Research Article

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Anti-asthmatic activity of Saudi herbal composites from plants *Bacopa monnieri* and *Euphorbia hirta* on Guinea pigs

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Abstract: Asthma, the respiratory disorder associated with bronchial hyper-responsiveness, affected 300 million people across the globe, with a prevalence of 4.05% in Saudi Arabia and causing 61.6% of hospital emergency room annual visits. Increased side effects of conventional drugs demand the necessity for the development of natural drugs. In this study, an herbal composite from *Bacopa monnieri* and *Euphorbia hirta* was prepared and characterized by Fourier transform infrared spectroscopy (FT-IR) and gas chromatography-mass spectrometry (GC-MS) analysis. *In vitro* bacterial inhibition and anti-asthmatic activity were evaluated using animal models. Ethanolic herbal composite (EHC) showed significant anti-pathogenic activities. GC-MS analysis identified potential bioactive compounds and FT-IR analysis revealed functional groups corresponding to plant composites. The EHC increased the preconvulsive time against 1% histamine aerosol compared to control animals. In sensitized + EHC-treated animals, total leukocyte, eosinophil, lymphocyte, neutrophil, and monocyte counts were found to be reduced as compared to sensitized and control groups. EHC decreased malondialdehyde and bicarbonate levels denoting the reduced oxidative burden and increased the antioxidant activity by increased intracellular glutathione (GSH) level. The EHC-treated group showed decreased inflammatory cell infiltration compared to the sensitized. A significant anti-asthmatic effect was observed in the EHC-treated group ($P < 0.05$). Thus, herbal composites are used in the treatment of asthma and can be used as an alternative to commercially available pharmaceutical drugs.

Keywords: anti-asthmatic activity, *Bacopa monnieri*, *Euphorbia hirta*, Saudi herbal composites, antibacterial activity

1 Introduction

Asthma is a life-threatening enduring respiratory disorder, resulting in the inflammation and narrowing of airways. Exacerbation includes bronchospasm, edema, mucus secretion, cellular infiltration, and damage of the airway epithelium [1]. Asthma is associated with wheezing, breathlessness, chest tightness, and coughing brought about by inflammation, bronchial constriction, and excessive mucus secretion due to bronchial hyperresponsiveness [2]. The occurrence of asthma worldwide is found to be around 300 million people [3]. An asthma attack is estimated to be around 11% among children and 6% among adults worldwide. In Saudi Arabia, asthma is the 19th disability-adjusted life years and 26th in causing mortality [4]. Different researchers have found that the prevalence of asthma in Saudi Arabia is more than 4% with a higher rate of asthma attack [5,6]. The children in Saudi Arabia (with a prevalence rate ranging from 8 to 25% in various parts of the Kingdom, reported by different researchers) are at a comparatively higher risk [7], but the ground reality is that asthma still has a high prevalence with an increasing pattern and many of the chemical drugs used in the treatment of asthma in Saudi Arabia have been found to have side effects [7,8].

Pathologically, the development of asthma is characterized by the liberation of Th2 or Th1 cells against specific allergens leading to the production of cytokines,

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such as IL-4, IL-5, IL-9, and IL-13, with an accumulation of collagen deep in the epithelium, resulting in the hyperplasia of the structural elements of all the cells. Interleukins are responsible for the production of immunoglobulin E which binds to mast cells in the airway mucosa and stimulates the release mediators such as histamine, tumor necrotic factor-α, chymase, prostaglandins, leukotrienes C4 and D4, prostaglandin D2, tryptase and amphiregulin [9]. These mediators diffuse through the airway mucosa triggering the bronchial smooth muscle spasm, increased mucus secretion, and vasodilation causing obstruction in the airways. Edema, scarring, and fibrosis buildup lead to thickened basement membranes, which tend to be irreversible [5].

Even though the effect of asthma on Covid-19 patients is not fully understood [10], several scientific investigations are on the way. Some of these studies have concluded that the incidences of Covid-19 have slightly increased in asthma patients [11] and asthma does not create a remarkable burden on Covid-19 patients as some virus infections do [12]. At the same time, an investigation [13] conducted in the Kingdom of Saudi Arabia found that asthmatic patients with Covid-19 infections showed a higher ICU admission rate and oxygen supply requirement compared to other Covid-19 patients.

Pharmaceutical drugs contribute to the major treatment for asthma given to patients worldwide. The increased number of asthma patients in every country led to drug unavailability, expensive drug prices, and exorbitant [14]. The commonly used medications for asthma are inhaled corticosteroids, short- and long-acting beta antagonists, oral steroids, leukotriene modifiers, mast cell stabilizers, and immunomodulators. Though they possess many side effects on treatment, there is a need for a search for an alternative to conventional drugs in the treatment of asthma [15]. Ayurveda, a traditional practice of medicine from India, has described medicinal plants in the treatment of asthma. Using medicinal plants in the treatment of asthma is considered to be natural and safe [16]. Many plant species, such as *Rehmannia glutinosa* Libosch, *Guiera senegalensis*, *Alisma orientale* (Sam.) *Juzepcz*, *Atractylodes ovate*, *Euphorbia thymifolia* L., *Paeonia suffruticosa* Andr, *Glycyrrhiza glabra*, *Zingiber officinale*, *Euphorbia thymifolia*, *Lablab purpureus*, *Coix lacryma-jobi*, *Psoralea corylifolia*, *Schisandra chinensis*, *Ziziphus jujube* Mill, *Ophiopogon japonicus tuber*, *Citrus aurantifolia*, and many other plant species, are used in the formulation of drugs against asthma [17]. Such medicinal plants possess various phytochemical compounds, which could result in the treatment of various diseases and infections [18].

