Anti-hemorrhoid, anti-inflammatory and anti-cancer activity of standardized leaf extract of *Eupatorium triplinerve Vahl*

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Abstract:

Objective: The primary objective of the present investigation is to evaluate the anti-hemorrhoidic, anti-inflammatory and anti-cancer potential of acetone leaf of *Eupatorium triplinerve.*

Materials and Methods: The acetone leaf extract were initially determined for *in vitro* cytotoxic potential against human colorectal adenocarcinoma cell line (HT-29) and to identify anti-inflammatory properties in macrophages (RAW 264.7 cells) through LPS stimulation with pro-inflammatory cytokines (IL-6 and TNF-α) expression study by flow cytometry. *In vivo* anti-hemorrhoidic activity and toxicity were evaluated by acute toxicity study and histopathological examination under close observation for 14 days on general behavior, body weight, food and water intake.

Results and discussion: The results demonstrated that the effect of herbal extract significantly inhibited the HT-29 cells with IC_{50} values of 44.89 µg/ml. In addition, the acetone leaf extract showed better anti-inflammatory effect in IL-6 cytokine of IC_{50} values 18.53 µg/ml with LPS stimulated macrophages. Histopathological study showed a reduction in the inflammation blood vessels of treated animals which may confirm the tissue recovery stages.

Conclusion: The results revealed that the leaf extract possessess anti-cancer, anti-inflammatory and anti-hemorrhoidic properties at different concentration. It could differ due to the composition and concentration of the bioactive compounds.

Keywords: anticancer, anti-inflammatory, antihemorrhoid, macrophages, cytokine

Introduction:

Hemorrhoids or hemorrhoidal disease is often considered as one of the most prevalent inflammatory disease which can be illustrated by alteration in vasculature of the anal canal region wherein comprises blood vessels, supporting tissues and muscles, with elastic fibers. It causes the development of three columns, namely the left lateral, right anterior, and right posterior, lined by

a cluster of vascular connective tissue, smooth muscle, and elastic fibers. Hemorrhoid is the fourth leading proctological disease and is the main cause of hematochezia, colorectal cancer, Inflammatory Bowel Disease (IBD), and colitis(1,2). The Hemorrhoids are most common gastrointestinal disorders which can be characterized by inflammation of the anorectum including submucosal, fibrovascular and arteriovenous sinusoids. During defecation, painless rectal bleeding is a common complaint particularly when the tissue prolapse appears and the other symptoms include anal pruritus, pain and a lump at the anal verge due to intra-abdominal pressure caused by pregnancy, prolonged straining or constipation, diarrhea, aging, spicy diet and chronic straining and weakened muscular support are the various risk factors for developing hemorrhoids. Colorectal cancer and hemorrhoids contribute several similar risk factors such as low fiber intake, obesity and lack of adequate exercise, and mostly comparable symptoms like presence of blood in the stool. In some previous studies, hemorrhoids significantly increase the risk of distal colon cancer of the sigmoid colon, rectum, anus and other common sites which implicates that a localized inflammation may lead to the tumor formation. Colorectal cancers are diagnosed in both male and female and the third leading cause of cancer related deaths.

Several plant derived drugs are currently used for the treatment of hemorrhoid and related disorders. Current medical treatment for hemorrhoids on comprise to therapeutic treatment ranges from dietary and lifestyle modifications and oral therapy which depends on the degree and severity of symptoms. The plant *Eupatorium triplinerve vahl* belongs to the Asteraceae family and is locally known as *Ayapana* in Tamil. Traditionally, the leaf extracts are consumed orally to reduce hemorrhage and it also heals induced ulceration in any place within or inside gut. It helps in treatment of ailments like gastric and duodenal ulcers, Crohn's disease, and hemorrhoids(3–6). The leaves are very useful in reducing pain and inflammation. The plant serves as a natural therapeutic agent for neurodegenerative and hepatotoxic disorders due to the presence of unique bioactive components. *Eupatorium triplinerve vahl* is widely used in folk medicine and it's antiparasitic, anthelmintic, sedative, antifungal, antibacterial, anticoagulant, and analgesic. The plant is useful for various ailments such as cardiac, dyspepsia, gastropathy, haematemesis, hemoptysis, haematuria, hemorrhages, menorrhagia, poisonous bites, pruritis, skin eruptions, stomatitis, wound healing, cholera, and bites of venomous reptiles(7–9).

Coumarin as (7-methoxy coumarin) is used as an anti-tumor agent and is considered to be a component of the general defense response to abiotic and biotic stresses which exhibit anti-

inflammatory activity(3). Coumarin also named as hernarin was toxic to cancer cells including multi drug resistant cancer cells and leukemic cells.

Materials and Methods:

Preparation of extracts: The plant *Eupatorium triplinerve Vahl* (Family-Asteraceae) were collected from the Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore,India and the plant was authenticated by the Botanical Survey of India (BSI), Coimbatore, Tamilnadu, India. The Voucher number of the plant specimen identified and reported is BSI/SRC/5/23/2019/TECH/250.The leaves of *Eupatorium triplinerve Vahl* (270 g wet leaf) were weighed and washed thoroughly with sterile distilled water. The leaves were then shade-dried to prevent the loss of volatile compounds. The dried leaves were finely ground using a mortar and pestle into coarse powder and stored at room temperature in an air tight container until further use.

Cell preparation and culture

Since hemorrhoids associate itselfwith similar symptoms which are comparable to Colorectal Cancer (CRC) such as low water intake, weakened muscular support, intra-abdominal pressure and so on. So we used the Human Colorectal Adenocarcinoma (HT-29) cell line as a model for evaluation. These cell lines used in the study were procured from NCCS (National Centre for Cell Science), Pune, India. The DMEM (Dulbecco's Modified Essential Medium) with high glucose media supplemented with 10% Fetal Bovine Serum (FBS) and the 1% antibiotic (antimycotic) solution was used to maintain the HT-29 cells. The cells were seeded in a 96-well plate with a volume of 200 µl of cell suspension at the required cell density (2×10^4 cells / ml), allowing the cells to grow for approximately 24 hours in an atmosphere of 5% CO_2, 18–20% O_2, and 37°C temperature in the CO_2 incubator and were sub cultured every two days.

