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SCREENING OF *RANDIA DUMETORUM* LAMK. EXTRACTS FOR ANTI-INFLAMMATORY ACTIVITY USING MOUSE MODEL OF HCL-INDUCED ALI

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Keywords:

Acute lung injury, Traditional medicine, Anti-inflammation, Antioxidants, Bioassay-guided isolation

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ABSTRACT: Acute Lung Injury (ALI) and its more severe form Acute Respiratory Distress Syndrome (ARDS) is a clinical syndrome with a high mortality rate (27-41%). There is no pharmacological therapy for ALI treatment so far because of the complex mechanism and involvement of multiple pathways. So, the present study was designed to test the role of different extract/fractions of fruits of a traditional plant *Randia dumetorum* Lamk. utilizing a mouse model of acid aspiration (HCl) mediated ALI. Hydromethanolic extract (HME) was screened for anti-inflammatory activity at 100, 200, and 400mg/kg doses employing total and differential cell count in bronchoalveolar lavage fluid. Our data demonstrated that oral administration of HME in mice before induction of ALI resulted in reducing the number of BALF inflammatory cells, particularly neutrophils, in a dose-dependent manner. Further, HME was partitioned into ethyl acetate soluble (EASF) and ethyl acetate insoluble fraction (EAISF) to extract phytoconstituents based on differential polarity. Our data revealed that EAISF showed better efficacy than EASF for the amelioration of lung inflammation. Similarly, bioactive EAISF was partitioned into ethanol-soluble (ESF) and ethanol insoluble fraction (EISF), where ESF had more potent anti-inflammatory activity in mice w.r.t. EISF as depicted by the number of total cells and neutrophils in the lungs of mice. Overall, our results validate the traditional use of *Randia dumetorum* fruits in managing respiratory ailments, and further fractionation/investigation of ESF is encouraged to unveil the bioactive ingredient possessing anti-inflammatory potential.

INTRODUCTION: Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are two terms used interchangeably to depict pulmonary inflammation. ALI/ARDS can be triggered by direct (pneumonia, aspiration of gastric contents, pulmonary contusion, near-drowning, inhalation injury, reperfusion pulmonary edema, *etc*.) or indirect means (sepsis, multiple traumas, acute pancreatitis, cardiopulmonary bypass, transfusion of blood products, drug overdose, *etc*.) [1]. It is characterized by rapid onset of respiratory failure, decreased static respiratory system compliance, and severe hypoxemia [2-4]. This clinical condition is associated with a poorer prognosis and higher mortality in cases of increased severity. The activation of both immune cells and structural cell types is involved in the pathogenesis of ALI. Neutrophils and macrophages are known to play a predominant role during ALI manifestation [3-5].

A complex network of cytokines and other pro-inflammatory molecules is responsible for initiating and amplifying the inflammatory response in ALI [6]. It has been more than half a decade since the initial description of ALI, yet only a little progress has been made in developing novel therapies. The only option available for treatment so far is mechanical

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ventilation, which cannot be considered the real treatment as it aims to improve respiratory compliance. It helps the physicians to buy enough time to wait for the resolution of the underlying disease. Moreover, ARDS is one of the leading causes of COVID-19 associated mortality [7, 8]. COVID-19 is a respiratory infection imitating viral pneumonia, which ultimately leads to ARDS in a majority of the severe cases [9, 10]. The lack of effective treatment strategies against SARS-CoV-2 mediated ALI resulted in a higher mortality rate. Hence, ALI represents an unmet medical need, and there is an urgent need to develop new therapies to treat patients with this condition.

Since ancient times, traditional medicines and phytopharmaceuticals have been used to treat inflammation and other disorders, especially in the Asian subcontinent. Around 80% of the global population depends on herbal-based medicines for their primary health care needs according to the World Health Organization (WHO) [11]. The success of plant-based drug development for the management of several diseases in the past inspires and encourages many researchers to investigate and validate the uses of traditional medicinal plants. It is estimated that around 70% of medicines available to date are directly or indirectly derived from plant sources [12]. *Randia dumetorum* Lamk. is such a plant with traditional medicinal properties. It is a small tree found in tropical and subtropical regions of India. It belongs to the Rubiaceae family and is commonly known as Madana (Sanskrit), Madanphal (Hindi), and emetic nut (English) [13, 14].