Euphorbia hirta a pantropical weed belonging to the plant family Euphorbiaceae is a hairy herb found on roadsides and pathways in India. The bioactive compounds present in *E. hirta* are found to be quercitrin, afzelin, kaempferol, protocatechuic acid, and gallic acid. The other phytochemicals present are flavonoids, quercitrin, sterols, 24-methylene-cycloartenol, β-sitosterol, triterpene, β-amyrin, and myricitrin, which are responsible for the anti-inflammatory effect. Tannins and tannic acid present in plant extracts are considered to possess antiseptic effects. The triterpenoids, taraxerone, and 11α,12α oxidotaraxerol also exhibit anti-microbial activity against various bacterial and fungal species [19]. *Bacopa monnieri* (water hyssop) is a perennial herb used in traditional Ayurveda for various ailments. Bioactive compounds present in *B. monnieri* are tannin, saponin, flavonoid, phenol, alkaloid, and phlobatannin, which are responsible for antifungal and antibacterial activities. Recent studies have described that *B. monnieri* also possesses anti-inflammatory activity, inhibits mast cell degradation, and thus can be used in the treatment of asthma [20]. Several studies investigated the anti-inflammatory and anti-asthmatic activities of *E. hirta* [21,22] and *B. monnieri* [23,24]. They significantly reduced the pro-inflammatory cytokine expressions compared with the commercial drugs. Thus, the present study concentrates on the synergistic activity of the *E. hirta* and *B. monnieri* extracts against bronchoconstriction and bronchospasm. Based on the literature review, this is the first reported work on the anti-asthmatic activity of the *E. hirta* and *B. monnieri* composite extracts in guinea pigs. The herbal composite was prepared by the ethanolic extracts of *E. hirta* and *B. monnieri*. Its bacterial resistance, histamine-induced bronchoconstriction, eosinophil and macrophage counts in the bronchoalveolar fluid, serum bicarbonate level, malondialdehyde (MDA) level, intracellular glutathione level, total protein level, and histopathology studies were evaluated.

2 Materials and methods

2.1 Collection of plants and extraction and preparation of herbal composite

The two selected herbs (*E. hirta* and *B. monnieri*) were collected from the territory desert of Riyadh. The ethanolic herbal composite (EHC) was prepared by extracting 50 g of each medicinal herb (leaves) with 125 mL of ethanol

using the Soxhlet extraction apparatus for 8 h. The extract was evaporated to dry using a hot air oven kept at 50°C. The obtained powder was stored in a cool dry place. For the preparation of the composite: two selected herbal extracts were mixed in equal ratios (1:1) and used for further studies.

2.2 Gas chromatography-mass spectrometry (GC-MS) analysis of plant extracts

GC-MS analysis of ethanol extracts of *B. monnieri* and *E. hirta* was performed using Agilent GC 7890A/MS5975C. The capillary column used was Agilent DB5MS. The column length used for the analysis was 30 m/0.25 mm internal diameter, 0.25 µm film thickness. One microliter of extracts were injected into the instrument and the temperature was raised as follows: 60°C for 2 min; followed by 300°C at the rate of 10°C·min^{-1}; and 300°C, for 6 min. Mass spectra were taken at a scanning interval of 0.5 s. The spectrum with the retention time, percentage composition, and peak area was given. The components were identified by using GC-MS NIST (2008) library.

2.3 Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR analysis was utilized to identify the functional groups corresponding to the plant extracts. The analysis was carried out in a KBr disk medium interconnected to a spectrometer with a resolution of 4 cm^{-1} between the range of 400 and 4,000 cm^{-1}. The plant extracts and composites were analyzed. The emission spectrum was recorded under a fluorescence spectrophotometer, and the corresponding functional groups identified were reported.

2.4 Microbial resistivity of EHC

From the resultant herbal composite extract, three different concentrations (1×, 2×, 3×) were prepared to evaluate the antibacterial activity. The antibacterial activity of different herbal composite ratios was evaluated against the significant organisms (*Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Acinetobacter* spp., and *Klebsiella pneumoniae*) by well diffusion method. About 50 µL of each prepared composite in 5% dimethyl sulfoxide (Sigma-Aldrich) was loaded into the well. The plates were incubated at 37°C for 24–48 h. The antibacterial activity was estimated with respect to the zone of inhibition around the wells of each extract in all the inoculated nutrient agar plates. The inhibition zones were measured and recorded in millimeters.

2.5 Anti-asthmatic activity

2.5.1 Experimental animals

Adult *Cavia porcellus* (guinea pigs) weighing from 400 to 450 g was obtained from a local bird and animal market at Aziziya in Riyadh. The animals were housed in polypropylene cages at 25 ± 2°C with an 8 h light/8 h dark condition. The animals were fed with standard diet and water (carrot with additional vitamin C). The work was carried out according to the ethical guidelines of the Department of Pharmacology and Toxicology, College of Pharmacy, Prince Sattam bin Abdulaziz University, Al-Kharj, Kingdom of Saudi Arabia (Bioethics Committee, Prince Sattam Bin Abdulaziz University; No: 20/2009/002). About 21 guinea pigs were used for the present study and they were divided into seven groups (three guinea pigs in a group) randomly. Groups I, II, III, and IV were used for the analysis of histamine-induced bronchoconstriction, and Groups V, VI, and VII were used for the ovalbumin (OVA)-induced bronchospasm studies.

2.5.2 Histamine-induced bronchoconstriction

The *in vivo* anti-histaminic activity of EHC was studied by inducing bronchospasm using histamine aerosols in guinea pigs [25]. Guinea pigs (Groups I, II, III, and IV) selected for this study were fasted for 24 h. Then, all the test animals were exposed to 1% histamine (Sigma-Aldrich) aerosol at a pressure of 300 mmHg using a nebulizer (Kent Scientific Corporation, Torrington, CT, USA) in an airtight chamber of dimension 24 cm × 14 cm × 24 cm. On exposure to 1% histamine, animals showed preconvulsive dyspnea (PCD). The duration of onset of PCD is considered preconvulsive time (PCT). After the onset of PCD, the animals were transferred to fresh air. This PCT was recorded as a 0th-day value. After 15 days, Group I was given 2 mL·kg^{-1} of normal saline, Group II was given 2 mg·kg^{-1} of chlorpheniramine (Sigma-Aldrich), Group III was given 100 mg·kg^{-1} of EHC, and Group IV was given

200 mg·kg^{-1} of EHC by oral administration. PCD was recorded after 2 h of administration using the following formula:

$$\text{Percent increase PCD (\%)} = (1 - T_1/T_2) \times 100 \quad (1)$$

where T_1 is the duration for PCD on the 0th day and T_2 is the duration for PCD after the 15th day.