MTT assay

MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay, a colorimetric assay for determining cell metabolic activity, was used to determine the cytotoxicity of *Eupatorium triplinerve Vahl* acetone leaf extracts(10). HT-29 cells per well were plated in a 96-well plate. After the cells had been incubated for 24 hours, the media was changed to 100 µl of *Eupatorium triplinerve* acetone leaf extract at various concentrations (5, 20, 80, 160, and 320 µg/ml) for 24, 48, and 72 hours respectively. The positive control was 5-fluorouracil. Samples of untreated cells are used as a checkpoint. The sample were rinsed with 5 mg/ml PBS before MTT reagent was added to each of the well at 0.5 g/ml and the incubated for 2-4 hours at 37ºC in 5%

CO_2. The MTT reagent was removed after 3 hours of incubation, and 100 µg/ml of solubilization solution (DMSO) was added. This was done after gentle swirling in a gyratory shaker to speed up the dissolution process. The formazan crystals were dissolved in 150 µl DMSO. The absorbance was determined using a microplate reader at 570 nm. The viability of the HT-29 cancer cell lines were examined by the formula:

$$\text{Percentage (\%) Cell Viability} = \frac{\text{Optical density values of treated cell}}{\text{optical density values of control samples}} \times 100$$

IC_{50} value of acetone leaf extract of *Eupatorium triplinerve Vahl*

The IC_{50} (50 % inhibition concentration) value is defined as the concentration of the acetone leaf extracts that decreased the viability of cancer cells by 50 %. A cell suspension (100 µl) containing 2×10^4 viable cells/ml were transferred to each of the wells of the microliter plate and incubated at 37ºC for 24 h in a CO_2 incubator. The cells were treated with various concentrations (5, 20, 80, 160, and 320 µg/ml) of acetone leaf extracts for another 24h and several concentrations (20 µM) of positive control with 5 – fluorouracil (5- Flu).

The percentage of growth inhibition was calculated using the following formula:

$$\text{Cell inhibition (\%)} = 100 - [(At\text{-}Ab) / (Ac\text{-}Ab)] \times 100$$

Where, At = Absorbance value of test compound

Ab = Absorbance value of blank

Ac = Absorbance value of control

Statistical analysis

The data is analyzed and presented as mean and standard errors (SE). Three distinct tests each carried out in triplicate for each dose. One-way ANOVA test for multiple comparisons or the t-test were both used to establish statistical significance. A p-value of 0.05 or lower was regarded as significant. The dosage curves were used to calculate the IC50 values. The statistical and graphical analyses were carried out using the American programme Graphpad Prism version 8.0.

Anti-inflammatory activity on acetone leaf extract of *Eupatorium triplinerve Vahl*: To measure the Pro-inflammatory cytokines (IL-6, TNF-α) expression study on Raw 264.7 macrophage cell lines by flow cytometry.

Maintenance of Cell lines

The RAW 264.7 cells (mouse monocyte/macrophage cell line) were processed from NCCS, Pune. RAW 264.7 cells cultured in Dulbecco's Modified Eagle Medium (DMEM) contain

high glucose media supplemented with 10% FBS (Fetal Bovine Serum) along with 1% antibiotic – antimycotic solution and 1% L- Glutamine (200mM) in the atmosphere of 5% CO_2, 18-20% O_2 at 37ºC temperature and maintained in a CO_2 incubator and sub cultured between two days interval periods.

Cells viability assay

The MTT (3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) test was used to assess the viability of the extract-treated RAW 264.7 Macrophages cell lines. Its intensity was spectrometrically assessed at 570 nm and is inversely correlated with the number of live cells(11,12). A 96-well plate was used in which 1×10^6 cells/well were seeded into each well and incubated for 24 hours. A volume of 100 µl of fresh medium and an extracts at concentrations (5, 10, 20, 40, 80,160,320µg/ml) were added to the cells after incubation. Before adding 0.5 g/ml of MTT reagent to each well and incubating the sample for 2-4 hours at 37° C in 5% CO2, the samples were washed with 5 mg/ml of PBS. After 3 hours of incubation, the MTT reagent was withdrawn and 100 µg/ml of solubilization solution (DMSO) was added. This was done after gentle swirling in a gyratory shaker to speed up the dissolution process. The colourful formazan crystals were dissolved in 150 µl DMSO and the absorbance was determined at 570 nm. The absorbance ratio between the treatment group and the control group was multiplied by 100 to get the percentage of cell viability.

The viability of the RAW 264.7 cell lines were examined by applying the formula:

$$\text{Percentage (\%) Cell Viability} = \frac{\text{Optical density values of treated cell}}{\text{optical density values of control samples}} \times 100$$

Table.1: Different concentrations of acetone leaf extract of *Eupatorium triplinerve Vahl* on RAW 264.7 cells

Sl.no	Test sample extract	Cell line	Concentration to treat cells (µg/ml)
1.	Untreated	RAW 264.7	-

2.	LPS (Lipopolysaccharide)	RAW 264.7	1 µg/ml
3.	Blank	-	Only media
4.	Acetone leaf extract	RAW 264.7	5, 10, 20, 40, 80, 160, 320 µg/ml

***In-Vitro* Pro-inflammatory activity on RAW 264.7 cells**

The cells were plated in a 6-well plate at a density of 3×10^5 cells/ 2 ml and incubation in a CO_2 incubator overnight at 37ºC for 24 hours (13–15). The cells were stimulated with 1 µg/ml LPS (except untreated) for 12 hours prior to drug treatment. Cells were treated with 1 µg/ml LPS used as a negative control for anti-inflammatory marker expression, and untreated cells served as control. A concentration of 40 µg/ml of acetone leaf extract of *Eupatorium triplinerve Vahl* treated cells were the test sample and blank consisted of 2ml culture medium. After 24 hours of incubation, the cells were harvested directly into centrifuge tube and centrifuged for 5 minutes at 25ºC and carefully discarded the supernatant and washed the cells twice with 1X PBS. A volume of 0.5ml of BD Cytofix/cytoperm solution was added and washed with 0.5% BSA in 1X PBS and 1% sodium azide. After incubation, the medium was aspirated. A volume of 20µl of mouse anti-human TNF-α antibody and rat anti-mouse IL-6 were added separately and mixed thoroughly and incubated for 30 minutes in dark at room temperature. Staining solutions were removed and 3.7% paraformaldehydes were added to fix the cells and absorbance was measured using a flow cytometer (BD FACS Calibur), Cell Quest Pro software (version: 6.0). Cells were mounted to a microscopic slide and examined under a fluorescence microscope.

Table.2: Concentration of acetone leaf extract of *Eupatorium triplinerve Vahl* to measure the expression of the pro-inflammatory cytokines on RAW 264.7 cell line

Sl.no	Test sample	Cell line	Concentration to treat cells (µg/ml)
1.	Control / untreated	RAW 264.7	-
2.	STD (LPS)	RAW 264.7	1 µg/ml
3.	Acetone + LPS	RAW 264.7	40 µg/ml

Evaluation of Anti-hemorrhoid activity (Croton oil-induced hemorrhoid model)

The anti-hemorrhoid activity of *Eupatorium triplinerve Vahl* acetone leaf extract was determined by the method (16).