Traditionally, the fruits of this plant are claimed to be used as an anti-dysenteric agent, a medical cure for piles, gonorrhea, jaundice, diarrhea, *etc.* [15]. In addition to this, it has been used in indigenous medicine in the treatment of several respiratory complications such as asthma, bronchitis, chest infections, common cold, cough, *etc.* [16]. Moreover, *Randia dumetorum* Lamk. is categorized as a drug having properties similar to "rasayanas" in Ayurvedic texts, *i.e.*, having immunomodulatory actions to promote normal health and longevity by boosting immunity and energy levels [17]. It has been quoted to have emetic, antispasmodic, abortifacient, anti-inflammatory, anodyne and expectorant activities in the ayurvedic texts [16]. Further, experimental animal-based studies show that the fruits of *Randia dumetorum* Lamk. possess antibacterial, immunomodulatory, anti-inflammatory and analgesic activity [18-20]. The traditional knowledge, phytochemical review and reported anti-inflammatory, antiallergic, analgesic and immunomodulatory activities of the fruits of this ayurvedic medicinal plant indicate its potential to inhibit the inflammatory and allergic mediators. Since, no study has been conducted w.r.t. *Randia dumetorum* in the treatment of ALI or any other pathological condition, we decided to evaluate the anti-inflammatory potential of the fruits of this plant via Bioactivity-guided fractionation in which the most potent fraction was selected based on total cell count and neutrophil infiltration in the lungs of mice to find new drug leads to treat ALI.

Material and Methods

Plant Material: Dried fruits of *Randia dumetorum* Lamk. were purchased from Dhanvantri Aushadh Bhandar, Gujarat, and authenticated from NISCAIR, Delhi (Authentication Number: NISCAIR/RHMD/Consult/2019/3456-57). The sample of the procured fruits has been deposited in herbarium-cum-museum of UIPS, Panjab University, Chandigarh (Voucher No.- Fr11/11/2021).

Chemicals and Reagents: All the chemicals used to carry out the study were of analytical grade and purchased from Sigma Aldrich Co. (St. Louis, MO, USA), Merck India (Mumbai, India), and Himedia Laboratories Pvt. Ltd. (Mumbai, India). The solvents such as methanol, ethyl acetate and ethanol were purchased from (Merck specialties Pvt. Limited, Mumbai, India), both laboratory and analytical grade. Dexamethasone (reference drug), HCl, DPPH, ABTS, TPTZ, and Ascorbic acid has been purchased from Sigma Chemical Company (St. Louis, USA). Distilled water was used throughout the study.

Preparation of Extract: The dried fruits of *Randia dumetorum* (1 kg) were coarsely powdered using mortar and pestle. The fruit powder was then subjected to exhaustive Soxhlet extraction using hydro methanol (70% methanol) as solvent. The solvent was evaporated using a rotary vacuum evaporator (Eyela N 1100), and procured extract was preserved in a vacuum desiccator containing

anhydrous silica gel blue until used for experimental procedures.

Solvent Partitioning: Hydromethanol extract (HME) was adsorbed on silica gel (mesh size) and dried. It was taken in a 500 ml round bottom flask and refluxed for 1 hr using ethyl acetate. Ethyl acetate with soluble constituents was procured. The process was repeated until no color was obtained in ethyl acetate. All the ethyl acetate fractions were pooled and subjected to vacuum rota evaporation using a rotary vacuum evaporator (Eyela N 1100). This fraction was labeled as ethyl acetate soluble fraction (EASF). The remaining adsorbed extract was reconstituted using hydromethanol (70%) and dried, labeling this fraction as ethyl acetate insoluble fraction (EAISF). Similarly, the ethanol-soluble fraction (ESF) and ethanol insoluble fraction (EISF) were obtained from bioactive EAISF.

