Innovation development and standardization of Novel Herbal Formulation
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Innovations on anti-inflammatory and protective activity of Ethyl acetate fraction isolated from *Vernonia Cinerea* (L) in Spargue Dawley rats

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Abstract

**Objective:** To evaluate effect of ethyl acetate fraction isolated from *Vernonia cinerea* (L) (EAVC) in acute lung inflammation in rats.

**Methodology:** Rats were pretreated with vehicle (CMC 0.5%), EAVC(25, 50 & 100 mg/kg po), roflumilast (1mg/kg po), once a day for 3 consecutive days. 2hrs after the last treatment the rats were intratracheally injected with lipopolysaccharide (LPS), (1mg/kg) while control group received saline(1ml/kg) under anesthesia. After 4hrs of intratracheal LPS injection, animals were sacrificed and BALF was collected. Lungs were perfused, dissected out and used for biochemical estimations and histological examination.

**Results:** EAVC (50 & 100 mg/kg po) significantly (p < 0.05) inhibited MPO, total protein, albumin, total cells, neutrophils and histamine content in BALF indicating suppression of lung permeability. Similarly, MOP, catalase enzyme activity in lung tissue homogenate were reduced as compared to positive control group. Histological evaluation revealed marked reduction in perivascular infiltration and edema in drug treated animals.

**Conclusion:** EAVC (50 & 100 mg/kg po) exhibited protective effect in LPS induced ALI in rats by inhibiting lung permeability and inflammation.

**Keywords:** vernonia cinerea, lipopolysaccharide, acute lung injury, cytokine, anti-inflammation

1. **Introduction**

Acute airway inflammation results in damage to the lung parenchyma including microvascular injury, diffuse alveolar damage with intrapulmonary hemorrhage and edema leading to increased frequency and severity of airway disorders as asthma and COPD. Acute Respiratory Distress Syndrome (ARDS) is characterized as one of the most dreadful form of Acute Lung Injury that leads to respiratory failure and death [1, 2]. Despite the newer advances in the treatment, the mortality rate still remains higher [3]. Inconsequence to the increasing mortality rate, newer treatment strategy for ARDS are now being explored. Most frequently used mechanisms for inducing ARDS consist of toxicant that promotes pulmonary edema, increased lung infiltration and tissue permeability resulting in lung inflammation. Such a disease pathology further alleviates the production and proliferation of different inflammatory mediators such as cytokines, arachidonic acid metabolites proteases, and free radicals. Increase in lung tissue permeability aggravates the severity of Pulmonary edema, which contributes to abnormal gas exchange. Over and above, the pathogenesis is accompanied by disassembly of several apoptotic cells, including neutrophils, alveolar epithelial cells, and endothelial cells resulting in acute lung injury in ARDS. Hence any therapeutic mechanism manifested to be effective in resolving these pathological abnormalities of lung injury could prove be a probable method for the treatment of ARDS [4].

A bacterial endotoxin, Lipopolysaccharide (LPS) found in the cell wall of a gram negative bacteria is most commonly used toxicant or stimulus for induction of local acute inflammation in the experimental models. LPS is instilled in the experimental models via Intratracheal route.
of administration. The LPS inducing inflammation in rats is widely accepted as an experimental model of ARDS [4, 5]. India is the hub for traditional and herbal medicines. Herbal remedies are forming a new and strong base in the prevention, mitigation and treatment of various disease and disorder due to the scientifically proven efficacy of its potential phytoconstituents. Soon in the near future traditional medicines are ought to take over major proportion of the global market of medicines, there are for exploring the newer. In years past, number of traditional weed like plants are being explored for its therapeutic application in the treatment of various disease and disorder. One of such traditional weed from asteraceae family is Vernonia cinerea. This herbaceous plant is widely distributed in the tropical regions such as in Southeast Asia, Thailand. The pharmacognostic and phytochemical analysis of the plant reveals its potential medicinal properties that could be used as folk medicine applications. The plant have reported therapeutic application to be used as a flattulence, anti-diarrhoeal, anti-pyretic, and in treatment of inflammation. It is also proved to be effective in treating the common cold, cough and chronic skin disease. It is also useful in promoting detoxification and general health care. Different extracts of the plant have also proven to possess different mechanism against immunological responses such as inflammation, pain, infection, wound healing, asthma and bronchitis [6, 7, 9]. The methanolic extract of Vernonia cinerea have a reported in vitro anti-inflammatory activity. Also the ethyl acetate fraction of the methanolic plant extract revealed the presence of various terpenoidal lactones [10, 11]. Thus the objective of this study is to investigate the anti-inflammatory and protective activity of ethyl acetate fraction of methanolic extract of vernonia cinerea