2.5.3 OVA-induced bronchospasm

Guinea pigs of Groups V, VI, and VII were used for the induction of bronchospasm. Group V was given normal water (control group), Group VI was given OVA (sensitized group), and Group VII was given EHC (200 mg·kg^{-1}). The guinea pigs of other groups (Groups VI and VII) were sensitized orally with OVA (Sigma-Aldrich) (1 mL, 10% w/v). The animals of Group VII were given ethanolic herbal extract (200 mg·kg^{-1}) orally for up to 15 days along with OVA. On the 15th day, all the test animals were given OVA orally (0.5 mL, 2% w/v) except Group I animals. After 3 h of the OVA administration, the animals were anesthetized with sodium pentobarbital (Sigma-Aldrich) (50 mg·kg^{-1}) ip [26]. The animals were sacrificed, and the bronchia was removed from the animals and washed with 15 mL of saline. The washed fluid is collected in a sterile container and centrifuged (Fisherbrand™ centrific model 225, Waltham, MA, USA) at 2,000 rpm for 5 min. Pellet was separated and dissolved in 0.5 mL of saline. Giemsa stain (0.2 mL) in buffered saline was added to the resuspended pellets.

2.6 Measurement of eosinophil, macrophages, serum bicarbonate, MDA, GSH, and protein levels

About 0.5 mL of the obtained fluid was observed under a microscope for leucocyte count (450× magnification). The results were recorded and compared with the other two groups (Groups V and VI). The specimen required for the estimation of bicarbonate level is serum. The complete blood of the animals was collected and should be tested within 1 h as they remain stable only for 1 h after collection. Bicarbonate level of serum was performed as described by Zilva and Pannall [27]. About 1 mL of lung tissue was added to 10 mL of ice-cold phosphate buffer and homogenized. MDA level of lung tissue was described by Buege and Aust. The amount of MDA was estimated and recorded [28]. GSH level and total protein level were calculated by the procedures described by Prajapati et al. [29]. Results are recorded in GSH mg^{-1} protein for GSH level and mg·mL^{-1} for proteins.

2.7 Histopathology of lung

The lungs of the test animals (Groups V, VI, and VII) were isolated after the collection of BALF and placed in 10% formaldehyde (HCHO). A microtome is used to cut the samples (paraffin-embedded lungs) into thin sections of 5 mm thickness. Later, the sections were stained using hematoxylin and eosin dyes and the sections were observed under a microscope for histopathological changes [30]. The histopathological slides were examined under a light microscope (Olympus BX51, Tokyo, Japan) by a pathologist who was unaware about the treatment that each of the animals had received. The pathological slides were graded histopathologically according to the severity of lung damage by using a modified scoring system [31]. In this scoring system, a leukocyte infiltration score was given between 0 and 4 (Table 1).

2.8 Statistical analysis to determine the anti-asthmatic effect of EHC on animal models

As all the tests were performed with three different animals of the same group, results were recorded and expressed in terms of mean ± standard deviation. A Chi-square non-parametric test using SPSS-9 for Windows 7 was used as a statistical tool to determine the anti-asthmatic effect of EHC on animal models. The hypothesis selected (H_0) was that "there is a significant anti-asthmatic effect on EHC treated group." The difference in the eosinophil and macrophage counts, serum bicarbonate level, protein, MDA, and GSH levels between the sensitized group

Table 1: Histopathological lung leucocyte infiltration according to modified scoring system

No.	Leucocyte infiltration	Score
1	Less than 10 cells	0
2	At least 10 cells	1
3	At least 25 cells	2
4	At least 50 cells	3
5	Over 75 cells	4

and the EHC treated group was statistically calculated with $P < 0.05$ considered significant.

3 Results

3.1 GC-MS analysis of plant extracts: bioactive compounds present in the plant extracts

E. hirta and *B. monnieri* were identified by using GC-MS analysis. Figures 1 and 2 show the GC-MS spectrum of the *E. hirta* and *B. monnieri* ethanolic extract. The compounds present in the plant extracts with their retention time are shown in Tables 2 and 3. About 26 bioactive compounds were identified from the GC-MS analysis of *E. hirta* and 19 from *B. monnieri*.

3.2 FT-IR of the plant composite

FT-IR analysis of plant composites revealed the functional groups corresponding to the plant extracts. Figure 3 shows the FT-IR spectrum of the composites. The medium band at 3309.85 cm^{-1} represents the N–H symmetric and asymmetric stretching of the amide functional groups. Weak bands at 2947.23, 2831.50, and 1651.07 cm^{-1}