Experimental animal

Wistar Albino rats of both male and female rats were used for this study. Rats with a weight of 150-180 g were housed in standard conditions of temperature 22 ± 3ºC, 55 – 65% relative humidity, and a constant 12-hour light-dark cycle was maintained before and after the study duration. The approved protocol (IAEC/KASC/2021-22/02) for the animal study was carried out as per the guidelines of IAEC (Institutional Animal Ethics Committee) and CPCSEA (Committee for Control and Supervision of Experiments on Animals).

Experimental design

Five groups of four Wistar albino rats (n=4) were formed in each of two sets of tests. The groups were divided into distinct groups as positive and negative control depending on the proportion of male and female rats in each group. A sterile cotton swab (4 mm in diameter) soaked in croton oil (100 µl) was inserted into the anal part of the rat (about 22 mm diameter) and held there for 10 seconds in order to induce hemorrhoids in all other groups of male and female rats besides the normal group. The croton oil preparation was made by mixing deionized water, pyridine, diethyl ether, and 6% croton oil. After 24 hours of induction, Evans blue (30 mg/kg) was injected into the tail part of the animals.

Group 1: Control (only distilled water) - The rat of group 1 served as normal control and was provided with only distilled water.

Group 2: Positive control - The rats of group 2 (positive control group) were studied on a single dosage working model with 5-fluorouracil (20 mg/kg) through oral gavage as a standard drug for induction of hemorrhoid.

Positive control Working model	Day 1- Day 5	Day 6- Day 15 Standard drug : (5- FLUOROURACIL)
	Induced hemorrhoid with croton oil	Dose 1
		80 mg

Group 3: Negative control

After five day's induction of hemorrhoids, in a further study, animals were not treated with any standard drugs or acetone leaf extract which served as negative control to compare the different dose level investigations.

Group 4: Dosage working model

For the acute toxicity study, the acetone leaf extract of *Eupatorium triplinerve Vahl* was kept in the air to dry until the solvent gets evaporated, and 50 ml of DMSO was added. All the animals were subjected to respective treatment as assigned to the group once daily for 10 days (from 6th day to 15th day) to observe the acute toxicity study.

Sample working model	Day 1- Day 5	Day 6 – Day 15 (acetone leaf extract sample)		
	Induced hemorrhoid with croton oil	Dose 1	Dose 2	Dose 3
		20 mg (84 µl)	80 mg (336 µl)	320 mg (1.344 ml)

Study of Hematological parameter

Blood samples were collected from the site of the recto-orbital sinus or in the tail or by cardiac puncture for the various hematological and biochemical analyses. Blood was collected from male and female rats about 0.5 ml into Ethylene Diamine Tetra Acetic acid (EDTA) treated at collection tubes on interval periods 1st, 5th, 7^{th,} and 14th days respectively. Blood samples were immediately processed for evaluation of the hematological parameters using an automated hematological analyzer. The following parameters was determined – White Blood Count (WBC), Red Blood Count (RBC), Hematocrit (HCT), Erythrocyte Sedimentation Rate (ESR), Hemoglobin (Hb), the body weight was checked from initial day to final day observation. For the coagulation profile, the blood was placed on a plain test plate allowed to stand and the clotting time was noted and noticed the bleeding time to screen for abnormal blood coagulation in the anal region.

Statistical analysis

The data from individual groups were represented as mean ± S.D. From the different groups, the collected data were illustrated using analysis of variance (ANOVA) followed by a comparisons test and minimum criterion for statistical significance at set $p < 0.01$ and $p < 0.05$.

Histopathologically examination of recto-anal tissue

All of the animals were sacrificed by exsanguinations on the fifteenth day, one hour after the treatment, while under profound isofluraneanaesthesia, and recto-anal tissues (4 mm in length) were separated.

Sample tissue was placed in a tube containing 10% buffered formalin for 24 hours and fixed tissues were dehydrated and cleared in graded series (80% isopropanol) by immersing overnight and followed by 100% isopropanol for one hour. The dehydrated tissue was cleaned by xylene and impregnated with histology-grade paraffin wax. The microscopic slides with poly-L-lysine coating were mounted with the paraffin-embedded tissue blocks. Sections that had been

paraffinized were dewaxed in xylene and rehydrated using ethanol and water (graded series). To avoid non-specific staining, recto-anal tissue was stained and then rinsed with 1% alcian blue (pH 2.5) in 3% acetic acid solution for 30 min. The sections were then dehydrated in alcohol and counterstained for 1 minute with neutral red (0.5% aqueous solution). The slides were then examined using a moderate power objective lens under the microscope (40 X).

This analysis for evaluation of the severity score was weighed and fixed in a 10% formalin solution for histological examination.

The RAC was calculated using the formula

$$\text{Rectoanal Co} - \text{efficient} = \frac{\text{Weight of Recto} - \text{anal tissue (mg)}}{\text{Body weight (g)}}$$

Histological observation of the rectoanal tissue was carried out in order to determine the appearance of hemorrhoids.

Results and Discussion:

Cell Viability assay

The biological activity included the anti-cancer activity of metabolites evaluated by bioassays and *in-vitro* techniques. Currently various cytotoxicity assays, trypan blue dye assay, MTT (3-(4,5-dimethylthiazol-2-YL)-2,5-diphenyltetrazolium bromide) assay, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, and sulforhodamine B assay based on quantifying cell proliferation, assessing cell membrane integrity or measuring protein content in cells. MTT assay is a widely used method to quantify metabolically viable cells through their viability to the reduction of soluble yellow MTT to blue purple formazan crystals. Percentage growth of inhibition was identified to increase in a dose-dependent manner.

In MTT assay, absorbance (cell density) between 0.9 and 1.0 is specific for measuring both stimulation and inhibition of cell proliferation in the linear range. The cytotoxicity of the methanol, ethanol and acetone leaf extract of *Eupatorium triplinerve Vahl* against HT-29 colorectal cancer cell lines were evaluated by treating the cells with various concentrations and time intervals. 5-Fluorouracil was used as a positive control.

Table.3: The IC_{50} value of *Eupatorium triplinerve Vahl* methanol, ethanol, and acetone leaf extract against two colon carcinoma cell lines – HT-29 cells. The values are expressed as mean ± SEM. The IC_{50} values of *Eupatorium triplinerve Vahl* acetone leaf extract against HT-29 cancer cell line.

