PHARMACOLOGICAL STUDIES OF *Justicia adhatoda*

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ABSTRACT

Asthma is a clinical syndrome of chronic airway inflammation characterized by recurrent, reversible, and airway obstruction. Allergens, infections, and irritants are the trigger factors that aggravate asthma exacerbation. Traditional medicines may have the potential to treat any kind of disease. The plant *Justicia adhatoda* has already been proven for establishing biological activities. The methanolic extract of *Justicia adhatoda* leaves was prepared using soxhlet apparatus. The different concentration of the herbal extract was evaluated for antioxidant activity by DPPH radical scavenging assay, anti-inflammatory activity byalbumin denaturation assay, anti-microbial activity by agar well diffusion method and anti-asthmatic activity by degranulation assay. The IC50 value was calculated using GraphPad Prism software. The yield of the methanolic *Justicia adhatoda* leaves extract was weighed and the *in vitro* study results reveal that the prepared extract having the anti-oxidant, anti-inflammatory, anti-bacterial, anti-fungal, anti-asthmatic property in a concentration-dependent manner. Hence, the methanolic extract of *Justicia adhatoda* leaves will be subjected to preclinical and clinical studies to treat asthmatic patients as well as to sustain human health.

Keywords: *Justicia adhatoda,* Asthma, DPPH, albumin denaturation, degranulation, anti-microbial

INTRODUCTION

Asthma is a disease of the human respiratory system in which the airways constrict and become narrow, often in response to a trigger such as exposure to an allergen, cold air, exercise, or emotional stress. Asthma is one of the most common chronic diseases in the world. It is perhaps the most commonly occurring respiratory disease after the common cold. The major characteristic features of asthma include intermittent and reversible airway obstruction leading to recurrent episodes of breathlessness, wheezing, chest tightness, cough, bronchial hyper-responsiveness (BHR) (increased sensitivity to Broncho constrictor such as histamine or cholinergic agonists), and airway inflammation. Asthma can simply be defined as recurrent reversible bronchospasm due to a trigger, characterized by episodic wheezing, cough,and chest tightness associated with airflow obstruction, airway hyper-responsiveness, and multicellular inflammation. According to the guidelines of the national asthma education and prevention program (NAEPP), asthma is a multifactorial disease associated with allergy, environmental, infectious, genetic, emotional, and nutritional components. It is defined as a chronic condition of the inflammation of the airways in which many cells and cellular elements play an important role, chiefly, mast cells, epithelial cells, T- lymphocytes, eosinophils, neutrophils, and macrophages; which results in a decrease in forced expiratory volumes (FEV) and flow rates, hyperinflation of the lungs and thorax, increase in airway resistance, increased breathing force, modification in respiratory muscle function, variations in elastic recoil, abnormal circulation of both ventilation and pulmonary blood flow with mismatched ratios and altered arterial blood gas concentration (McFadden 2003; Holgate 1997). Air pollution- Air pollution does not appear to cause asthma, but may trigger an asthma attack. Outdoor pollutants include industrial and photochemical smog. Indoor pollutants arise from cooking and heating sources, as well as materials used in building construction and furnishings. Smoking- Active smoking and passive smoking contribute to respiratory morbidity in children less than two years of age. Exposure of the fetus, infant, and young child to tobacco smoke increases the risk of asthma. Active smoking, when combined with occupational sensitizers, increases the risk of developing asthma. Low income- Asthma is more prevalent among low-income adults over 35 years of age (Erzenet al., 1997).Young boys appear to develop asthma more often than young girls, probably as a result of their smaller

airways. This imbalance reverses with age; more adult women than men develop asthma. Many researchers believe in the role of sex hormones in asthma, however, the exact mechanism has yet not been known. In general, the studies on the role of sex hormones in asthma have been inconclusive. Theories include sex-related differences in immune cells, estrogen's involvement in signaling pathways marked decrease in bronchial responsiveness observed in boys but not in girls, older than age 11 years (Poznanskiand Cydulka, 2016). Asthma is more common in families where at least one parent has asthma. The link is stronger if it is the mother who has been suffering from asthma. The relationship toparental allergies follows a similar, but weaker, Colds or chest infections (rhinovirus, influenza) , exercise or sports, tobacco smoke, allergens (pollen, flowers, grass, dust, fungal spores, cockroaches, etc.), cold air, dampness or humidity, animals, air pollution, anxiety, and stress, mold *(Alternaria, Aspergillus, Aureobasidium (pullularia), Cladosporium (hormodendrum), Epicoccum, Fusarium, Helmin-thosporium, Mucor, Penicillium, Rhizopus)*, temperaturechange, feathers, perfumes, fumes from wood heating, occupational chemicals, certain foods and certain medicines such as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), β-blockers, etc. can trigger an asthma attack history of either asthma or various allergies including hay fever, eczema, and dermatitis. On the other hand, the intrinsic type develops at around 40 years of age and occursbecause of non-specific factors (common cold, exercise, or emotion) which may trigger the asthmatic attack(Linzer, 2007; Prasad et al., 2009). The pathogenesis of asthma is multi-factorial and multi-cellular since macrophages, mast cells, eosinophils, neutrophils, and platelets are involved in its pathogenesis (Joseph et al., 1983). These cells produce an arsenal of mediators such as bradykinin, histamine, leukotrienes, platelet- activating factor, prostaglandins, and thromboxane, which interact in a complex way to produce pathological effects such as the constriction of airway smooth muscles, increased microvascular leakage, mucus secretion, and recruitment of inflammatory cells into the airways (Sasaki et al., 1993; Cookson, 1999). Histopathological studies of patients with asthma have shown inflammation in the airways with infiltration of inflammatory cells, particularly, eosinophils, disruption of the airway epithelium, and mucus hypersecretion, thus indicating thatairway inflammation may underlie bronchial hyperresponsiveness (Dunnill, 1960). The current pharmacotherapy of asthma is guided by the fact that it is a chronic