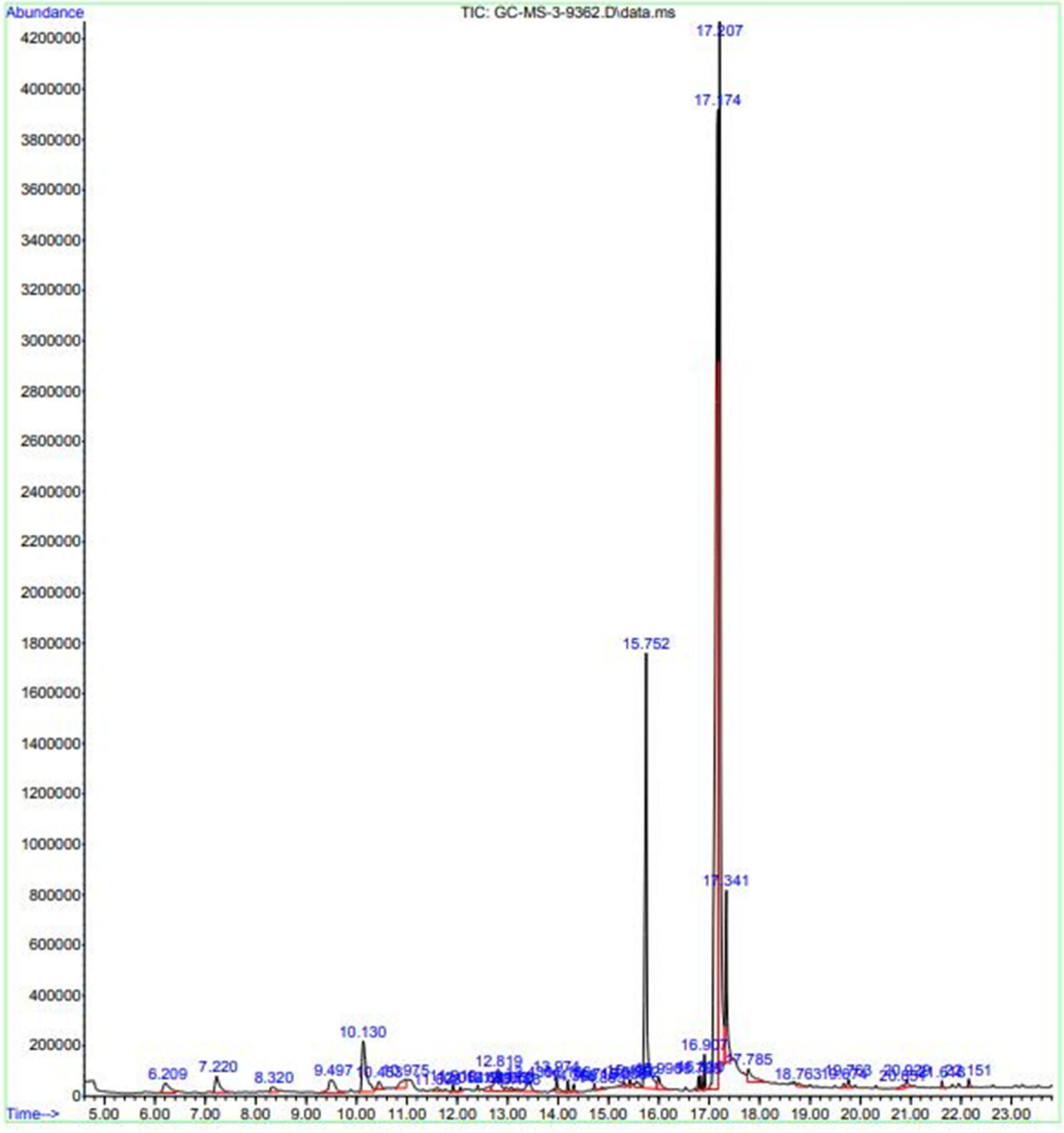

Figure 1: GC-MS spectrum of *E. hirta* ethanolic extracts.

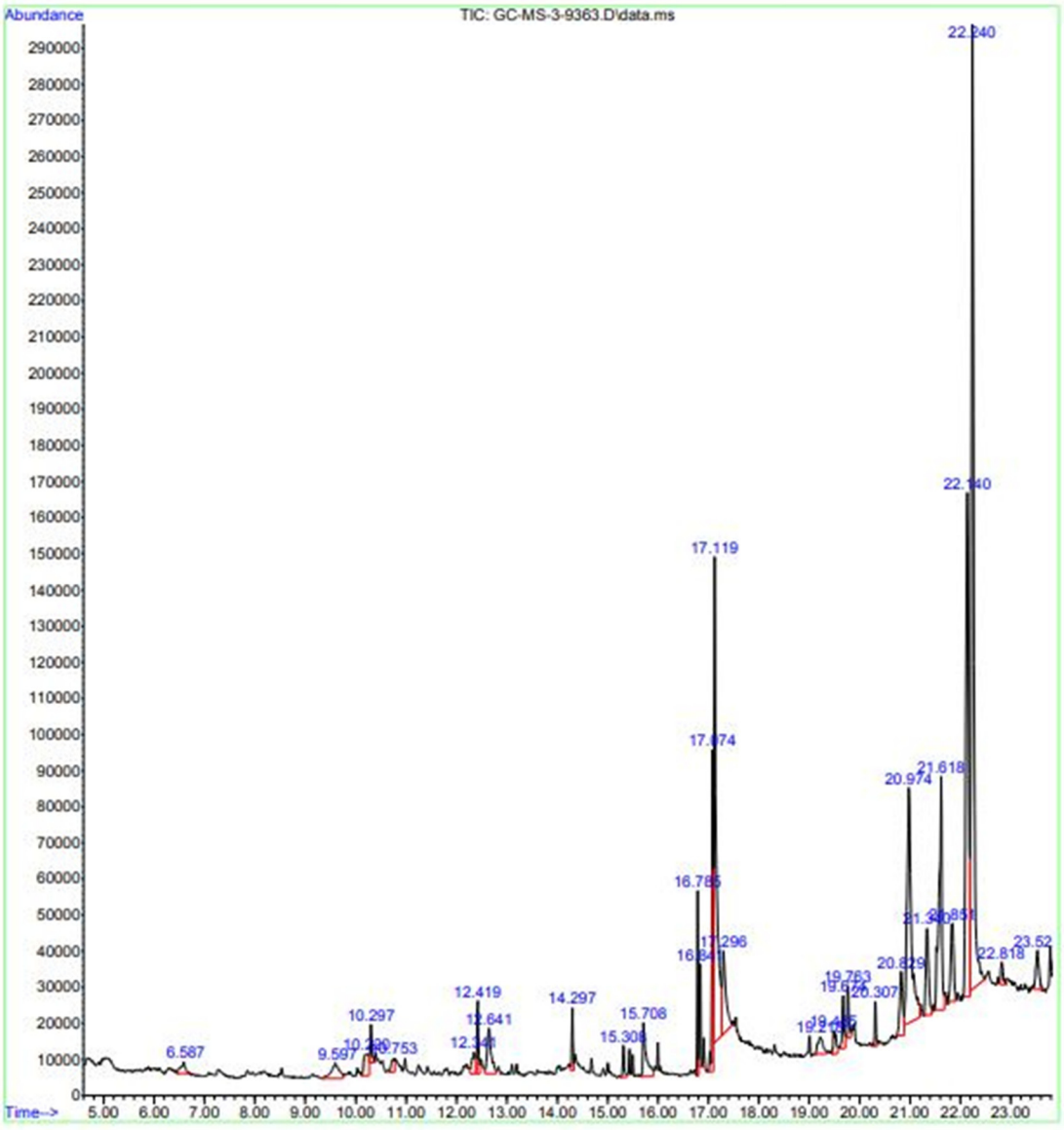

Figure 2: GC-MS spectrum of *B. monnieri* ethanolic extracts.