2. Material and Methods

2.1. Selection of the plant

Aerial part of Vernonia cinerea (L), family: Asteraceae was collected from the premises of Bombay college of Pharmacy, Kalina and authenticated from Botanical Survey of India, Pune.

Extraction, fractionation and characterization of the crude from the plant:

The shade dried and powdered plant material (200g) was extracted with methanol (1L) in soxlet extractor for 24 hours at 50°C. The extract was concentrated using rotary vacuum evaporator at temperature not higher than 50°C to get the yield of 15.97% w/w. Further the suspension of crude methanolic extract in water (50mg in in 150ml) was sequentially fractionated with petroleum ether (3x 50ml), ethyl acetate (3 x 50 ml) using a separating funnel. The resulting fractions were then concentrated to get petroleum ether fraction of V. cinerea (PEFVC), ethyl acetate fraction of V. cinerea (EAFVC) and water fraction of V. cinerea (WFVC). The polled fractions were then preserved in airtight glass container at 4°C for further use. HPTLC analysis of ethyl acetate fraction was performed using hexane: ethyl acetate (78:22) as mobile phase and anisaldehyde sulphuric acid reagent to confirm the presence of sesquiterpene lactone. EAVC was further used in the study.

2.2. Experimental model and grouping: The experimental study was carried out in consonance with the Institutional Animal Ethics Committee. Thirty six female Sprague Dawely rats weighing 150-170 g were used for the study. The experimental design was developed by randomly dividing the rats in six groups with six animals each viz; Vehicle control group, Disease control group, High dose receiving group, Low dose receiving group, Intermediate dose receiving group [12].

2.3 Experimental protocol: Rats were pretreated with vehicle (CMC 0.5%), EAVC(25,50&100 mg/kg po), roflumilast (1mg/kg po), once a day for 3 consecutive days, 2hrs after the last treatment the rats were intratracheally injected with LPS (1mg/kg) while control group received saline(1ml/kg) under ketamine: xylazine (80:20) anesthesia. After 4hrs of intratracheal LPS injection, the animals were sacrificed under high dose of urethane. The bronchoalveolar lavage fluids (BALF) was retrieved and the following experimental paradigm was evaluated for myeloperoxidase activity (MPO), total protein, albumin, total cells, neutrophils and histamine content. For the retrieval of bronchoalveolar lavage fluids (BALF), airways were flushed with 2.0 ml PBS. Further at an interval of 4 hour, the Bronchoalveolar lavage (BAL) was performed after intratracheal instillation. Lungs were perfused, dissected out and used for estimation of different biochemical parameters viz. MPO, catalase activity and histological examination [12, 13, 14].

3.1 Total cell count: Total cell count of BALF was performed using equal volume of turk’s solution.

3.2 Differential cell count: Smears were stained with Giemsa stain and DLC of 200 cells was performed using standard morphological criteria.