Animal Ethical Approval: The experimental protocols for animal studies were approved by the Institutional Ethics Committee (Approval No. PU/45/99/CPCSE/IAEC/2019/256). Male balb/c mice weighing 20-25 g were procured from the Central Animal House of Panjab University, Chandigarh. The animals were housed in polypropylene cages bedded with sterilized rice husk and acclimatized for a week before the commencement of the study. All the experiments were conducted according to the Indian National Science Academy Guidelines for the use and care of animals in scientific research. The animals were fed on a standard mouse chow pellet diet (Ashirwad Industries, Punjab, India) and water *ad libitum*.

Preparation of Suspension of Extract / Fractions: Tween 80 (5%) in aqueous carboxymethyl cellulose (CMC 0.5% w/w) was used as a vehicle for preparing the suspension of extracts. Doses of various test substances were prepared by suspending appropriate quantities in the vehicle and administered to the mice orally (0.2 ml/mice).

Acute Toxicity Studies: Acute toxicity studies of hydromethanol extract of *R. dumetorum* fruits were carried out on mice as per OECD 423 guidelines. After 12h of fasting, different groups of mice were administered a single oral dose (500, 1000, and 2000 mg/kg) of hydromethanol extract. Immediately after dosing, mice were observed for signs of toxicity during the first 0.5, 1, 2, 4, 8, and 12 h and at every 24 h for 14 days. Behavioral parameters, tremors, lethargy, death, amount of water and feed intake were observed.

Anti-inflammatory Activity:

Experimental design: Control (Normal saline treatment intratracheally; 60µl). HCl: A volume of 2 ml/kg *b.wt.* 0.1 N HCl (60µl) dissolved in saline was instilled intratracheally (*i.t.*) to induce ALI [21]. HCl + Extract/ fraction's (X mg/kg b.wt.): Single-dose 60 mins prior to HCl instillation (0.2 ml orally). HCl + Dexamethasone: Single-dose (3mg/kg bw; *i.p.* injection) 30 min prior to HCl instillation [22].

X is the dose of the extract/fraction tested for anti-inflammatory activity. For mother extract *i.e.*, hydromethanol extract, dose-response studies were performed at 100, 200, and 400 mg/kg. Doses of subsequent fraction(s) were determined proportionately based on the fraction w.r.t its mother fraction yield and are shown in **Table 1** [23]. Mice were euthanized 24 h after the HCl instillation for the analysis of anti-inflammatory activity using the following parameters:

Bronchoalveolar Lavage Fluid (BALF) Procurement and Analysis: After 24 h of ALI induction, BALF was procured by injecting 1.5 ml of phosphate buffer saline (PBS) into the lung followed by gentle aspiration of the contents. The fluid recovery rate was more than 90%. The BALF samples were centrifuged to obtain the cell pellet. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of PBS. The total cell count was determined using a hemocytometer [24]. Cyto-slides were prepared using cytospin centrifuge and stained with hematoxylin and eosin for differential analysis of the cells [24]. The photomicrographs of cytoslides were captured using a microscope attached with a camera (Nikon Eclipse Y-TV 55) and analyzed with NIS-Elements software.

Antioxidant Capacity Assays: Evaluation of the antioxidant capacity of different extracts was done using standard antioxidant assays [Ferric Reducing

Antioxidant Power (FRAP), 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays] [25-27]. The result of the FRAP assay was expressed as "mM AAE/gm of DE", where AAE and DE stand for ascorbic acid equivalents and dry extract, respectively. Results of ABTS and DPPH assays were expressed as IC_{50} values (µg/ml).

Estimation of Total Phenolic and Flavonoid Content: Different extracts' total phenolic and flavonoid content was estimated using standard assays [28, 29].

The results of total phenolic content were expressed as "mg GAE/gm of DE", where GAE and DE stand for gallic acid equivalents and dry extract, respectively. The results of total flavonoid content were expressed as "mg QE/gm of DE", where QE and DE stand for quercetin equivalents and dry extract, respectively.