correspond to C–H stretch alkane compounds, O–H stretch carboxylic acids, and C=C stretch alkenes, respectively. Sharp absorption bands at 1450.47 and 1111.00 cm^{-1} correspond to C=C stretch aromatic compounds and C–O stretch alcohols, respectively. A very strong band at 1018.41 cm^{-1} shows the C–F stretch alkyl fluorides. The medium peak at 671.23 cm^{-1} represents alkynes of ≡C–H bend. The weak band at 555.50 cm^{-1} is due to the presence of alkyl bromide (C–Br) stretching. The observation of two alkyl halides is due to the composite formation of plant extracts (Table 4).

3.3 Microbial resistance of EHC

Among the given concentration, the 3× concentrates showed more significant antibacterial activity than the other two concentrates. A maximum zone of inhibition was observed against *E. coli* and *S. aureus* (31 and 30 mm, respectively). About 28 mm was observed against *E. aerogenes* and *Acinetobacter* sp. Herbal composites showed 27 mm of inhibition zone against *K. pneumoniae*. The zone of inhibition of three concentrates of EHC is presented in Table 5.

Table 2: GC-MS analysis of *E. hirta*

No.	Compound name	Retention time
1	4*H*-pyran-4-one,5-hydroxy-2-methyl	6.209
2	4*H*-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	7.220
3	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	8.320
4	Phthalic anhydride	9.497
5	1,2,3-Benzenetriol	10.130
6	α-[Di-*n*-butylamino methyl]-2-ethoxy-4-quinoline methanol	10.453
7	5-Fluoro-6-methyl-5-hepten-2-one	10.975
8	Pyrazole-5-carboxylic acid, 3-methyl	11.608
9	2(4*H*)-benzofuranone,5,6,7,7*a*-tetrahydro-4,4,7*a*-trimethyl-	11.919
10	3-Deoxy-D-mannoic lactone	12.641
11	1,2,3,5-Cyclohexanetetrol,(1. α.,2. β.,3. α.,5. β.)	12.819
12	4-*O*-β-D-Galactopyranosyl-D-glucopyranose	12.963
13	2-Amino-4-hydroxypyrrolo[2,3-D]pyrimidine	13.063
14	1,2,3-Thiadiazole-4-carboxylic acid, hydrazide	13.186
15	2,2-Diethoxytetrahydrofuran	13.430
16	5-Ethylcyclopent-1-ene-1-carboxylic acid	14.186
17	2-Pentadecanone, 6,10,14-trimethyl	14.719
18	D-chiro-inositol, 3-*O*-(2-amino-4-((carboxyiminomethyl)amino)-2,3,4,6-tetradeoxy- α-D-arabino-hexo	15.308
19	Inositol	15.430
20	Neo-inositol	15.552
21	5-Eicosene, (*E*)-	15.996
22	Methyl 7,12-octadecadienoate	16.785
23	10-Octadecenoic acid, methyl ester	16.830
24	Phytol	16.907
25	*Cis*-vaccenic acid	17.207
26	3-(3,4-Dimethoxyphenyl)propylamine, PFP	22.151

Table 3: GC-MS analysis of *B. monnieri*

No.	Compound name	Retention time
1	3-Acetoxy-4-cyano-2,5-dimethylpyri dine	10.753
2	Phenylethyne	12.341
3	2-Tetradecene, (*E*)-	12.419
4	Octane, 1,1′-oxybis-	12.641
5	Trichloroacetic acid, tetradecyl ester	14.297
6	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	15.308
7	9,12-Octadecadienoic acid (*Z*,*Z*)-	17.296
8	Cholest-2-ene-2-methanol, (5.α)-	19.218
9	1,*E*-6,*Z*-11-hexadecatriene	19.485
10	1-Octadecanesulphonyl chloride	19.674
11	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	19.763
12	Behenyl chloride	20.307
13	2-Myristynoyl-glycinamide	20.829
14	γ-Sitosterol	20.974
15	17-OH-pregnenolone 17-α-hydroxypregnenolon	21.340
16	2,6,10,14,18,22-Tetracosahexaene	21.618
17	9,19-Cyclolanost-24-en-3-ol, ta.)-	22.140
18	Lupeol	22.240
19	1,2-Bis(trimethylsilyl)benzene	22.818

3.4 Histamine-induced bronchoconstriction

Treatment with EHC showed a significant delay in PCT on exposure to histamine compared to control normal animals. The percentage increase in PCT after treatment with 100–200 mg·kg^{-1} of EHC was observed as 47.36 ± 4.38% and 64.59 ± 2.94%, respectively, whereas the control group showed only 3.61 ± 2.50% in PCD on histamine aerosol (Table 6).

3.5 Eosinophil and macrophage counts

Significant differences in white blood cells were observed in three groups. There are increased total leukocyte, eosinophil, monocyte, polymorph, and lymphocyte counts in sensitized guinea pigs compared to the control group. EHC-treated animals showed a decrease in levels of total leukocyte, polymorph, eosinophil, monocyte, and lymphocyte counts compared to sensitized animals (Table 7).