INTERVAL	**24 hrs**	**48 hrs**	**72 hrs**
HT – 29 Cell line			
IC_{50} value for ACETONE leaf extract	**125.37 (µg/ml)**	**90.95 (µg/ml)**	**44.89 (µg/ml)**

Morphology of cells, at the lower concentration (5 µg/ml) inhibited HT-29 cells by 96.5% viability at 24 and 48-hour exposure and at 72-hour incubation acetone leaf extract had a significant around 90 percentage of cell viability. At the higher concentration (320 µg/ml) of acetone leaf extract inhibited with HT-29 cells by 32.1% at 24 and 48 hour and for 72 hour inhibition were around 11.5% cell viability and compared with positive control. The results showed that acetone leaf extracts showed lower IC_{50} values 44.89 µg/ml respectively at 72-hour treatment against the HT-29 cell line. In the different concentration of acetone leaf extracts of *Eupatorium triplinerve*, had significantly cytotoxic effect against Human Colorectal Adenocarcinoma (HT-29) cell line respectively.

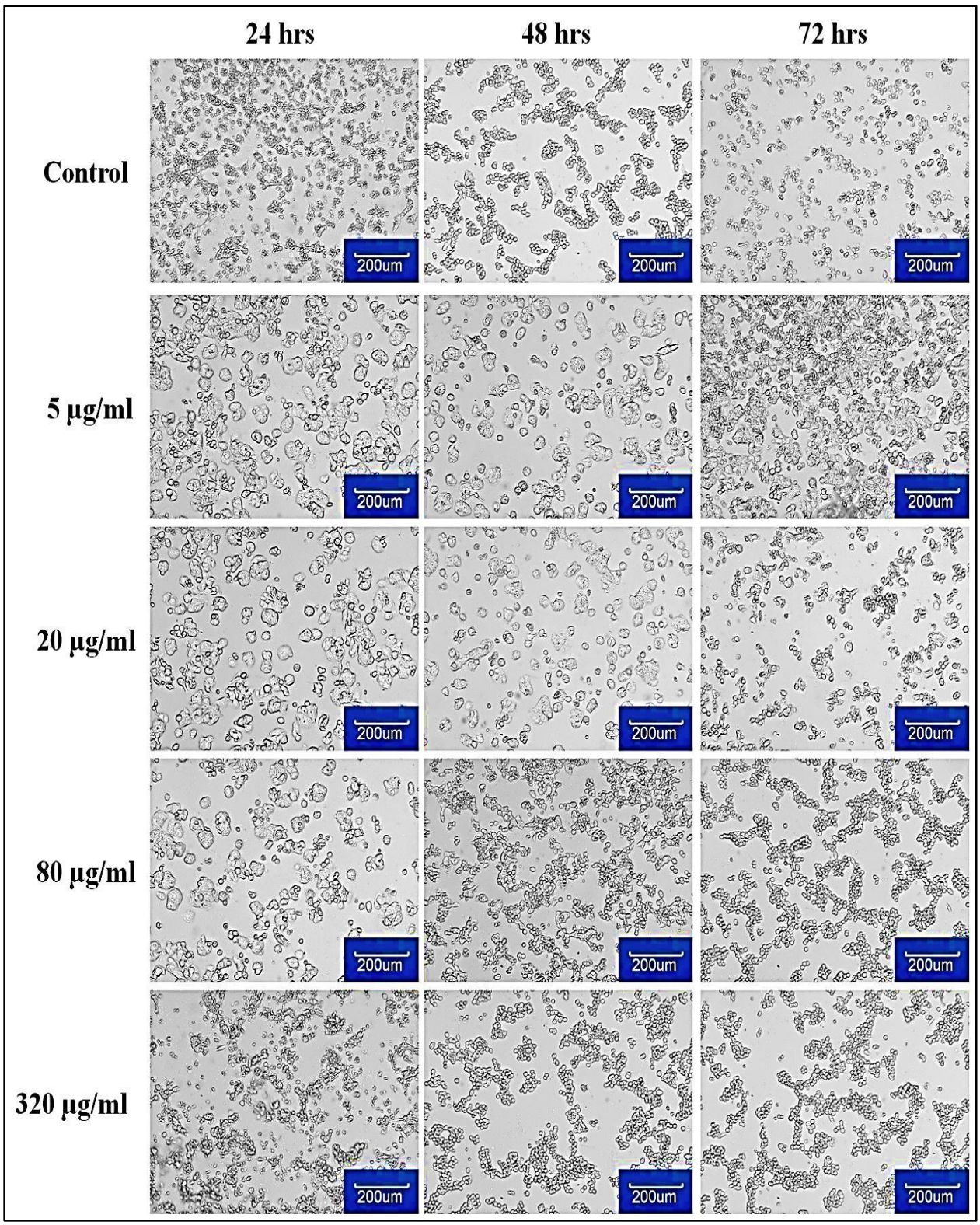


Figure.1: Morphological characteristics HT-29 colorectal cancer cell lines treated with different concentrations of acetone leaf extract of *Eupatorium triplinerve Vahl* and the standard.

Anti-inflammatory activity

The inflammatory response is controlled primarily by cytokines which is an acute phase response that protects the host against infection and direct injury. The major role starts with the release of pro-inflammatory cytokines (IL-6, TNF-α) produced predominately from activated macrophages and involved in the up-regulation of inflammatory reactions. This reaction is to communicate with surrounding tissues that can perform cell stimulation. Macrophages are the first line of host defense against bacterial infection and play a role in the intimation of adaptive immune responses.

Eupatorium triplinerve acetone leaf extract has been demonstrated to exhibit anti-inflammatory activity through the inhibition of IL-6, and TNF-α in an LPS – stimulated mouse macrophage RAW 264.7 cells which are widely used as *an in vitro* inflammatory model by dose-dependent inhibition. A flow cytometry was performed to quantify secreted pro inflammatory cytokines.

Table.4: IC_{50} concentrations of the acetone leaf extract against the Raw 264.7 cells.

