LIEA has a powerful antitumor property, particularly at a dose of 300 mg/kg and without adverse effects such as myelo toxicity as seen in cisplatin.

CONCLUSION

This study concluded that the potent fraction of *Lawsonia inermis* root extract LIEA showed significant activity on *in vitro* HCT-116 (Colon cancer), and *HeLa* (Cervical cancer) cancer cell lines and it was determined by means of SRB Assay. This was followed by *in-vivo* studies on mice using EAC induced liquid tumor models which gave equivalent results. Hence further aspects should be looked upon to identify the gaps and progress further into research.

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