inflammatory airway disorder and the control of airway inflammation is the key to effectively control the disease (Lodha and Kabra, 2004). The NAEPP guidelines (2007) categorize anti- asthmatic medications into quick relief agents (short-acting inhaled β2 agonists, anti-cholinergic and short-term systemic corticosteroids) and long-term control agents (inhaled and systemic corticosteroids, long-acting β2 agonists, methylxanthines, mast cell stabilizers, and leukotriene modifiers). Corticosteroids are the most potent anti-inflammatory drugs used for the treatment of asthma. Inhaled corticosteroids (ICS) are the most effective long-termcontrol therapy for persistent asthma while systemic corticosteroids are used primarily as a rescue medication for acute asthma exacerbations (NAEPP, 2007). Long-term treatment with high doses of ICS produces adverse effects like hypothalamic-adrenal axis suppression and adrenal insufficiency, decreased bone mineral density, easy bruising, cataracts, growth retardation, dysphonia, etc. β2-adrenergic agonists are the safest and most effective bronchodilators and are meant for symptomatic relief (as a rescue) only as they do not inhibit the late phase inflammatory response nor do they prevent a subsequent increase in bronchial hyper reactivity. The most common adverse effects are tachycardia, skeletal muscle tremor, hypokalemia (Guhan et al., 2000). Excessive long-term use of β2 agonists associated with worsening of asthma control and death due to asthma (Sears, 2002). Sustained release theophylline preparations have been used in the treatment of nocturnal asthma (Barnes et al., 1982) but the narrow therapeutic index and its variable pharmacokinetics depending on hepaticmetabolism limit the use of this drug. Severe, life-threatening toxicity of theophylline includes seizures and arrhythmias (Hendeles et al., 1977). Cromones or mast cell stabilizers like cromolyn sodium and nedocromil are recommended as second-line therapy only in mild persistent asthma (NAEPP, 2007). Anticholinergics (ipratropium bromide and oxitropium bromide) have been used only for relief of acute bronchospasm and remain ineffective against allergen- and exercise-induced bronchospasm (EIB). Leukotrienes modifiers (zileuton, zafirlukast and montelukast) are used for the treatment of mild persistent asthma (Zeiger et al., 2005). Drug interactions occur with zileuton and zafirlukast but have not been found with montelukast (Bisgaard, 2001). Reports of Churg-Strauss syndrome and liver dysfunction have occurred in patients treated with leukotrienes modifiers .

MATERIALS AND METHODS

Collection of Leaves

The leaves were collected from the selected plants *Justicia adhatoda* in the place of Attur, Salem District, Tamil Nadu. The collected leaves were washed thoroughly using ethanol. These clean leaves were shade dried for 3-4 days. About 100 grams of leaves were powdered using a mixer grinder.

Extraction

Weigh appropriately 50 grams of powdered leaves and extracted with 120 ml of the solvent methanolusing Soxhlet apparatus for 5-6 hrs. The obtained extract was then evaporated at the temperature of 70^{o}C for 1 hr. Weighed and reported the appropriate yield of the leaves toextract obtained.

Antioxidant activity by DPPH radical

solution in methanol and add 100 µl of this solution to *Justicia adhatoda* leaves extract at different concentrations (500, 250, 100, 50, and 10 µg/mL). The mixtures have to be shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance has to be measured at 517 nm using a UV-VIS spectrophotometer.(Ascorbic acid can be used as the reference).Lower absorbance values of the reaction mixture indicated higher free radical scavengingactivity. The capability of scavenging the DPPH radical can be calculated by using the following formula. DPPH scavenging effect (% inhibition) = [(absorbance of control-absorbance of reaction mixture)/absorbance of control] X 100. Prepare 0.1 mM of DPPH solution in methanol and add 100 µl of this solution to *Justicia adhatoda* leaves extract at different concentrations (500, 250, 10 0, 50, and 10 µg/mL). The mixtures have to be shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance has to be measured at 517 nm using a UV-VIS spectrophotometer.(Ascorbic acid can be used as the reference).Lower absorbance values of the reaction mixture indicated higher free radical scavenging activity. The capability of scavenging the DPPH radical can be calculated by using the following formula. DPPH scavenging effect (% inhibition) = [(absorbance of control-absorbance of reaction mixture)/absorbance of control] X 10

Anti-inflammatory activity by albumin denaturation assay

Denaturation of proteins is the main cause of inflammation. Inhibition of

protein denaturation was evaluated by the method of Mizushima and Kobayashi and Sakat *et al*. with slight modification. 500 µL of 1% bovine serum albumin was added to *Justicia adhatoda* leavesextract (500, 250, 100, 50, and 10 µg/mL) of the test sample. This mixture was kept at room temperature for 10 minutes, followed by heating at 51°C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Acetylsalicylicacid was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

% Inhibition=100− $((A_1-A_2)/A_0)*100)$.