Phytochemical Screening: The bioactive extract/fractions, namely hydromethanol extract, ethyl acetate insoluble fraction and ethanol-soluble fraction, were screened for different classes of phytoconstituents such as alkaloids, tannins, flavonoids, saponins, phenols, carbohydrates, proteins, terpenoids/steroids, and glycosides using the standard procedures [30, 31].

Statistical Analysis: Results are depicted as mean ± Standard deviation (S.D.). Statistical analysis was performed by one-way analysis of variance (ANOVA) tests followed by Tukey's multiple comparisons using graph-pad Prism software (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION:

Acute Toxicity Study and Dose Selection for Extract / Fraction: The use of pharmacological substances (pure and extracts) has increased to an innumerable amount. These substances may result in chronic toxicity if used over a long period and may also exhibit acute toxic effects depending on the dose and nature of the substances administered.

Hence, it is important to evaluate the acute toxicity effects of new pharmacological substances to ensure none or minimal side effects of the test substance in experimental animals before the initiation of the study.

The acute toxicity studies showed no toxic effects of *Randia dumetorum* fruit up to the dose of 2000 mg/kg body weight. In mice, no changes in behavioral parameters, tremors, lethargy, death, amount of water, and feed intake were observed. In addition to this, the doses of different extract/fractions administered to mice were selected proportionately based on the yield w.r.t. their mother extracts, as shown in **Table 1** [23].

TABLE 1: YIELD AND ADMINISTERED DOSES OF DIFFERENT EXTRACTS/FRACTIONS

Mother Extract/Fraction (Weight in gms)	Extract/Fraction	Yield (%)	Yield (gms)	Proportionate dose for bioactivity
Dried fruit (1 kg)	HME	15%	150	400 mg/kg (most potent)
HME (150 gms)	EASF	15%	22	50 mg/kg
	EAISF	80%	120	350 mg/kg
EAISF (120 gms)	ESF	38%	45	150 mg/kg
	EISF	55%	66	200 mg/kg

Anti-inflammatory Activity of HME: The infiltration of neutrophils in the lungs is a paramount event in the pathophysiology of ALI, which ultimately leads to the aggravation of subsequent inflammatory events [32]. Hence, we depicted the total cell and neutrophils in BALF of mice as parameters for the evaluation of the anti-inflammatory effect of the *Randia dumetorum* Lamk. fruit. BALF analysis among different groups of mice revealed a dose-dependent reduction of the total as well as differential cells with maximum efficacy at 400 mg/kg dose. BALF from the HCl group had a prominently higher number of total cells than the control group (***$p<0.001$; three-fold change). Next, administration of HME before HCl treatment resulted in a dose-dependent reduction in the number of total cells w.r.t HCl treated animals and maximum reduction was observed at the dose of 400mg/kg ($^{###}p<0.001$; twofold change) **Fig. 1A**. The differential analysis

of BALF cytosildes revealed that the majority of inflammatory cells were neutrophils **Fig. 1B**. Interestingly, pre-treatment of HME at different doses prior to HCl administration resulted in the reduction of neutrophils in BALF of mice in a dose-dependent manner **Fig. 1C**. These results showed the efficacy of HME of *Randia dumetorum* fruit at 200 and 400mg/kg doses, with the latter being most potent. Hence, the dose of 400mg/kg was selected for further evaluation.