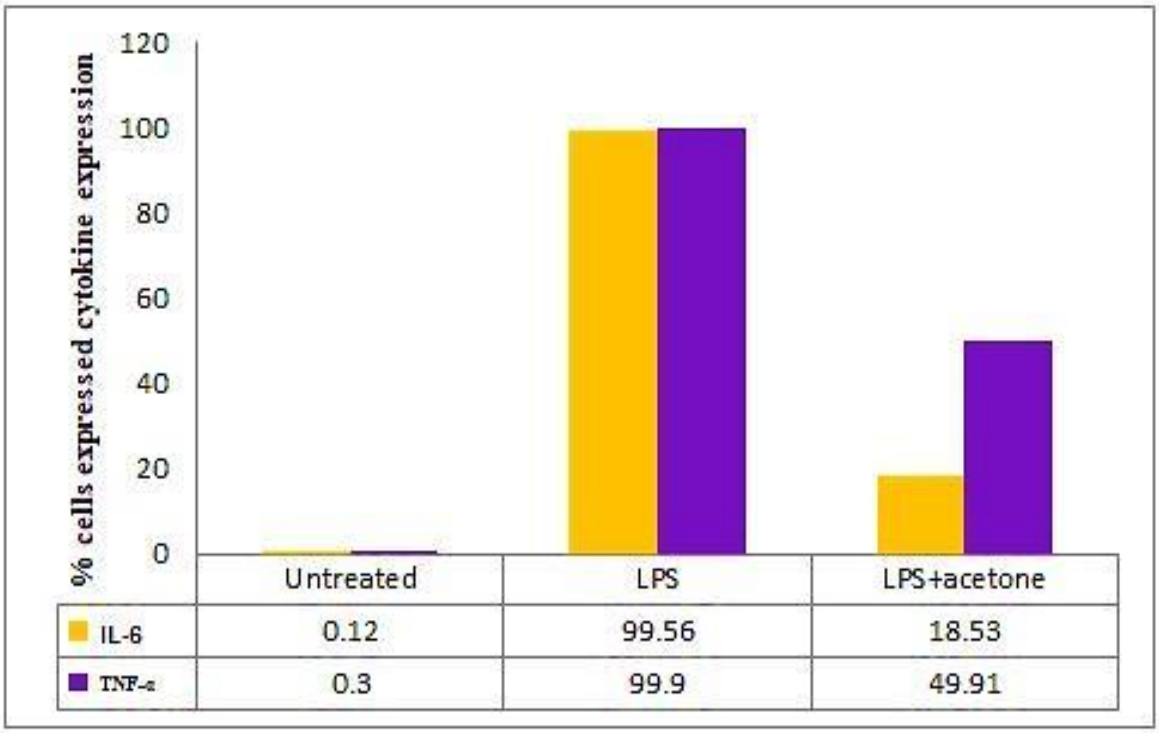
S.No	**Leaf Extract**	**IC_{50} Concentration (µg/ml)**
RAW 264.7 CELL LINE		
1.	**IC_{50} value for ACETONE leaf extract**	248.49

Human Interleukin – 6 expression study of acetone leaf extract *Eupatoium triplinerve* treated with RAW 264.7 cell line

Omnidirectional processing of IL-6 in the human body is what makes it distinctive. It is believed that cytokines significantly increase inflammatory response and strongly engage the immune system. The glycoprotein IL-6 has 184 amino acid residues and is enhanced by a significant post translational mechanism to form an interconnected structure.

To investigate the anti-inflammatory activity of acetone extract of *Eupatorium Triplinerve Vahl* leaf extracts were activated with LPS stimulated RAW 264.7 cells. The concentrations of acetone leaf extract of 40 µg/ml were able to inhibit IL-6 production in LPS-induced RAW 264.7 cells. The LPS induction successfully increases the concentration of IL-6 and significantly showed a high level in positive control (LPS) compared to the negative control (untreated) and treated leaf extract (acetone sample). After pre-stimulation with LPS macrophages treated with acetone leaf

extract reduction showing 99.56 %, for acetone sample 18.53 %, only with untreated 0.12% of RAW 264.7 cells expressed the positive observation of IL-6 respectively. The results revealed that the acetone leaf extract works as an efficient anti-inflammatory agent by inhibiting the production of IL-6 in macrophage cells and then playing a major key role in oxidative stress and inflammation.

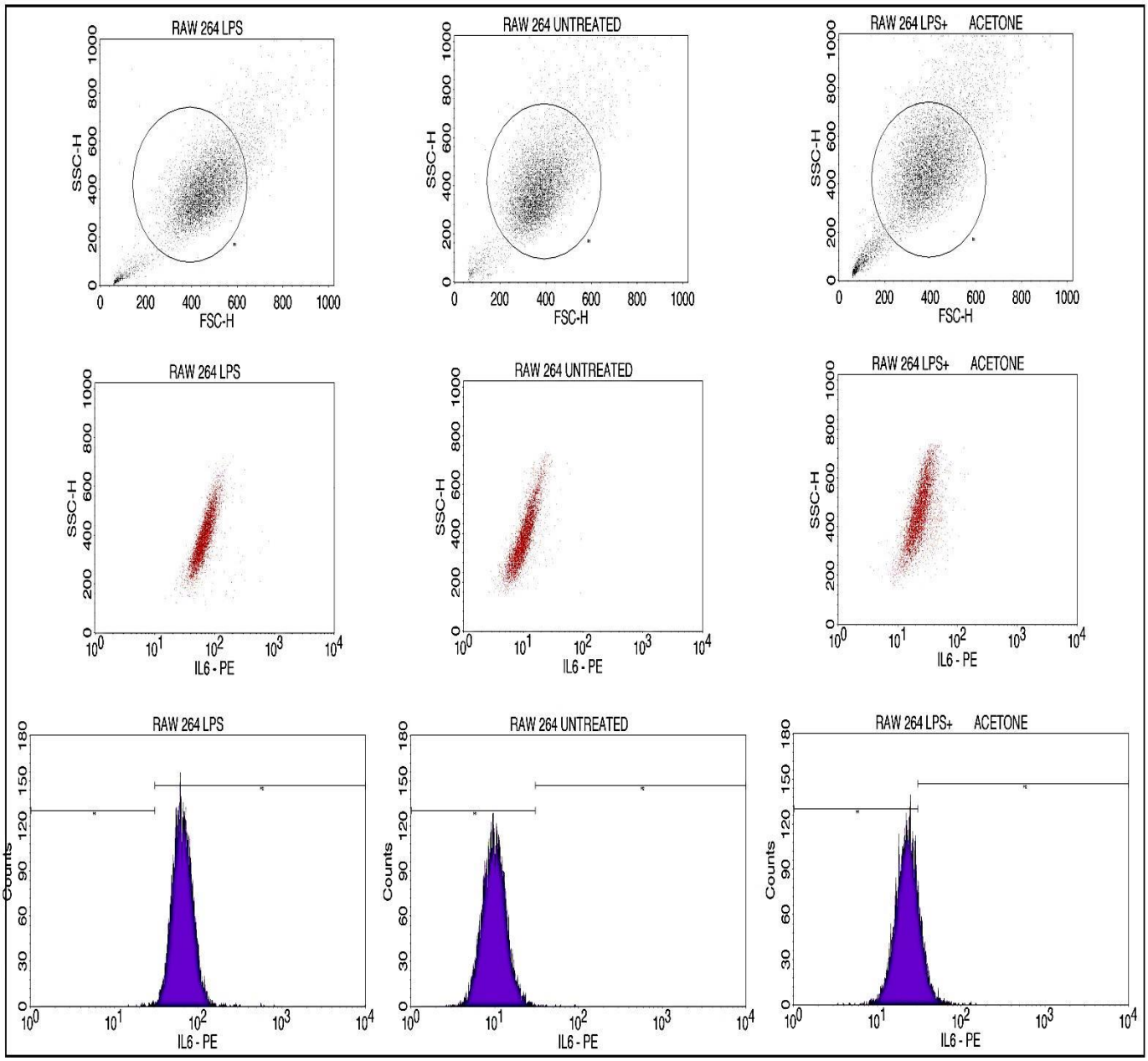


	Untreated	LPS	LPS+acetone
IL-6	0.12	99.56	18.53
TNF-α	0.3	99.9	49.91

Figure.2: Comparison of control LPS, untreated and treated group (acetone leaf extract) test compounds showing the % of viable, late, and early apoptotic and necrotic cells against the pro-inflammatory cytokines.