3.3 Myeloperoxidase activity (MPO) in BALF: MPO activity assay, estimates the degranulation of neutrophils in to the BALF due to airway inflamation using microarray colorometric assay. The assay was performed by incubating 50 μl sample with 100 μl substrate buffer (0.167 mg/ml O-dianiside dihydrochloride and 0.4 mM H₂O₂, 50 mM in sodium phosphate buffer, pH 6.0 containing, 0.5 % hexadecyl trimethyl ammonium bromide) for 5 minutes at room temperature. The reaction was stopped with 100 μl of 5 % w/v sodium azide in distilled water and the optical density was read at 450 nm [11].

3.4 Total protein content in BALF: The total protein content is determined using BIURET protein estimation kit. Increase in its concentration in BALF is indicated by blue-violet complex formation in the solution that marks the airway epithelial damage and increase in lung permeability.

3.5 Albumin assay: The albumin content in BALF is estimated by addition of the bromocresol (BCG) dye that causes a shift in the absorbance of the yellow BCG dye when measured photometrically between 580-630nm.

3.6 Myeloperoxidase assay of the lung tissue: Lung tissues were homogenized and the supernatant was added to a solution containing 10 ml ice-cold 50mM potassium phosphate buffer (pH 6.0), 0.5% hexadecyl trimethyl ammonium bromide (HETAB) and 10mM EDTA and centrifuged for 20min. The activity was measured spectrophotometrically at 460 nm.

3.7 Assessment of oxidant and antioxidant parameters in lung tissue:

3.7.1 Reduced Glutathion: Glutathion reduces hydrogen
peroxides and hydroperoxides like DNA peroxides and lipid peroxides inside the body into water, alcohol etc. Thus protect bio-membranes and cellular components against oxidative stress. Assessment of total non-protein sulphhydryl content is an indirect measure of reduced GSH. The tissue was homogenized and the supernatant was further added to a solution containing tris-EDTA buffer, dithiobis (2 nitrobenzoic acid) i.e. DTNB and methanol. The mixture was incubated and then centrifuged at 4000 rpm for 15 min. The supernatant was separated and the intensity of color developed was determined at 412nm against blank treated in the same way replacing tissue supernatant with distilled water and the GSH values were obtained by interpolation of standard plot of GSH concentration and absorbance.

3.7.2 Superoxide dismutase (SOD): The assay procedure is by the addition of 0.5ml of epinephrine solution to the supernatant of tissue homogenate. Auto oxidation of epinephrine to adrenochrome at pH 10.5 was measured by following change in O.D at 480nm against reagent blank. Results were expressed as units of SOD activity (per mg tissue protein). One unit of SOD activity induced approx. 50% of inhibition of adrenochrome formation.

3.7.3 Catalase: Catalase exerts its antioxidant effect by catalyzing the decomposition of hydrogen peroxide (H₂O₂) to water and oxygen there by protecting the cell from the oxidative damage. Catalase activity can be measured by following either the decomposition H₂O₂ or O₂. The reaction was initiated by addition of homogenate to the solution containing phosphate buffer, distilled water and hydrogen peroxide at 25 °C & pH 7.3. The change in absorbance was recorded at 240nm for 3 min. The results were expressed as U/mg tissue protein.

4. Results and Discussion

HPTLC analysis of EAVC reveled the presence of sesquiterpene lactone at 254nm.

**Table 1:** Effect of EAFVC, WFVC and Roflumilast on total cell count in BALF in LPS induced lung inflammation.

| Group   | Treatment                      | % Neutrophils | % Mast cells | % Macrophages | % Monocytes |
|---------|--------------------------------|---------------|--------------|---------------|-------------|
| Group I | Sham-operated vehicle control  | 19.81±2.91    | 17.13±1.72   | 44.05±1.63    | 19.01±3.07  |
| Group II| Positive control               | 71.07±6.50#   | 7.43±1.99#   | 10.00±3.18#   | 11.51±6.20# |
| Group III| Roflumilast (1.5 mg/kg)       | 22.84±1.38*   | 14.85±2.15*  | 40.02±8.08*   | 22.30±3.12* |
| Group IV | EAFVC (25 mg/kg, p.o.)        | 57.10±4.99*   | 7.39±0.85    | 12.32±1.75*   | 23.19±6.16* |
| Group V  | EAFVC (50.0 mg/kg, p.o.)      | 48.31±4.4*    | 9.69±1.73    | 18.87±3.56*   | 23.13±5.71* |
| Group VI | EAFVC (100.0 mg/kg, p.o.)     | 31.17±2.48*   | 11.48±0.73   | 37.27±2.30*   | 20.09±3.51* |