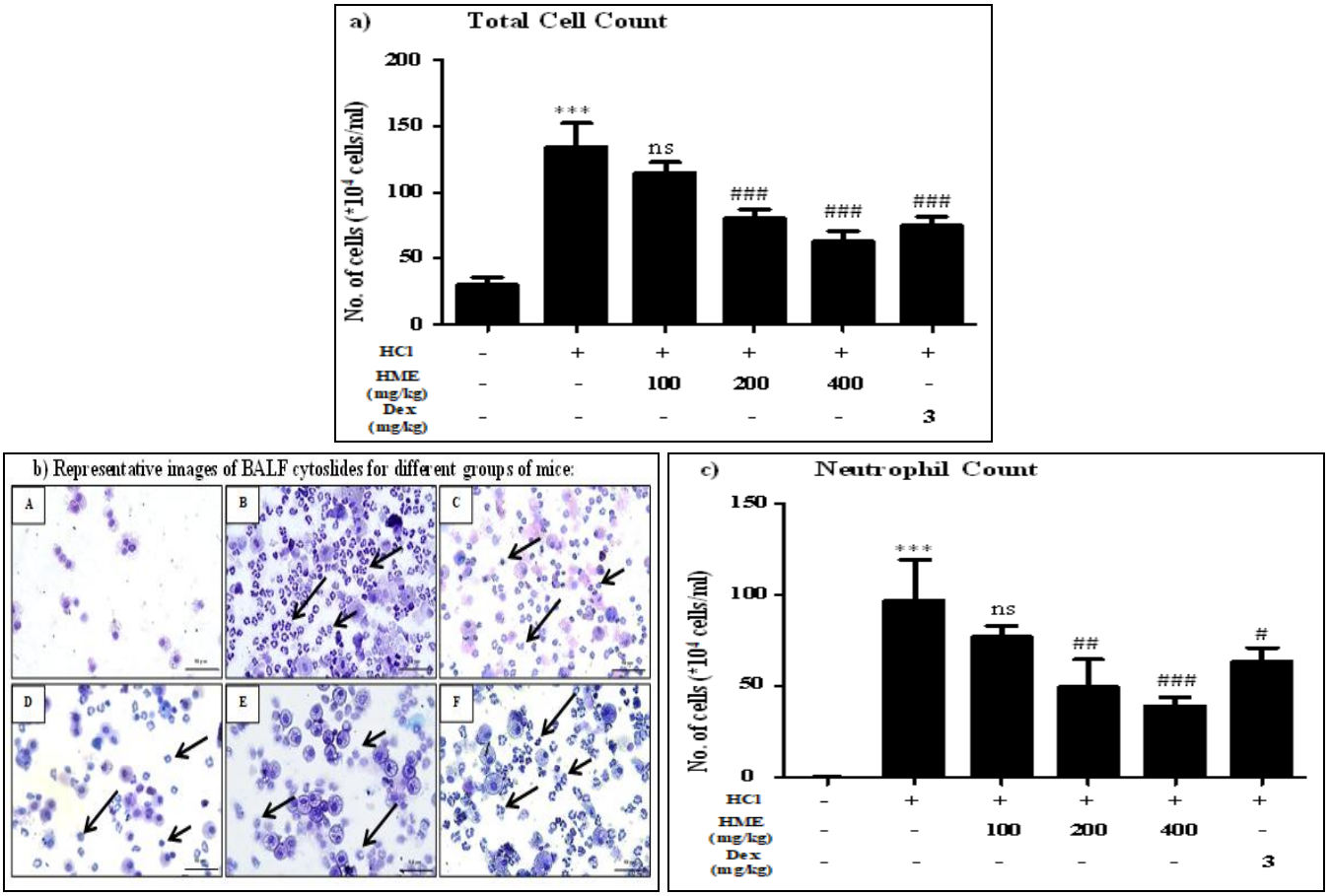

FIG. 1: DOSE-RESPONSE EVALUATION OF HME ON HCL-INDUCED ALI. Total cell count was done using a hemocytometer (a). The cytolsides were prepared and observed under a microscope at 400X magnification (Scale bar- 50 µm) (b). Differential cell count was done by counting the cells from the representative images of BALF slides (c). Results are depicted as Mean±SD of 5 animals in each group. ***p<0.001 *vs* Control, $^{###}$p<0.001 *vs* HCl group, ns- non-significant

Antioxidant Capacities of Different Extracts: Solvent partitioning is a technique used in drug discovery and development from crude extracts, which works on the principle 'like dissolves like', where different classes of phytochemicals can be extracted based on the polarity of the solvents.

The HME of *Randia dumetorum* fruits was partitioned using ethyl acetate, and the resultant fractions obtained were termed EASF and EAISF **Fig. 2.** Plant products are known to have anti-inflammatory properties due to the presence of a large number of antioxidants.

The antioxidant assays like FRAP, DPPH and ABTS are commonly used to evaluate the antioxidant capacities of crude plant extracts.