Where A1 is the absorbance of the sample, A2 is the absorbance of the product control and A0 is the absorbance of the positive control. A dose response curve was plotted to determine the IC50 values. IC50 is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were run in triplicate and averaged.

Anti-bacterial activity by agar well diffusion

Petri plates containing 20 ml nutrient agar medium were seeded with 24hr culture of bacterial strains (*Staphylococcus aureus*- 902 *and E. coli*-443*)* Wells were cut and different concentrations of *Justicia adhatoda* leaves extract (500, 250, 100, and 50 µg/ml) was added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Gentamicin antibiotic was used as a positive control. The values were Calculated using Graph Pad Prism 6.0 software(USA

Anti-fungal activity by agar well diffusion

Petri plates containing 20ml potato dextrose agar medium were seeded with 24hr cultureof fungal strain *(Aspergillus niger and Aspergillus flavus)* wells were cut and different concentration of *Justicia adhatoda* leaves extract (500, 250, 100 and 50 µg/ml) was added. The plates were then incubated at 37°C for 24 hours. The anti-fungal activity was assayed by measuring the diameter of the inhibition zone formed around the wells. Amphotericin B was used as a positive control. The values were calculated using Graph Pad Prism 6.0 software (USA).

Anti-asthmatic activity by Degranulation Assay

About 2 ml of peripheral blood was collected through venipuncture of human and transferred into a 15 ml centrifuge tube containing 50 µl of sodium carbonate (anticoagulant). To this solution mixture, add 2 ml of PBS and mix well. This entire solution mixture was addedalong the sides of the 15 ml centrifuge tube containing 2 ml of LSM. Then, the centrifugal force was applied at 1500 rpm for 10 minutes. The separated cells (1 ml) were collected and transferred into a fresh tube. Makeup to 10 ml of volume with PBS and this cells suspension was subjected to centrifugation process at 1500 rpm for 5 minutes. The obtained pellet was separated. Different concentrations such as 500, 250, 100, 50, and 10 µg/ml of *Justicia adhatoda* leaves were added into different fresh Eppendorf tubes containing 500 µl of cells. 500 µl of cells with 50 µl of histamine was taken as a control. The value of optical density was measured at the wavelength of 405 nm using a microplate reader.

RESULT AND DISCUSSION

The collected leaves of *Justicia adhatoda* were successfully extracted using methanol as a solvent by Soxhlet extractor. The obtained yield of the leaves extract from *Justicia adhatoda* by soxhlet extraction was found to be 4.4 grams . Antioxidant activity, anti-inflammatory activity, and anti-asthmatic activity of methanolic leaves extract of *Justicia adhatoda* were analyzed by DPPH radical scavenging activity, albumin denaturation assay, and degranulation assay respectively as shown in 1.2, 2.1, and 3.1. All the assay results reveal that methanolic leaves extract of *Justicia adhatoda* causes concentration-dependent effects. The IC50 value for antioxidant activity anti-inflammatory activity and anti-asthmatic activity of methanolic leaves extract of *Justicia adhatoda* was found to be 249.2 µg/ml, 35.65 µg/ml, and 203.6 µg/ml respectively. The antimicrobial effect of methanolic leaves extract of *Justicia adhatoda* was evaluated by *the* agar well diffusion method as shown in 2.2. The methanolic leaves extract of *Justicia adhatoda* having the antibacterial and antifungal effect in a concentration-dependent manner. The zone of inhibition was measured as high in the treatment of 500 µg/ml of the methanolic leaves extract of *Justicia adhatoda*. These results reveal that there is an effect of the methanolic leaves extract of *Justicia adhatoda*. against the selected strains of pathogens *Staphylococcus aureus* (MTCC No. 902), *Escherichia coli* (MTCC No. 443), *Aspergillus niger,* and *Aspergillus flavus.* The zone of inhibition for all the bacterial and fungal strains was compared to the antibiotic-treated pathogens. Thus,

the *in vitro* study shows that methanolic leavesextract of *Justicia adhatoda* having an anti-oxidant, anti-bacterial, anti-fungal, anti-inflammatory, and anti-asthmatic effect.