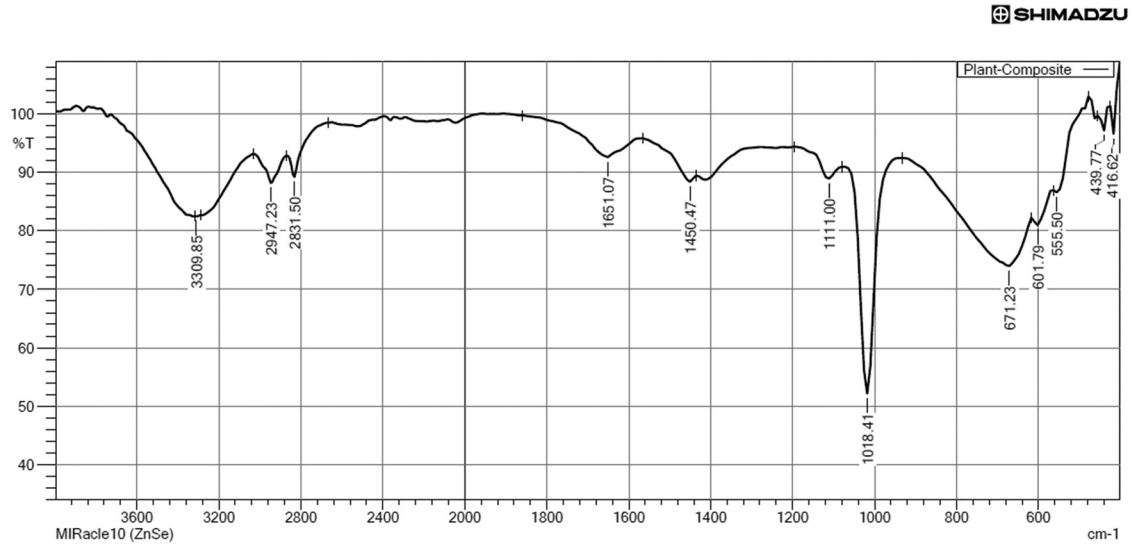

Figure 3: FT-IR spectrum of the plant composites.

Table 4: FT-IR characterization of the plant composite

No.	Absorption peaks	Functional group
1	3,309.85	N–H symmetric and asym. stretch
2	2,947.23	C–H stretch
3	2,831.50	O–H stretch
4	1,651.07	C═C stretch
5	1,450.47	C═C stretch
6	1,111.00	C–O stretch
7	1,018.41	C–F stretch
8	671.23	≡C–H bend
9	601.79	═C–H bend
10	555.50	C–Br

Table 5: Zone of inhibition of EHC against test organisms

No.	Test organism	Zone of inhibition (mm)		
		1×	2×	3×
1	*S. aureus*	22	25	30
2	*E. coli*	24	26	31
3	*E. aerogenes*	19	23	28
4	*Acinetobacter* sp.	21	25	28
5	*K. pneumoniae*	18	23	27

3.6 Serum bicarbonate level, protein, MDA, and GSH levels

Sensitized animals showed an increase in serum bicarbonate level (62.21 ± 0.2 mEq·L^{-1}) compared to the control group (38.36 ± 0.68 mEq·L^{-1}). The group treated with EHC showed a significant reduction in the serum bicarbonate level (35.73 ± 1.06 mEq·L^{-1}). Guinea pigs treated with EHC (0.072 ± 0.0026 mEq·L^{-1}) showed a decrease in the MDA level compared to the sensitized group (0.29 ± 0.0007). Similarly, the EHC-treated groups showed a decrease in levels of protein (7.55 ± 0.072) and GSH (16.87 ± 0.008) compared to protein (6.24 ± 0.041) and GSH (18.43 ± 0.002) levels in the sensitized group (Tables 8 and 9).

Table 6: Histamine-induced bronchoconstriction

No.	Treatment group	% Increase in time of PCD*
1	Control	3.61 ± 2.50
2	Chlorpheniramine	82.87 ± 1.63
3	100 mg·kg^{-1}	41.36 ± 4.38
4	200 mg·kg^{-1}	64.59 ± 2. 94

*All values are calculated using mean ± SD.

3.7 Histopathological study

No changes were observed in the normal control group (Figure 4). Massive infiltration of inflammatory cells in the interstitial space was observed indicating the airway inflammation in the sensitized group (Figure 5). The sensitized group also showed emphysema, hyperplasia, and necrosis. The EHC-treated group showed decreased inflammatory cell infiltration compared to the sensitized group (Figure 6). The treatment with the EHC prevented hyperplasia and bronchoconstriction in sensitized + EHC-treated animals (Group III). Score values were given for the leucocyte counts. Based on the score values, the EHC-treated group

Table 7: Eosinophil and macrophage count in the bronchoalveolar fluid

No.	Cell count	Normal group*	Pathological score#	Sensitized group*	Pathological score*,#	EHC-treated group* (200 mg·kg^{-1})	Pathological score#
1	TLC/cmm	6,912.17 ± 207.86	NA	16813.42 ± 467.33	NA	9348.12 ± 362.02[a]	NA
2	Polymorph count/cmm	22.25 ± 1.85	1 ± 0.0	40.74 ± 0.36	2 ± 0.0	33.2 ± 0.79[a]	2 ± 0.0
3	Lymphocyte count/cmm	46.18 ± 0.43	2 ± 0.0	54.36 ± 1.75	3 ± 0.0	49.26 ± 0.66[a]	2 ± 0.0
4	Eosinophil count/cmm	8.05 ± 0.16	0 ± 0.0	18.11 ± 0.47	1 ± 0.0	11.49 ± 0.27[a]	1 ± 0.0
5	Monocyte count/cmm	5.5 ± 0.72	0 ± 0.0	15.52 ± 0.44	1 ± 0.0	9.32 ± 0.76[a]	0.5 ± 0.3

cmm – cells per cubic millimeter.
*All values are calculated using mean ± SD.
#Pathological score described by Yamanel et al. [33].
[a]$P < 0.05$ – significant difference in comparison with the sensitized group.

Table 8: Serum bicarbonate level and protein levels

No.	Treatment group	Serum bicarbonate level* (mEq·L^{-1})	Protein* (mg·mL^{-1})
1	Normal control group	38.36 ± 0.68	6.24 ± 0.041
2	Sensitized group	62.21 ± 0.2	10.08 ± 0.026
3	EHC treated (200 mg·kg^{-1})	35.73 ± 1.06	7.55 ± 0.072

*All values are calculated using mean ± SD.

showed a significant decrease in leucocytes, which are found to be active in inflammation. Detailed pathological scores and comparisons between groups are shown in Table 7.