Dot plot: refers to all the cells that were collected for the study as well as some gated cells that were chosen to be in the centre of this portion plot. Clumps of cells or possibly double- or triple-clumps of cells are located above gated cells. Dead cells or detritus are thought to be located below gated cells. A dot plot will show the size and granularity of the cells based on Forward Scattering Light (FSC), Side Scattering Light (SSC), shape, and granularity of the cells. A histogram showing the number of living and dead cells in treated, untreated, and control samples on RAW 264.7 cells is called a histogram. The mean fluorescence intensity of the M1 populations is modest and the cells are still alive. M2 populations, on the other hand, are apoptotic cells that can absorb inflammatory cytokines and have high mean fluorescence intensities.

Figure.3: Pro-inflammatory cytokines RAW 264.7 LPS, RAW 264.7 LPS untreated, RAW 264.7 LPS + acetone, - IL-6 expression study by flow cytometry: 1. Dot plot, 2. Annexin V-PI expression study, 3. Histogram. Here M1 phase refers to the negative expression/region, and M2 refers to the positive expression/region.



Tumor Necrosis Factor-Alpha (TNF-α) expression study of acetone leaf extracts of *Eupatorium triplinerve* on RAW 264.7 cell line

TNF-α also known as cachectin, acts on several signaling pathways through two cell surface receptors (Trans membrane receptor). It plays a major role in inflammatory and neuropathic hyperalgesia. TNF- α is associated with intracellular signaling pathways and downstream cellular targets and effects on superfamily receptor members. TNF-α is produced as a pro-hormone consisting of 233 amino acids that are closely linked in cell membrane with a 157 residue.

Both acetone leaf extract treatments were able to lower TNF-α levels in LPS-stimulated RAW 264.7 cells, according to the analysis of the impact on TNF-α production. A flow cytometeric analysis was performed to quantify the secreted proinflammatory cytokines TNF-α. Acetone leaf extract, after pre-stimulation with LPS (1 µg/ml) showed a positive inhibitory effect of 49.91(%).

Additionally, the findings showed that the distribution of TNF receptors on target RAW 264.7 cells may determine the physiological action of TNF-α, with a focus on a positive impact on tolerance induction. This mechanism could be the cause of a few reported problems with anti-TNF-α therapy. TNF-α, like IL-6, is a pro-inflammatory cytokine with a wide range of actions, including cytotoxic and cytostatic effects against cancer cells. TNF-α has a significant impact on the metabolism and operation of adipose and muscle tissue. TNF-α levels need to be higher in order for macrophages to get activated and acquire the pro-inflammatory M1 phenotype, to phagocytose microorganisms, to scavenge dead cells, and to promote better cutaneous regeneration. A group of immune regulatory molecules known as anti-inflammatory cytokines can regulate the pro-inflammatory cytokine response. Together with cytokine inhibitors and soluble cytokine receptors, cytokines work to control the human immune response. More people are becoming aware of their physiological and pathological functions in inflammatory conditions. Focus is placed on the characteristics of soluble cytokine receptors and anti-inflammatory cytokines with an eye towards potential therapeutic applications.

Pro-inflammatory cytokines FITC expression study: Using BD FACS Calibur and Cell Quest Pro software, cytokine expression was examined in RAW 364.7 cells that had been exposed to the test chemical both untreated and treated (Version 6.0). The expression percent of several cell types was displayed in quadrants: The percentage of viable cells is shown in the lower left quadrant (LL), the early apoptotic cells are shown in the lower right quadrant (LR), the necrotic cells are shown in the upper left quadrant (UL), and the late apoptotic cells are shown in the upper right quadrant (UR).