Antioxidant assay by DPPH radical scavenging assay

A. OD Value at 517 nm

Control Mean OD value: 1.814

S. No	Tested sample concentration (µg/ml)	OD Value at 517 nm(in triplicates)		
1.	Control	1.74	1.967	1.736
2.	500 µg/ml	0.704	0.779	0.735
3.	250 µg/ml	1.359	0.778	0.849
4.	100 µg/ml	0.855	1.129	1.062
5.	50 µg/ml	1.371	0.927	0.945
6.	10 µg/ml	0.948	1.098	1.311
7.	Ascorbic acid	0.08	0.11	0.12

Table 1.1: OD value of *Justicia adhatoda* methanolic leaves extract for anti-oxidantactivity by DPPH radical scavenging assay

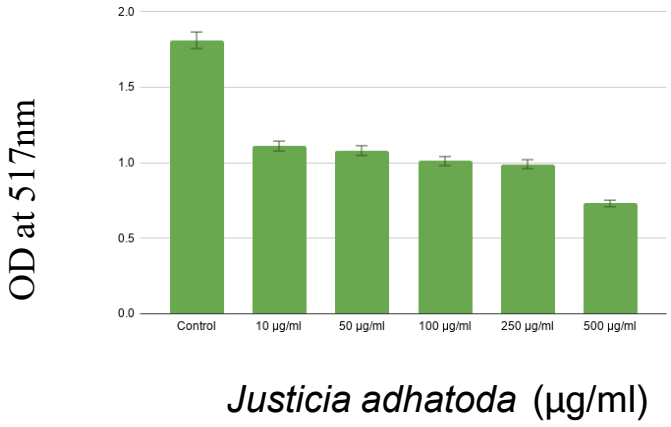


Figure 1.1: OD value of *Justicia adhatoda* methanolic leaves extract for anti-oxidant activity by DPPH radical scavenging assay

B. Percentage of inhibition

Table 1.2: Percentage inhibition of *Justicia adhatoda* methanolic leaves extract for anti-oxidant activity by DPPH radical scavenging assay

S. No	Tested sample concentration (µg/ml)	Percentage of inhibition (in triplicates)			Mean value (%)
1.	Control	100	100	100	100
2.	500 µg/ml	61.19	57.05	59.48	59.24
3.	250 µg/ml	25.08	57.11	53.19	45.13
4.	100 µg/ml	52.86	37.76	41.45	44.02
5.	50 µg/ml	24.42	48.89	47.90	40.40
6.	10 µg/ml	47.73	39.47	27.72	38.31
7.	Ascorbic acid	95.58	93.93	93.38	94.30

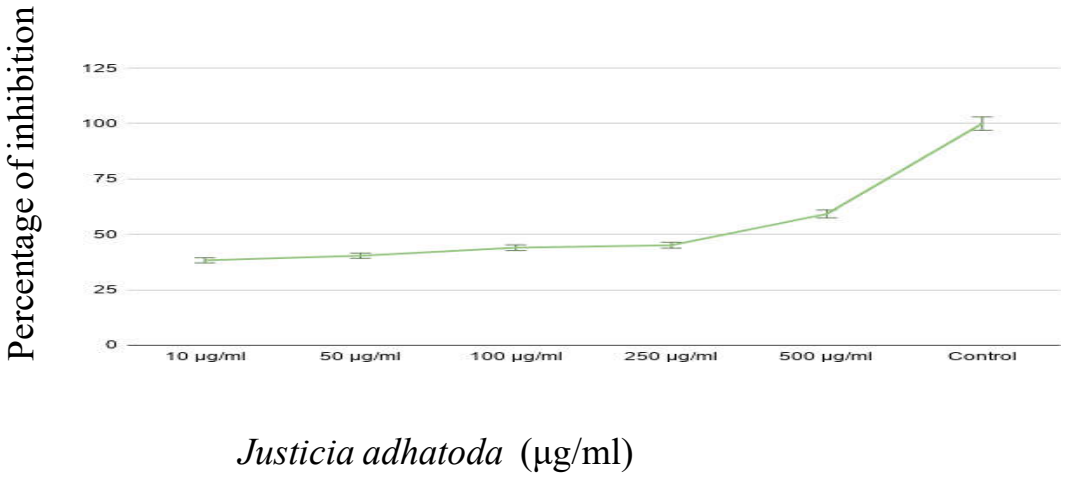


Figure 1.2: Percentage inhibition of *Justicia adhatoda* methanolic leaves extract for anti- oxidant activity by DPPH radical scavenging assay

IC50 Value of tested sample: 249.2 µg/ml

log(inhibitor) vs. normalized response -- Variable slope	*Justicia adhatoda* µg/m
Best-fit values	
LogIC50	2.396
	-2.201
IC50	
Std. Error	0.1867
LogIC50	2.001
HillSlope	
95% Confidence Intervals	1.993 to 2.800
LogIC50	-6.523 to 2.121
HillSlope	98.43 to 630.7
IC50	
Goodness of Fit	13
Degrees of Freedom	0.3211
R square	33094

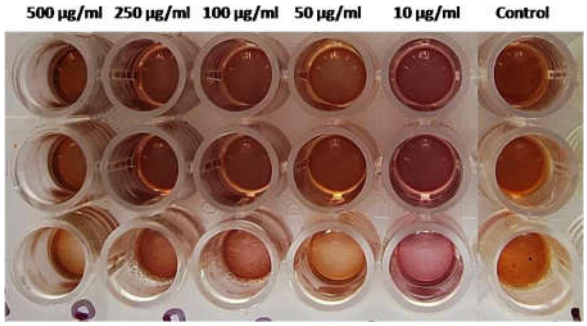


Figure 1.3: Anti-oxidant activity by DPPH assay

Anti-inflammatory activity by albumin denaturation assay

A. OD Value at 660 nm

Control Mean OD value: 2.955

S. No.	Tested sample concentration (µg/ml)	OD Value at 660 nm (in triplicates)		
1.	Control	2.973	2.934	2.959
2.	500 µg/ml	0.157	0.226	0.183
3.	250 µg/ml	0.362	0.348	0.284
4.	100 µg/ml	0.346	0.325	0.413
5.	50 µg/ml	0.717	0.875	0.728
6.	10 µg/ml	2.318	2.379	2.343