We analyzed the antioxidant capacities of HME, EASF, and EAISF to examine if antioxidants present in *Randia dumetorum* fruits can explain its anti-inflammatory action.

The results showed a significant difference between the antioxidant capacities of both EASF and EAISF depicted by standard antioxidant assays [FRAP ((***p<0.001; two-fold change), DPPH [(***p<0.001; two-fold change) and ABTS ((***p<0.001; three-fold change)] with EASF presenting higher antioxidant capacity w.r.t. EAISF **Table 2**.

Hence, as per our data, EASF had more antioxidant capacity in comparison to EAISF.

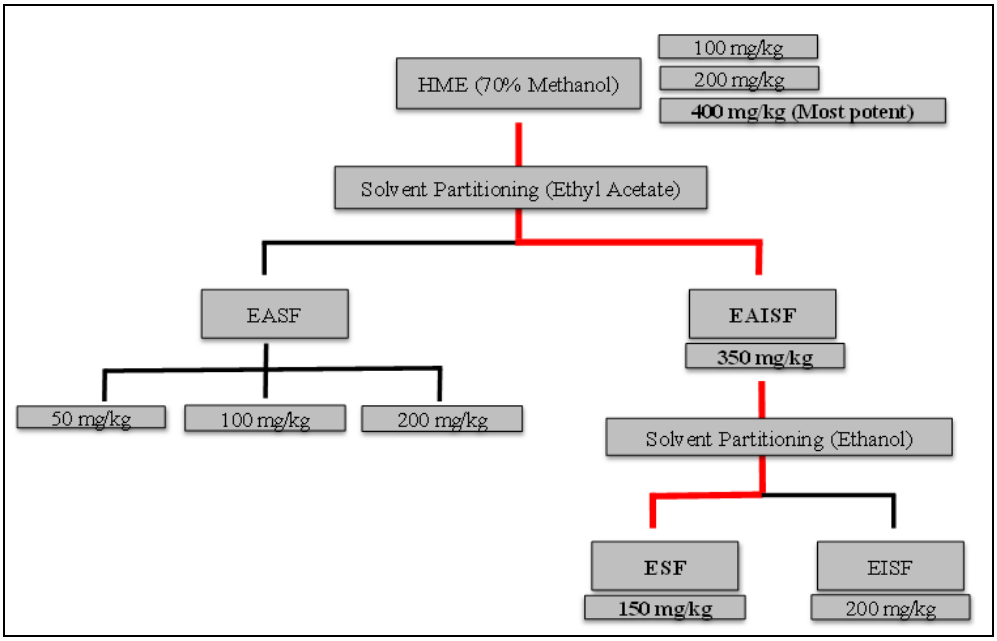

FIG. 2: SCHEME OF EXTRACTION AND FRACTIONATION OF *RANDIA DUMETORUM* FRUITS

TABLE 2: *IN-VITRO* ASSAYS FOR DIFFERENT EXTRACTS/FRACTIONS

A. Antioxidant assays			
Assay	**Hydromethanol Extract**	**Ethyl Acetate Soluble Fraction**	**Ethyl Acetate Insoluble Fraction**
FRAP assay (mM AAE/gm of DE)	948.6 ± 118.8	1504 ± 206.34	878.2 ± 103.3***
DPPH assay (µg/ml)	162 ± 5.06	96.33 ± 2.8	215 ± 8.42***
ABTS assay (µg/ml)	103.43 ± 4.23	27.30 ± 3.12	99.24 ± 5.39***
B. Total Phenolic and Flavonoid content			
Total Phenolic content (mg GAE/gm of DE)	72 ± 9.8	158.4 ± 13.65	54.92 ± 6.74***
Total Flavonoid content (mg QE/gm of DE)	4.52 ± 0.60	3.9 ± 0.28	4.56 ± 0.42^{ns}

The values are depicted as Mean ± SD (n=3). ***p<0.001 *vs* EASF.