Values are expressed as means ± SD where n=6. Statistical evaluation was undertaken by analysis of variance (ANOVA) followed by Dunnets test. * Differences were considered statistical significant as compared to control at p < 0.05

Fig. 1 demonstrates differential cell count of inflammatory cells in BALF from different experimental group. The percent recovery of neutrophils, macrophages, mast cells, macrophages prominently increased after 4 hours of LPS induction. There was prominent fall in the number of these cells in the experimental group receiving higher dose of EAFVC which is comparable to the group receiving the roflumilast. The recovery rate of the cells in the group receiving low dose of EAFVC is lesser as compared to the intermediate and high dose receiving group. The differential counting of inflammatory cells in BALF sham operated group was found to be increasing than the expected level.
The total protein and albumin levels in BALF act as a marker for lung permeability. The protein and albumin level in BALF increased after the LPS instillation. Oral administration of EAFVC attenuated the increased protein and albumin levels in BALF. Higher and intermediate dose of EAFVC have proved to be more effective in attenuating the increases in the protein levels than roflumilast. The BALF protein and albumin levels of low dose receiving experimental group are comparable to the disease control group.

Effect of ethyl acetate fraction of methanolic extract of Vernonia cinerea on LPS-induced lung hyperpermeability:
Fig 4: Effect of ethyl acetate fraction of methanolic extract of *Vernonia cinerea* on LPS-induced MPO activity in BALF and lung tissue

Table 4: Effect of EAFVC, WFVC and Roflumilast on Myeloperoxidase (MPO) content in lung tissue and BALF in LPS induced lung inflammation.

| Group    | Treatment                  | Myeloperoxidase in lung tissue (ng/ml) | Myeloperoxidase in BALF (ng/ml) |
|----------|----------------------------|---------------------------------------|---------------------------------|
| Group I  | Sham-operated vehicle control | 27.61±2.36                           | 7.14 ± 0.58                     |
| Group II | Positive control            | 128.96±7.80*                         | 46.36 ± 2.37*                   |
| Group III| Roflumilast (1.5 mg/kg)     | 37.19±0.66*                          | 10.22 ± 3.46*                   |
| Group IV | EAFVC (25 mg/kg, p.o.)      | 124.06±6.48                          | 38.98 ± 2.62                    |
| Group V  | EAFVC (50.0 mg/kg, p.o.)    | 103.38±4.64*                         | 27.41 ± 2.18*                   |
| Group VI | EAFVC (100.0 mg/kg, p.o.)   | 74.81±6.79*                          | 19.30 ± 2.82*                   |

MPO is a chemical marker indicating degranulation of neutrophils in ALI. These are the cytoplasmic granules released by neutrophils. The MPO activity was remarkably decreased in the high and intermediate dose receiving group and comparatively increased in roflumilast treated group. Whereas MPO activity was found to be increasing in group receiving no drug treatment which was comparable to the low dose receiving experimental animals.