Table 2.1: OD value of *Justicia adhatoda* methanolic leaves extract for anti-inflammatory activity by albumin denaturation assay

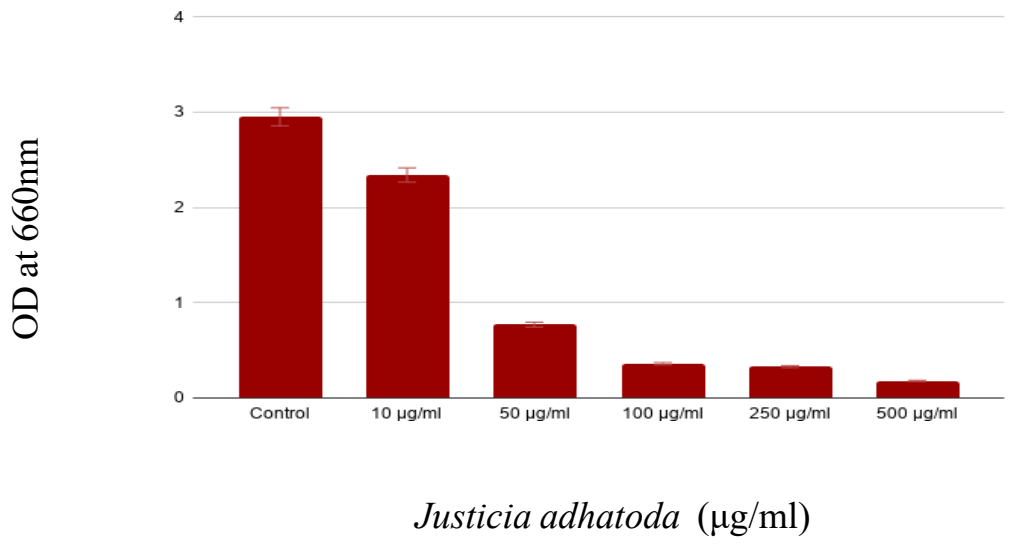


Figure 2.1: OD value of *Justicia adhatoda* methanolic leaves extract for anti-inflammatory activity by albumin denaturation assay

B. Inhibition percentage of albumin denaturation (%)

Table 2.2: Percentage inhibition of *Justicia adhatoda* methanolic leaves extract for anti-inflammatory activity by albumin denaturation assay

S. No.	Tested sample concentration (µg/ml)	Inhibition percentage of albumin denaturation (%) (in triplicates)			Mean (%)
1.	Control	100	100	100	100
2.	500 µg/ml	94.68	92.35	93.80	93.61
3.	250 µg/ml	87.74	88.22	90.38	88.78
4.	100 µg/ml	88.29	89.00	86.02	87.77
5.	50 µg/ml	75.73	70.38	75.36	73.82
6.	10 µg/ml	21.55	19.49	20.71	20.58

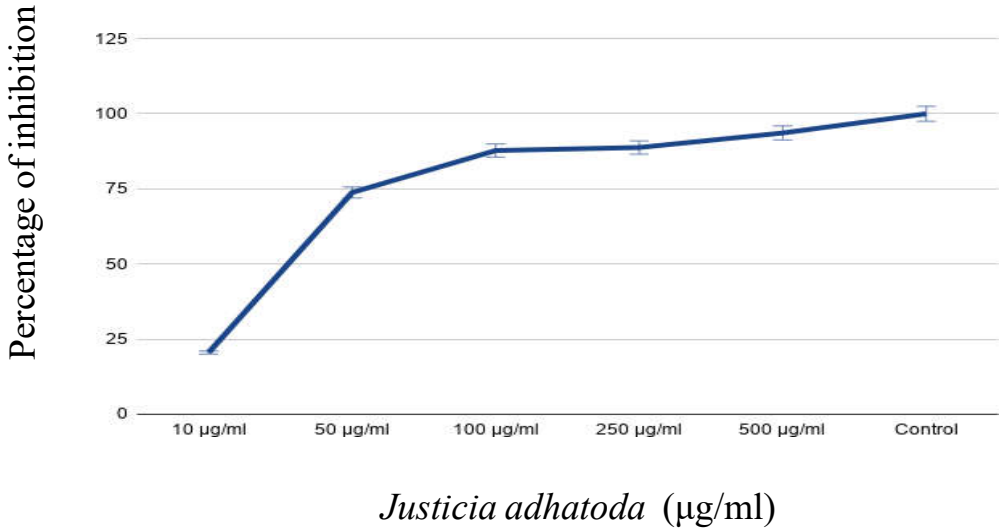


Figure 2.2: Percentage inhibition of *Justicia adhatoda* methanolic leaves extract for anti- inflammatory activity by albumin denaturation assay