Total Phenolic and Flavonoid Content: Next, we carried out the quantitative evaluation of the most common phytochemicals that are known to exert antioxidant properties i.e., phenols and flavonoids. Our data showed that EASF has higher phenolic content than EAISF (***p<0.001; threefold), whereas flavonoid content showed no significant difference among both the fractions **Table 2**. Hence, it appears that the phenolic content present in the extract/fractions might explain the higher antioxidant capacity of EASF.

Anti-inflammatory Activity of EASF and EAISF: Next, we conducted a comparative analysis of EASF and EAISF for their respective anti-inflammatory properties by analysis of total cells as well as neutrophils in BALF upon HCl administration. Much to our surprise, EAISF suppressed the HCl-induced lung inflammation more potently than EASF despite having lesser phenolic content and subsequent antioxidant capacity. Hence, we hypothesized that the dose of EASF that was administered (50 mg/kg b.wt) used by us might be low for exerting its anti-inflammatory action under *in-vivo* conditions. Therefore, we further performed the dose-response studies with EASF to check the anti-inflammatory effect at higher doses. Surprisingly, EASF did not show any improvement in anti-inflammatory action (depicted by total cell and neutrophil count) despite increasing its dose upto 200 mg/kg (data not shown). These results indicate that the sole presence of antioxidants might not be responsible for the anti-inflammatory action of *Randia dumetorum* fruit. There might be an additional

active ingredient/metabolite that may provide a beneficial effect independent of antioxidants. Hence, we decided to proceed with EAISF for further analysis following the Bioactivity-guided approach to find the potential bioactive component.

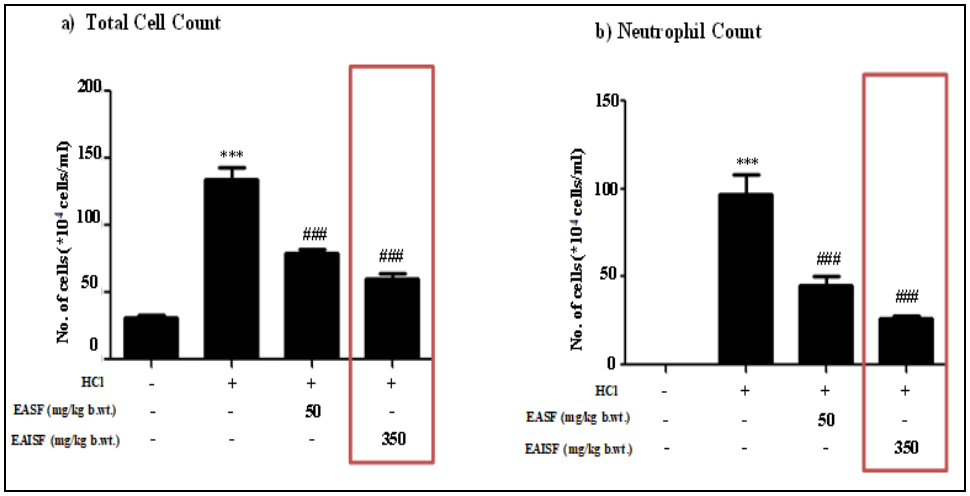

FIG. 3: ANTI-INFLAMMATORY EFFECT OF EASF AND EAISF ON HCL-INDUCED ALI. Total cell count was done using a hemocytometer (a). Differential cell count was done by counting the cells from the representative images of BALF slides (b). Results are depicted as Mean±SD of 5 animals in each group. ***p<0.001 *vs* Control, ###p<0.001 *vs* HCl group.

Anti-inflammatory activity of ESF and EISF: Further, partitioning of bioactive ethyl acetate insoluble fraction (EAISF) was done using ethanol to get ethanol-soluble (ESF) and an ethanol-insoluble fraction (EISF) to separate phytoconstituents based on differential polarity **Fig. 2.** HCl administration significantly increased both total cells and neutrophil count w.r.t control group (***p<0.001) **Fig. 4A, 4B**. Interestingly, administration of ESF at a dose of 150mg/kg b.wt. significantly suppressed the HCl-induced infiltration of inflammatory cells, particularly neutrophils in the lungs (***p<0.001) **Fig. 4A, 4B**. On the other hand, administration of EISF at a dose of 200mg/kg b.wt. failed to ameliorate the HCl-induced ALI as reflected by a modest reduction in total cells as wells as neutrophils in lungs **Fig. 4.**