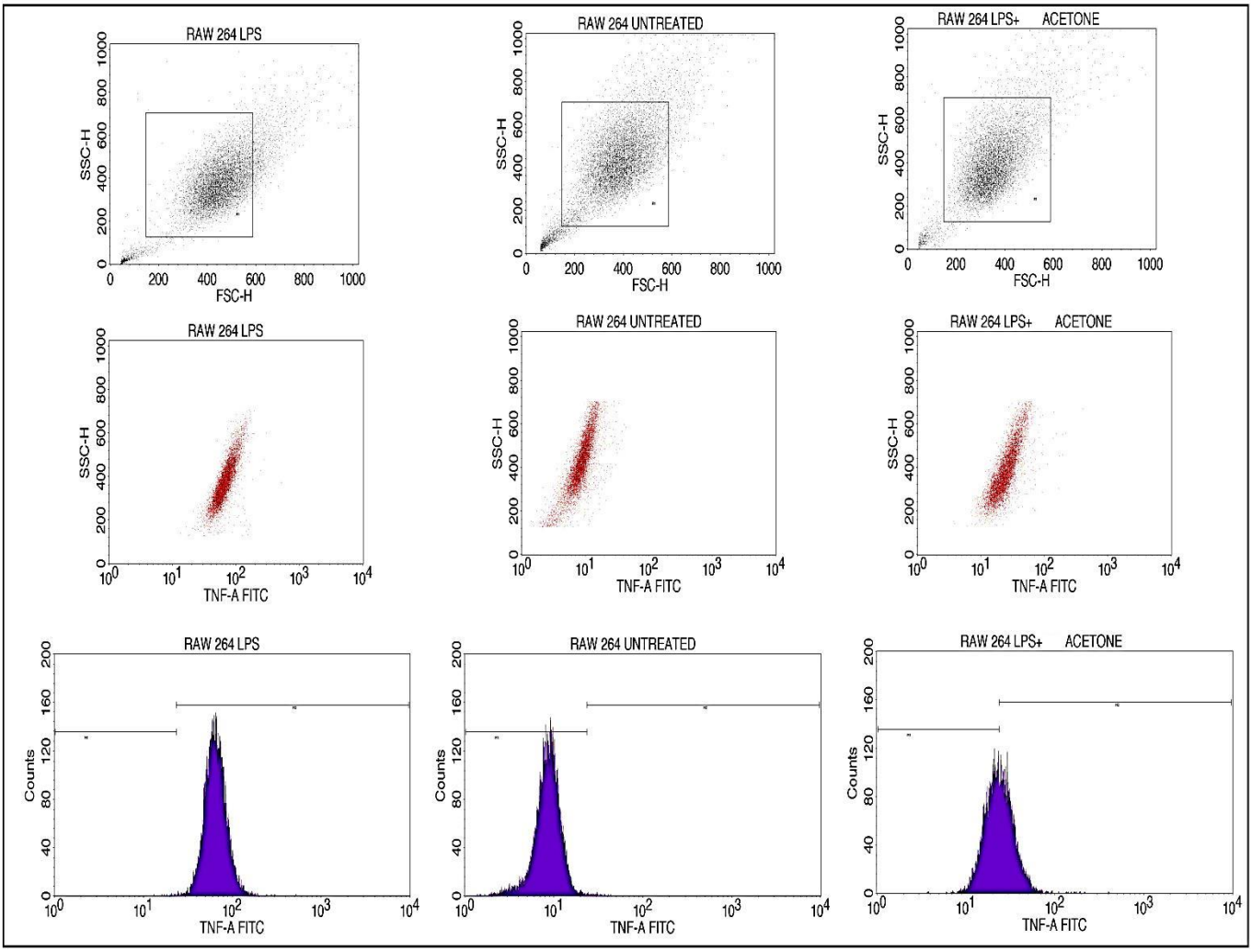


Figure.4: Pro-inflammatory cytokines RAW 264.7 LPS, RAW 264.7 LPS untreated, RAW 264.7 LPS + acetone - TNF-α expression study by flow cytometry: 1. Dot plot, 2. Annexin V-PI expression study, 3. Histogram. Here M1 phase refers to the negative expression/region, and M2 refers to the positive expression/region.

Evaluation of *in vivo* anti-hemorrhoid property for acetone leaf extracts of *Eupatorium triplinerve* through animal experiments.

Haematological parameter study:

Eupatorium triplinerve is traditionally used in folklore medicine for the treatment of various ailments. To evaluate its use in traditional medicine, it is mainly important to identify its toxicity in the animal system by the *in vivo* method. Therefore, this study was aimed to validate the toxicological effects of oral administration of acetone leaf extract of *Eupatorium triplinerve* in Wistar Albino rats. Acute toxicity test studies were conducted by the oral administration of 20, 80, and 320 mg/kg body weight of the animal.

Scientifically, the plant *Eupatorium triplinerve* is reported to have potent anti-inflammatory and antioxidant activity (17,18). While traditionally, the leaf of the plant is used to treat hemorrhoids. Blood is a better indicator to identify the health of an organism. It also acts as a pathological reflector of the body, hence the study of hematological parameters are important in diagnosing the functional status of animals exposed to toxicants(19)(20).

In the acute study, animals were administered with various doses of acetone leaf extract of *Eupatorium triplinerve* to evaluate its toxicity for a period of 15 days. The effects of acetone leaf extract of *Eupatorium triplinerve* on body weight and hematological and histopathologically methods were analyzed. In acute toxicity, no deaths were recorded after 14 days of administration. Clinical signs of toxicity were observed after the acute administration. No mortality was seen in the 15 days of oral administration of acetone leaf extract. No signs of neurological and behavioral changes were noticed during 72 hours of observation. There was some significant difference between the control and sample treated rats in terms of food, water intake. The extract intake induced changes in hematological parameters such as body weight, clotting time and bleeding time, Red blood Count (RBC), White Blood Count (WBC), Hemoglobin concentration (HB), Erythrocyte Sedimentation Rate (ESR), was also compared with positive control and negative control group rats. There are no acute or sub chronic toxicity observed and our results indicate that extract lacks any toxic risk factor.

Acetone leaf extract of *Eupatorium triplinerve* was evaluated for acute toxicity in rats and it was observed that the leaf extract it safe even at higher concentrations of 320 mg/kg body weight. Croton oil application in the rectoanal region caused significant increases in RAC ($P<0.001$) in the comparison of normal group, positive control group, negative control group animals. On the first

day of application, initial body weights differed based on male (133.7 kg) and female (124 kg) rats. After 15 days of administration of the leaf extract resulted in dose-dependent effects on body weight, with significant increase in oral dosage group at 320 mg (158.7 for male and 159.4 for female) compared to normal, positive and negative control groups. Similarly food intake significantly decreased in oral dosage leaf extract group. An increase in body weight ratio is based on indication of inflammation.

The mean values of hematological parameters of male and female rats are closely similar to each other based on the statistical comparison. RBC count ranged between ($4.35 – 5.65×10^{12}$ /L) followed by the positive control (5-Fluorouracil) , normal control, negative control, and acetone leaf extract 320 mg respectively. The treated group of *Eupatorium triplinerve* acetone leaf extract 320 mg/kg body weight had a significantly ($P < 0.01$) higher RBC count compared with normal control group rats. White Blood Count (WBC) is to measure the number of white blood cells, it may also be called a leukocyte test. The reference WBC count range of adults is $4.5 – 11×10^{9}$/μL. All groups of rats varied in the reference range; 12.90/ μL of normal control, 14.75 μL positive control (5-Fluorouracil), 13.8 μL negative control, 15.25 μL of treated group (80mg acetone leaf extract) significant changes were observed on oral administration. The HB concentrations were 14.6 g/dL for normal control, 15g/dL positive control, 15.4 g/dL of negative control and 15.4g/dL for treated group (320 mg of acetone leaf extract) respectively. The treated group had a significantly ($P < 0.001$) higher hemoglobin level than compared to the control group. The hemoglobin reference level varied from 11.6 – 16.6 g/dL (for both males and females). The ESR (Erythrocyte Sedimentation Rate) normal range is about 0 to 22±6 mm/hr for both male and female. In the normal group rats, 6 mm/hr for male and 5.3 mm/hr for female, treated group of 7 mm/hr for male rat and 8 mm/hr for female rat. Values are expressed as the mean ±, S.D, statistical significance calculated by ANOVA followed by Dennett's $p < 0.001$, $p< 0.01$, and $p < 0.05$ by comparing the treated group with induced hemorrhoid rats.

Throughout the experimental phase, all the male and female rats gained body weight in the final stages of the observation. After hemorrhoid induction, at the end of 15^{th} day of treatment with acetone leaf extract group, body weight of the rats increased compared to the positive group and normal control group. The mechanism behind excess of body weight and it may be increased risk factor of cancer(21,22). The acetone leaf extract of topical herbal gel (80mg and 160mg) showed a clotting time and bleeding time significantly increased which compared to positive control for

both male and female rats for the coagulation parameter test. The results of the coagulation profile showed that the hemorrhoid state in the rats increased clotting and bleeding time as shown by a reduction in the times observed for blood coagulation parameters. This was effectively countered by the anticoagulation action of *Eupatorium triplinerve* leaf extracts and fractions.