IC50 Value of tested sample: 35.65 µg/ml

log(inhibitor) vs. normalized response -- Variable slope	*Justicia adhatoda* µg/ml
Best-fit values	
LogIC50	1.552
HillSlope	-2.750
IC50	35.65
Std. Error	
LogIC50	0.02862
HillSlope	0.4033
95% Confidence Intervals	
LogIC50	1.490 to 1.614
HillSlope	-3.621 to -1.879
IC50	30.92 to 41.10
Goodness of Fit	
Degrees of Freedom	13
R square	0.9892
Absolute Sum of Squares	220.8
Sy.x	4.121
Number of points	
Analyzed	15

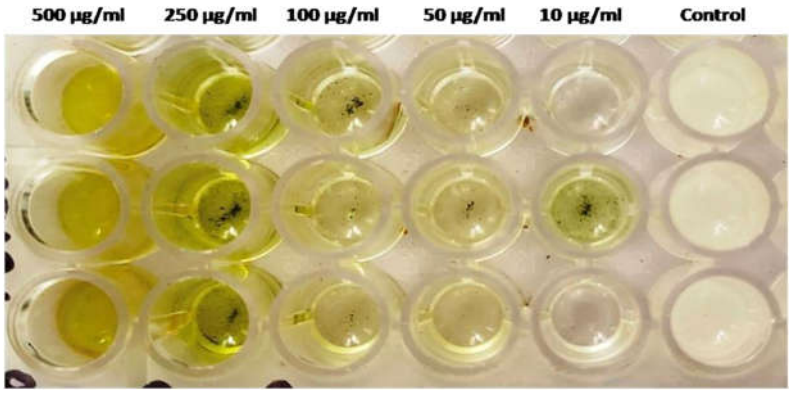


Figure 2.3: Anti-inflammatory activity by albumin denaturation assay

Anti-microbial activity by agar well diffusion method

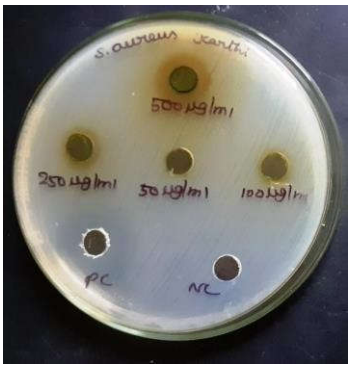


Figure 3.1: Effect of *Justicia adhatoda* leaves extract against *Staphylococcus aureus*

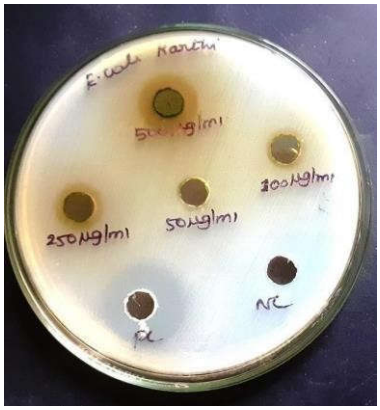
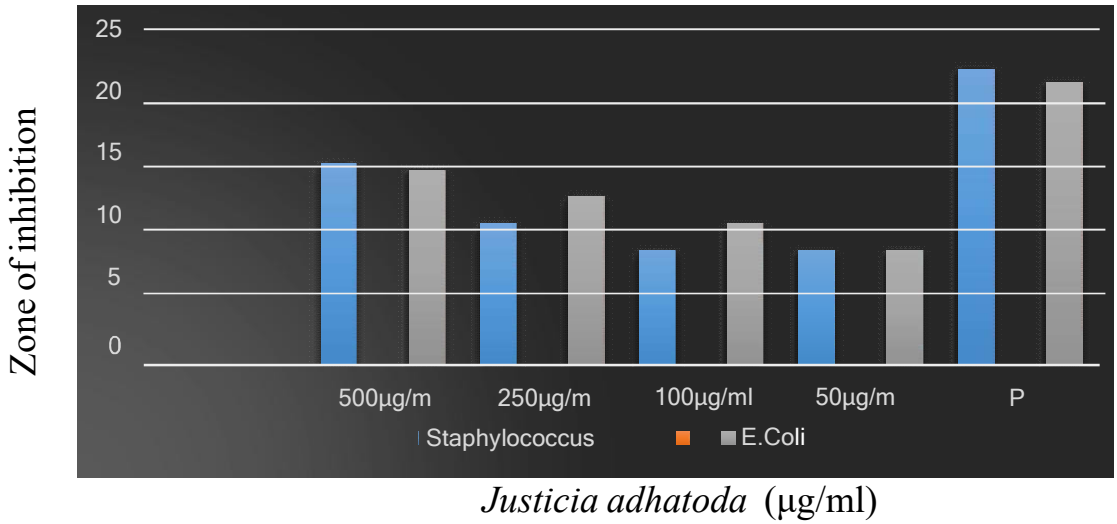


Figure 3.2: Effect of *Justicia adhatoda* leaves extract against *E. coli*

Means of zone of inhibition obtained by sample against the pathogens *Staphylococcus aureus and E.Coli*