3.8 Statistical analysis to determine the anti-asthmatic effect of EHC on animal models

The difference in the eosinophil and macrophage counts, serum bicarbonate level, protein, MDA, and GSH levels between the sensitized group and the EHC treated group was taken as the statistical experimental design. The hypothesis selected was "there is a significant anti-asthmatic effect on EHC treated group." The difference in the eosinophil and macrophage counts, serum bicarbonate level, protein, MDA, and GSH levels between the sensitized group and the EHC-treated group was statistically calculated with $P < 0.05$ considered significant. For all the data, the values of the EHC treated group (Tables 7–9) were less than that of the sensitized group, which confirmed the anti-asthmatic effects of EHC on the treated group of animals. Hence, the assigned hypothesis was accepted ($P < 0.05$).

4 Discussion

This study attempts to establish the anti-asthmatic property of an ethanol leaf extract of *E. hirta* and *B. monnieri* composite. As the anatomy of the airways, anaphylactic bronchoconstriction on antigen challenge, and inflammatory mediator responses are similar to humans, Guinea pigs are used for histopathological works [32]. The process of inflammation in asthma is classified into seven phases: sensitization, stimulation, cell signaling, migration, cell activation, tissue damage, and resolution. In

Table 9: MDA and GSH levels

No.	Treatment group	MDA level* (μg·mg^{-1}/protein)	GSH level* (μg·mg^{-1}/protein)
1	Normal control group	0.089 ± 0.0013	18.43 ± 0.002
2	Sensitized group	0.29 ± 0.0007	5.7 ± 0.032
3	EHC treated (200 mg·kg^{-1})	0.072 ± 0.0026	16.87 ± 0.008

*All values are calculated using mean ± SD.

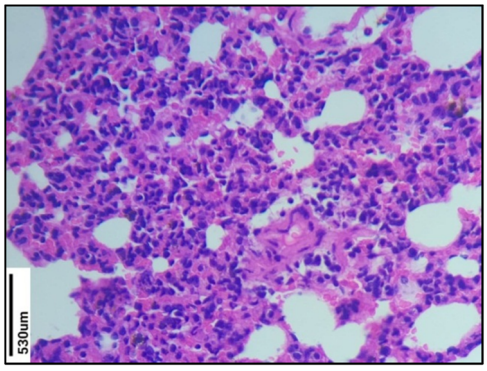

Figure 4: Histopathological study of Group V lung section (control group). No changes were observed in the normal control group.

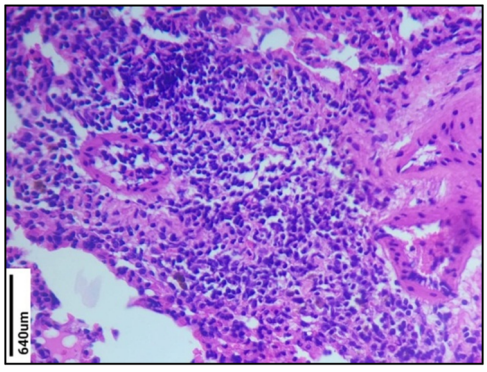

Figure 5: Histopathological study of Group VI lung section (sensitized group). Massive infiltration of inflammatory cells in the interstitial space was observed in the sensitized group. The sensitized group also showed emphysema, hyperplasia, and necrosis.

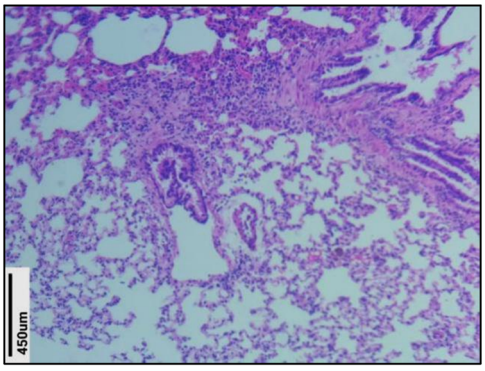

Figure 6: Histopathological study of Group VII lung section (EHC-treated group). The EHC-treated group showed decreased inflammatory cell infiltration compared to sensitized group. The treatment with the EHC prevented hyperplasia and bronchoconstriction in sensitized + EHC-treated animals.

humans, eosinophil counts are increased in the asthmatic airways and these cells release proteins and growth factors that may damage airway epithelium and cause airway remodeling. Moreover, neutrophil numbers are usually found to be elevated in the airways and in the sputum of patients with severe asthma. Hence, it is useful to evaluate these cell counts to determine if an anti-asthmatic product (EHC) is effective [29].

In this study, histamine is used as a spasmogenic agent to induce PCD. Histamine increases sensitivity by stimulating H1 receptors in airways leading to bronchoconstriction. The EHC increased the PCT against histamine aerosol when compared to control animals; thus, it tends to possess the broncho-dilating activity and an antihistaminic effect. EHC and chlorpheniramine (H1 receptor antagonist) prevented the histamine-induced bronchoconstriction by delaying the onset of PCD. Induction of bronchial hyper-responsiveness by OVA in an animal model is the most commonly used method of allergic asthma model worldwide. Sensitization is carried out by intranasal or intra-tracheal administration. This helps in studying mechanisms and treatments of bronchial hyperresponsiveness as most of the pathophysiological mechanisms resemble those seen in asthma patients [34]. OVA has the ability to induce asthma in humans and causes chronic airway inflammation [33]. Also, OVA is a T-cell-dependent antigen that is non-toxic and inert [35], thus proving suitable for the study. The total leukocyte, eosinophil, polymorph, monocyte, and lymphocytes counts were found to be increased in the sensitized group (Group VI), thus indicating the inflammatory response. In the sensitized + EHC-treated animals (Group VII), the herbal composites reduced the total leukocyte, neutrophil, lymphocyte, eosinophil, and monocyte counts.

Thus, the herbal composite acts as a protective agent preventing the release of mediators resulting in bronchoconstriction. An increase in MDA level increases the oxidative stress and lowered glutathione decreases the antioxidant. This effect is seen in a sensitized group, while in the MHC-treated group, the MDA level is reduced and the GSH level is increased. Increased CO_2 concentration in the lungs resulted in increased bicarbonate levels. Decreased tissue inflammation was observed in the EHC-treated group. Massive infiltration of inflammatory cells in the interstitial space was observed indicating that airway inflammation was observed in the sensitized group. The EHC-treated group showed decreased inflammatory cell infiltration compared to the sensitized group. The treatment with the EHC prevented hyperplasia and bronchoconstriction in sensitized + EHC-treated animals (Group VII).