The number of RBC increased significantly in the treatment of acetone leaf extract group compared with the control group. RBC preventing oxidation of red blood cells and hemoglobin concentration that often leads to hemolysis. It may also stimulate formulation or secretion of erythropoietin in stem cells of animals as evidence by increased level of RBC(23,24). Low counts of WBC may indicate the body immune compromised. At the same, it a high WBC counts might indicate underlined diseases. The number of WBC in treatments of oral dosage application was simultaneously higher for 80 mg/kg concentration of acetone leaf extract group rats as compared to control as per reference normal range. The profile of the WBC count reflects balance between granulocyte and WBC rate. The raised WBC may also reflect for low-grade inflammation symptoms.

To summarize, from this study we conclude that oral administration of *Eupatorium triplinerve* increases red blood count, white blood count, hemoglobin concentration, body weight, and Erythrocyte sedimentation rate at higher doses when compared with the normal control group. The increase in leukocyte count showed that the extract has an immuno modulatory effect which raises the immune system. The increased level of hemoglobin may due to the antioxidant effect of *Eupatorium triplinerve* acetone leaf extract which prevents the destruction of RBC from free radicals formation.

Histopathological analysis of hemorrhoid in rats:

Histopathology is the study of standards for tissue examination either for research or diagnostic purpose, for both qualitative and quantitative measurement analysis. It is used to assess the inflammation or healing stage and to visualize the presence of tissues, cells, and their structural differences. In the present study, histopathological studies of the hemorrhoid anal tissues were carried out in normal control rats, croton oil-induced inflammation-bearing rats, and hemorrhoid-bearing rats treated with 5-fluorouracil and acetone leaf extract of *Eupatorium triplinerve* at the three different dosages.

A histopathological examination was carried out for the anal canal region epithelial tissue of all experimental rats. For all the rats, tissues were removed in a humane manner using isoflurane

anesthesia at the end of 15th for acute toxicity study. Anal tissues were examined microscopically and macroscopically. Macroscopically observation under a light microscope (100X) revealed a gross pathological change (lesion developed) due to exposure to the test extract as compared to the normal control group. This study demonstrated that the extract of *Eupatorium triplinerve* was not toxic at a given dosage as revealed from an acute toxicity study of epithelial tissue.

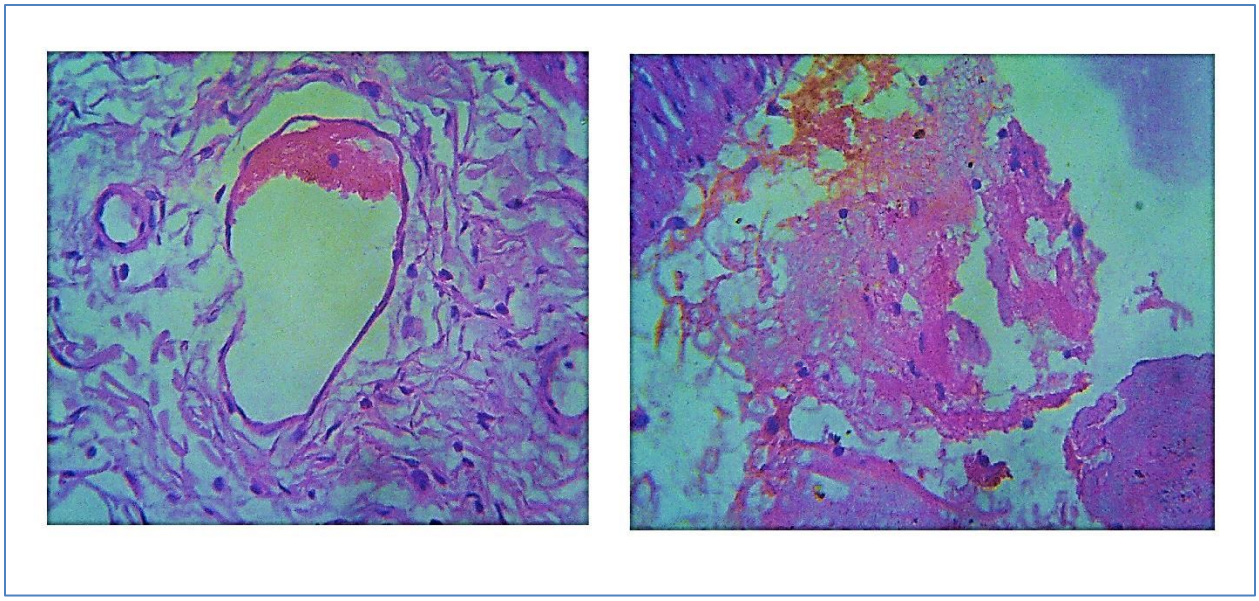


Figure. : Histopathological examination of anal canal region epithelial tissues of acetone leaf extract at 320 mg treated experimental group.

Sections studied show normal-appearing squamocolumnar junction. The lining of the epithelium showed congestion with marked inflammatory cells, few areas with degenerations and necrosis was observed in this section. Submucosa showed many dilated ecstatic blood vessels with hemorrhoids and minimal inflammation. The changes included venous dilation, vascular thrombosis, a degenerative process in fibroelstic tissue, and distortion of sub epithelial tissue. Multiple linear grey white soft tissues bit measuring about 3.2×2.5×1.4cms were noticed.

Conclusion:

The evaluation of anticancer activity of plant extract is essential for safe treatment begins and to identify the intrinsic toxicity of the plant. It is frequently used as *in vitro* model system to measure cytotoxic effects of toxic substances and plant extracts against cancer cell lines. *Eupatorium triplinerve Vahl* acetone leaf extract showed good anticancer potential with high cytotoxicity against the analyzed (HT-29) humancolorectal cancer cell line in a concentration dependent manner. The herbal acetone leaf extract showed high inhibition to secretion of pro-

inflammatory factor IL-6 better effect than compared to TNF-α and NO cytokines. The data describes that acetone leaf extract is able to reduce inflammation in LPS – stimulated mouse macrophage RAW 264.7 cells. To demonstrate the possible effects on arresting the cells between M1 and M2 specific phase of cell growth, the RAW 264.7 cells are treated with acetone leaf extract the IC_{50} concentrations of 18.53 % (for IL-6) expressed at 40 μg/ml concentration in positive observation. Results clearly showed that extract had significantly reduced the level of inflammation with major contributing factor due to the phytoconsituents. A detailed study on the isolated phytochemicals from the plants is required to know about the bioactive compounds responsible for the observed anti-hemorrhoid potential of *Eupatorium triplinerve Vahl* with specific mode of action. These findings further for the idea that a diet rich in herbs and species may contribute to the reduction of inflammation and prevention against related diseases.

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