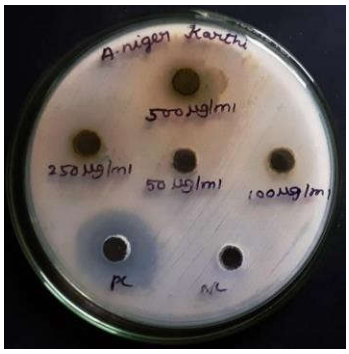


Figure 3.3: Effect of *Justicia adhatoda* leaves extract against *Aspergillus niger*

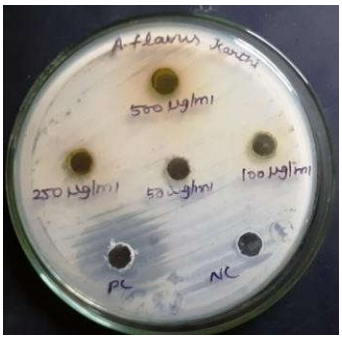


Figure 3.4: Effect of *Justicia adhatoda* leaves extract against *Aspergillus flavus*

Mean ± SD of zone of inhibition obtained by *Justicia adhatoda* leaves extract against

Aspergillus niger and Aspergillus flavus

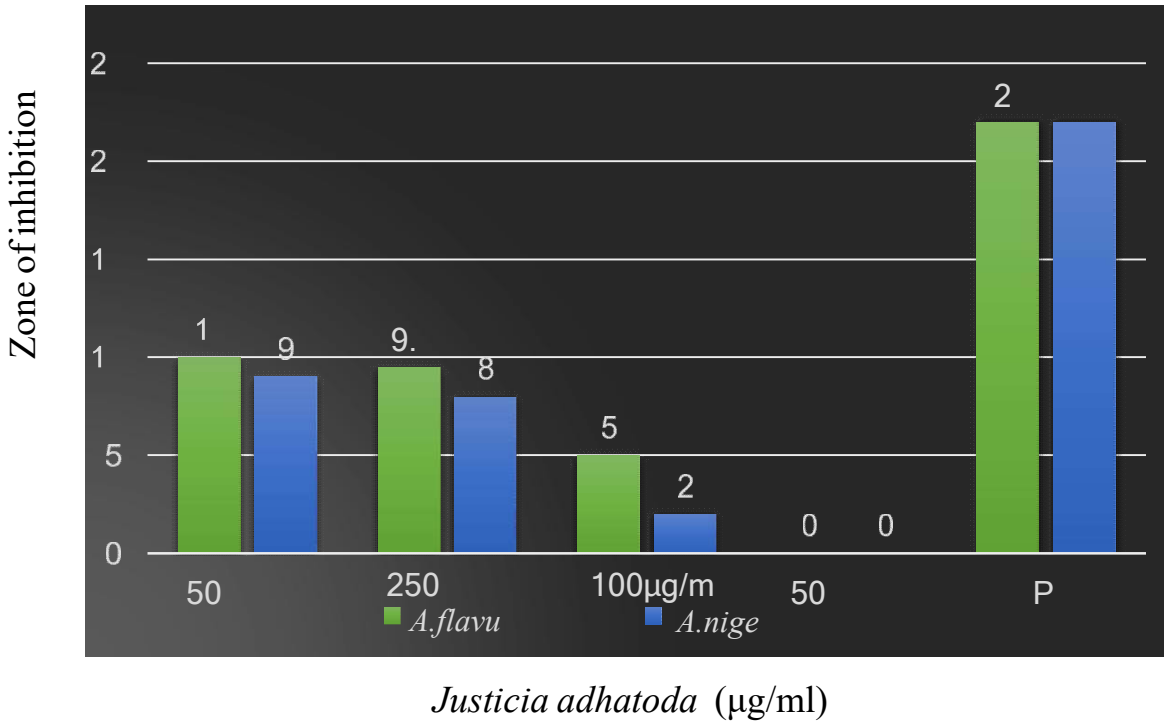


Table 3.1: Mean ± SD of zone of inhibition obtained by *Justicia adhatoda* leaves extract against the pathogens *Staphylococcus aureus and E. coli*

S. No.	Name of the test organism	Zone of inhibition (mm)Mean ± SD				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Staphylococcus aureus*	15±1.5	10.5±0.5	8.5±0.5	8.5±0.5	22±1.0
2.	*E. coli*	14.5±0.2	12.5±0.1	10.5±0.5	8.5±0.5	21±1.0

Table 3.2: Mean ± SD of zone of inhibition obtained by *Justicia adhatoda* leaves extract against *Aspergillus niger and Aspergillus flavus*

S. No.	Name of the test organism	Zone of inhibition (mm)Mean ± SD				
		500 µg/ml	250 µg/ml	100 µg/ml	50 µg/ml	PC
1.	*Aspergillus niger*	9±0.3	8±0.2	8±0.5	0	22±1.0
2.	*Aspergillus flavus*	10.5±0.2	9.4±0.2	8.7±0.2	0	22±1.0

Anti-asthmatic activity by Degranulation Assay

A. OD at 405 nm

Mean control OD value: 0.124

Table 4.1: OD value of *Justicia adhatoda* methanolic leaves extract foranti-asthmatic activity by degranulation assay