Similar work on the anti-asthmatic activity of *E. hirta* was carried out by Pretorius and Smit [25] on mice. They reported that the treatment with hydrocortisone and the plant extracts decreased the white blood counts such as neutrophils, eosinophils and basophils which were found to be active during inflammation. Platelets and fibrin networks also play a fundamental role in asthma. Pretorius and Smit [25] observed that untreated mice possess major thick fibers and minor thin fibers as well as normal tight round platelet aggregates. Though the hydrocortisone drug made the fibrin more fragile, the *E. hirta* extracts do not affect the fragility of the fibrin proteins and that it prevents the minor fibers from forming the dense layer over the major fibers, as observed in untreated control asthmatic mice [36]. The morphology of the platelets also remained unaltered on *E. hirta* extract-treated mice. Hence, *E. hirta* extracts reduced the inflammatory cells' level equal to hydrocortisone, indicating that the plant, indeed, has anti-inflammatory properties [37]. These results well correspond with the present work as the developed herbal composite reduced the inflammatory cells to a significant extent.

Upadhyay et al. [26] investigated the anti-inflammatory activity of *E. hirta* on rats. The anti-inflammatory activity was determined by the carrageenan-induced paw edema experiment. *E. hirta* extracts decreased the inflammation in a dose-dependent manner. Reduction in inflammation was found to be effective at 500 $mg \cdot kg^{-1}$ b.w. concentration. Moreover, *E. hirta* plant extracts decreased the production of PGE-2 and pro-inflammatory cytokines. Similarly, several works were also investigated on extracts of *B. monnieri*. Hossain et al. [27] studied the anti-inflammatory activity of *B. monnieri* by the carrageenan-induced paw edema method. Methanolic extracts of *B. monnieri* showed significant anti-inflammatory activity. The increased reduction in the paw volume at a dose of 400 $mg \cdot kg^{-1}$ body weight was 62.73%, which was comparable to that of the standard drug indomethacin (67.08%) at 5 h. *B. monnieri* extracts showed higher anti-inflammatory activity, i.e., 92% (100 $mg \cdot kg^{-1}$) when compared to standard anti-inflammatory drug aspirin, i.e., 68.62% (25 $mg \cdot kg^{-1}$). The study provides evidence that extracts of *B. monnieri* act as a potent anti-inflammatory agent in rats in an acute inflammation model. Phytochemical studies of *B. monnieri* examined by Jain Paras et al. [24] revealed the presence of several bioactive compounds, such as tannin, saponin, flavonoid, phenol, alkaloid, and phlobatannin. Quantitative analysis revealed the presence of 12.5 mg of tannin, 110 mg of alkaloid, 1.5 mg of saponin, and 24.75 $mg \cdot g^{-1}$ of phenol in dry plant. Thus, they exert antifungal and antibacterial activities. Recent studies have described that they also possess anti-inflammatory activity, inhibit mast cell degradation, and thus can be used in the treatment of asthma [25].

From the GC-MS analysis, several bioactive compounds responsible for the anti-asthmatic effect were identified. 4*H*-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl identified from *E. hirta* showed excellent anti-inflammatory potential equal to standard drug diclofenac (10 $mg \cdot kg^{-1}$) [36]. Other bioactive compounds from *E. hirta*, such as 2-furancarboxaldehyde [37], 5-(hydroxymethyl) [38], phthalic anhydride [39], 1,2,3 benzenetriol [40], 1,2,3-thiadiazole-4-carboxylic acid hydrazide [41], inositol [42], phytol [43], *cis*-vaccenic acid [44], and 3-(3,4-dimethoxyphenyl) propylamine [45], possess significant anti-inflammatory potential. Similarly, few bioactive compounds from *B. monnieri*, i.e., 1,*E*-6,*Z*-11-hexadecatriene [46], 1-octadecanesulphonyl chloride [47], gamma-sitosterol [48], and lupeol [49], exert anti-inflammatory potential. The enhanced anti-asthmatic activity is due to the presence of all the bioactive compounds exhibiting synergistic mode of action.

Thus, based on the aforementioned studies, the present work focused on the synergistic anti-asthmatic activity of *E. hirta* and *B. monnieri*. A significant reduction in inflammatory cells was observed in EHC extracts when compared to control groups. Moreover, based on the literature review, this is the first reported work on the anti-asthmatic activity of *E. hirta* and *B. monnieri* extracts on guinea pigs. As plant extracts are used in this study, the stability and the consistency of the plants showing anti-asthmatic activity need to be determined. This limitation could be evaluated in further studies.

5 Conclusion

EHC was prepared using *E. hirta* and *B. monnieri*. A maximum zone of inhibition was observed against *E. coli* and *S. aureus*. The EHC increased the PCT against 1% histamine aerosol compared to control animals; thus, it tends to possess broncho-dilating activity and an antihistaminic effect. In the sensitized + EHC-treated animals (Group VII), the herbal composites of *E. hirta* and *B. monnieri* significantly reduced the total leukocyte, neutrophil, lymphocyte, eosinophil, and monocyte counts. EHC decreased the MDA levels denoting the reduced oxidative burden and increased the antioxidant activity by increasing the GSH level. There is a significant anti-asthmatic effect on the EHC-treated group ($P < 0.05$). The difference in the eosinophil and macrophage counts, serum bicarbonate level, protein, MDA, and GSH levels between the sensitized group and the EHC treated group was statistically calculated with $P < 0.05$ considered significant. The EHC-treated group showed decreased inflammatory cell infiltration compared to the sensitized group, indicating the anti-asthmatic property of the developed herbal composite. GC-MS analysis identified several potential bioactive compounds, and FT-IR analysis revealed the functional groups corresponding to plant extracts. Thus, from the present research, it is evident that composites of *E. hirta* and *B. monnieri* (both of which are seen in different parts of the Kingdom) extracts are effective in the treatment of asthma and can also be used as an alternative to commercially available pharmaceutical drugs.

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