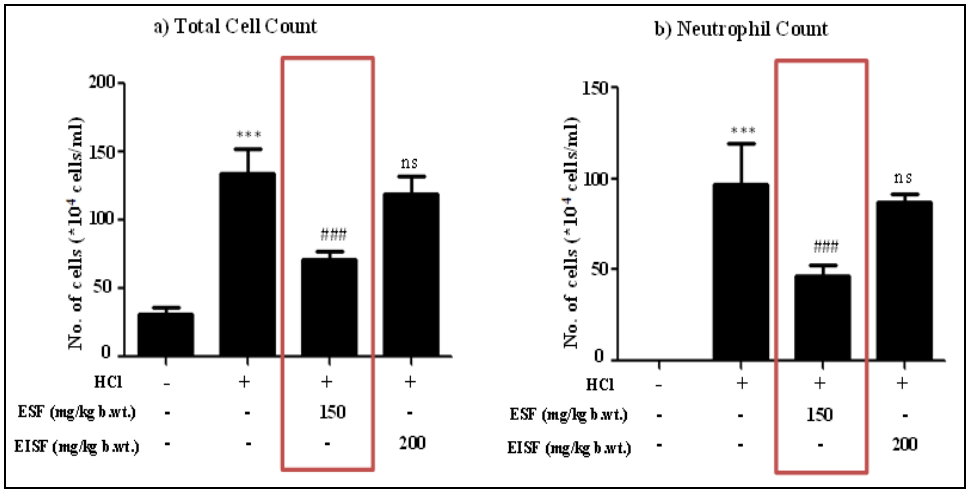

FIG. 4: ANTI-INFLAMMATORY EFFECT OF ESF AND EISF ON HCL-INDUCED ALI. Total cell count was done using a hemocytometer (a). Differential cell count was done by counting the cells from the representative images of BALF slides (b). Results are depicted as Mean±SD of 5 animals in each group. ***p<0.001 *vs* Control, ###p<0.001 *vs* HCl group, ns-non-significant.

Phytochemical Screening: We next conducted the phytochemical screening of bioactive fractions to know about the presence/absence of different classes of phytoconstituents **Table 2**. The bioactive ESF showed the presence of saponins and glycosides in abundance and a moderate presence

of flavonoids, phenols, carbohydrates, and terpenoids/steroids. There is a vast literature present on the medicinal importance of these phytochemical classes. Hence, it is difficult to comment on the exact nature of the bioactive ingredient (s) that might be responsible for exerting anti-inflammatory action at this stage. Therefore, further fractionation of bioactive ESF is required to unveil the active compound (s).

TABLE 3: PHYTOCHEMICAL ANALYSIS OF BIOACTIVE EXTRACT/FRACTIONS

Class of Phytochemicals	HME	EAISF	ESF
Alkaloids	-	-	-
Tannins	++	++	-
Flavonoids	++	+	+
Saponins	++	++	++
Phenols	++	+	+
Carbohydrates	+	+	+
Proteins	+	+	-
Terpenoids/ Steroids	++	+	+
Glycosides	++	++	++

Absence (-), Moderate presence (+), Abundant presence (++).

CONCLUSION: Foremost, the present study results validate the traditional use of *Randia dumetorum* fruit in the treatment of respiratory complications. In addition, HME/EAISF/ESF of *Randia dumetorum* fruit possess significant anti-inflammatory activity, which might not necessarily be because of medicinal plants' inherent standard antioxidant capacity.

Our data strongly suggest that ESF constitutes the active ingredient exhibiting anti-inflammatory properties devoid of antioxidant action. However, further studies are required to isolate the active ingredient(s) present in ESF of *Randia dumetorum* fruit that can be used as a treatment drug for ALI and other inflammatory pathologies.

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CONFLICTS OF INTEREST: The authors report no conflicts of interest.

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