S. No	Tested sample concentration (µg/ml)	OD Value at --405 nm (in triplicates)		
1.	Control	0.213	0.208	0.215
2.	500 µg/ml	0.121	0.126	0.127
3.	250 µg/ml	0.116	0.119	0.121
4.	100 µg/ml	0.053	0.051	0.043
5.	50 µg/ml	0.049	0.034	0.045
6.	10 µg/ml	0.012	0.012	0.013

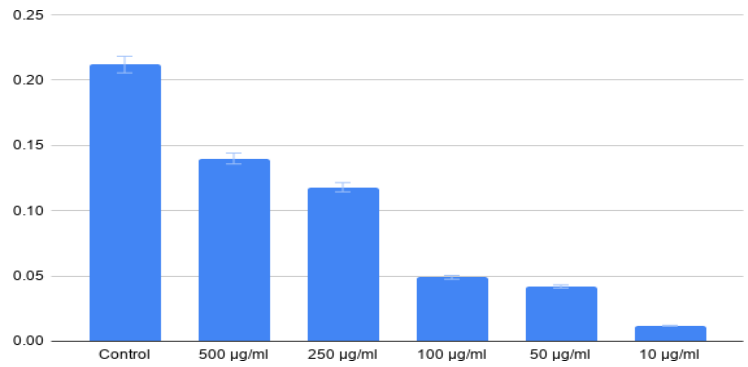


Figure 4.1: OD value of *Justicia adhatoda* methanolic leaves extract for anti-asthmatic activity by degranulation assay

B. Percentage of Inhibition (%)

Table 4.2: Percentage inhibition of *Justicia adhatoda* methanolic leaves extract for anti-asthmatic activity by degranulation assay

S. No	Tested sample concentration (µg/ml)	Inhibition percentage (in triplicates)			Mean (%)
1.	Control	100	100	100	100
2.	500 µg/ml	90.32	90.32	89.51	90.05
3.	250 µg/ml	60.48	72.58	63.70	65.59
4.	100 µg/ml	57.25	58.87	65.32	60.48
5.	50 µg/ml	6.451	4.03	2.41	4.30
6.	10 µg/ml	0	0	0	0

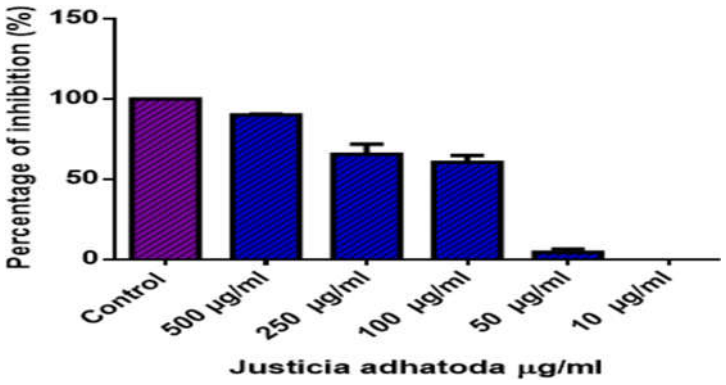


Figure 4.2: Percetntage inhibition of *Justicia adhatoda* methanolic leavesextract for anti-asthmatic activity by degranulation assay

C. IC50 value of the sample: 203.6 μg/ml

log(inhibitor) vs. normalized response -- Variable slope	Justicia adhatoda μg/ml
Best-fit values	
LogIC50	2.309
HillSlope	2.218
IC50	203.6
Std. Error	
LogIC50	0.03399
HillSlope	0.3293
95% Confidence Intervals	
LogIC50	2.235 to 2.382
HillSlope	1.507 to 2.929
IC50	171.9 to 241.1
Goodness of Fit	
Degrees of Freedom	13
R square	0.9463
Absolute Sum of Squares	1039
Sy.x	8.938
Number of points	
Analyzed	15

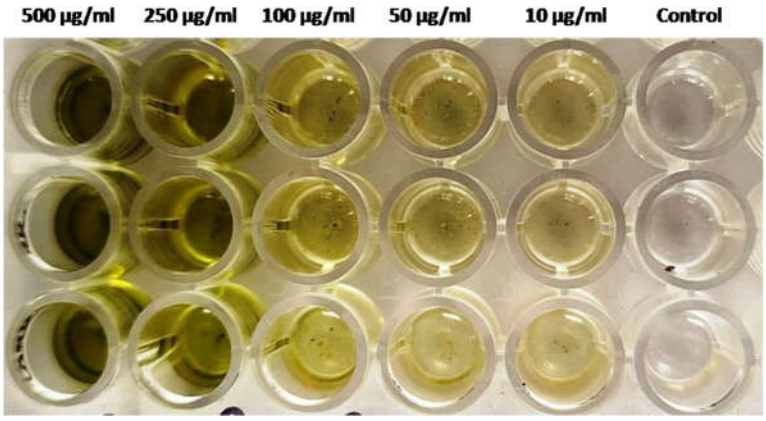


Figure 4.3: Anti-asthmatic activity by degranulation assay

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