Table 5: Effect of EAFVC, WFVC and Roflumilast on Reduced glutathione, Superoxidase Dismutase and Catalase content in lung tissue in LPS induced lung inflammation.

| Group    | Treatment                  | Reduced GSH (µg/mg protein) | SOD (U/mg protein) | Catalase (U/mg protein) |
|----------|----------------------------|----------------------------|--------------------|-------------------------|
| Group I  | Sham-operated vehicle control | 29.04±2.12               | 23.61±1.32         | 1.63±0.128              |
| Group II | Positive control            | 11.21±0.96*              | 1.98±0.63*         | 0.44±0.092*             |
| Group III| Roflumilast (1.5 mg/kg)     | 26.81±1.65*              | 17.61±1.97*        | 1.21±0.111*             |
| Group IV | EAFVC (25 mg/kg, p.o.)      | 14.33±1.45*              | 2.33±1.23*         | 0.53±0.077*             |
| Group V  | EAFVC (50.0 mg/kg, p.o.)    | 17.52±1.44               | 6.79±1.36*         | 0.65±0.068*             |
| Group VI | EAFVC (100.0 mg/kg, p.o.)   | 18.11±0.82               | 12.70±1.00*        | 1.03±0.050*             |
| Group VII| WFVC (25 mg/kg, p.o.)       | 16.53±1.29               | 8.82±0.65*         | 0.77±0.047*             |
| Group VIII| WFVC (50.0 mg/kg, p.o.)    | 20.58±1.38               | 12.60±1.66*        | 1.14±0.059*             |
| Group IX | WFVC (100.0 mg/kg, p.o.)    | 22.94±0.84               | 15.93±1.71*        | 1.18±0.095*             |
High and intermediate dose receiving experimental group showed considerable increase in the antioxidant levels which was comparable to the roflumilast treated group. The disease control group had a marked fall in the antioxidant level.

![Antioxidant Levels](image)

**Fig 5:** Effect of EAFVC, WFVC and Roflumilast on Reduced glutathione, Superoxidase dismutase and Catalase content in lung tissue in LPS induced lung inflammation.

**Effect on Histopathology of lung:** No histopathological changes were seen in the lung tissue of vehicle control group. LPS instillation caused thickening of alveolar wall, edema and congestion, infiltration of inflammatory cells. The pretreatment with EAFVC markedly reversed these alterations of the lung tissue.

A: alveoli; B: Bronchiole; LI: luminal infiltration; STI: Septal thickening and infiltration; PVE: Perivascular edema; PBE: Peribronchiolar edema; PBI: Peribronchiolar infiltration; HPB: hyperplasia of BALT (Bronchial Associated lymphoid tissue)

5. Discussion
The study employs LPS induced experimental model given through intratracheal route. LPS is an endotoxin secreted by gram negative bacteria. LPS instillation causes severe lung damage leading to ALI. It increases the lung tissue permeability that trigger the release of various inflammatory mediators [15, 16]. This plays an important role in promoting lung edema, pathological changes in the lung tissue, increases the lethality of lung epithelium and endothelium. These pathophysiological changes were manifested by measurement of various biochemical parameters in BALF and through lung histological changes. Pretreatment with EAFVC significantly decreased the abnormally high levels of various inflammatory mediators such as neutrophils, macrophages, monocytes, mast cells [17, 18, 19]. The protein and albumin levels were also found to be decreased in the pretreatment group thereby decreasing the lung vascular permeability, lung edema. Finally the MPO activity was assessed for confirming the accumulation of neutrophils in the lung tissue. The pretreatment with EAFVC prominently increased the SOD, catalase and GSH levels in BALF. Thus EAFVC pretreatment played a vital role in prevention of LPS induced airway inflammation severities [20, 21].

6. Conclusion
In conclusion this study proposes the significance of EAFVC in reducing the LPS-induced airway inflammation which attenuates lung injury thereby leading to a rise in the LPS-induced mortality rate. It also revealed the effective dose of EAFVC required to prevent the LPS-induced ALI. EAFVC (50 & 100 mg/kg po) exhibited protective effect in LPS induced ALI in rats by inhibiting lung permeability and Inflammation. It also suggests that Sesquiterpenes from EAVC may serve as effective alternative for the treatment of acute lung inflammation.

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