In vivo and In silico Anti Inflammatory Studies of Alstonia scholaris Bark Extract

M. Ganga Raju a*, R. Manisha a, NVL Suvarchala Reddy V. a and P. Samson Simharayullu a

a Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, Telangana, India.

ABSTRACT

The present research is focused on screening In vivo anti-inflammatory activity using carrageen and formalin induced paw edema model in rodents and In silico approaches like docking studies (mcule), Ramchandran plot (procheck) and PASS. The extract significantly inhibited inflammation caused by carrageenan and formalin at doses of 200 mg and 400 mg/kg body weight which is compared to the the effect of the standard drugs meloxicam and indomethacin. Docking studies for natural compounds were carried out against PDB ID: 2AZ5, PDB ID: 1IBC, PDB ID: 6COX, and PDB ID: 4NOS in order to assess the ligand-binding affinity of the active principles of the extract. The docking results showed that the phytoconstituents from the extract and standard drugs meloxicam and indomethacin had shown highest glide scores with all the selected proteins which indicate a greater affinity for binding between receptor and ligand. From the PASS results the possible interventions of selected active constituents of Alstonia scholaris were found to be anti-inflammatory intestinal, Prostaglandin-E2 9-reductase inhibitor, TNF expression inhibitor, Cyclooxygenase 1 and 2 inhibitors, NOS2 expression inhibitor, and Interleukin1 and 6 antagonists. From the prediction results of adverse effects the constituents like Stigmasterol, Diospyrolide, D-Friedoolean-14-en-3-one and Lupeol acetate were found to be free from any adverse effects. All the constituents of Alstonia scholaris were found to have interventions either as direct targets or possible targets with Histamine H2 receptor, Arachidonate 5-lipoxygenase, Interleukin-1 receptor-associated kinase 3 TNF-alpha, Cyclooxygenase 1, Prostanoid EP2 receptor, Prostaglandin E synthase, and Serotonin 1e (5-HT1e) receptor. From In vivo and In silico results it is evident that ethanolic bark extract of Alstonia scholaris possessed significant anti-inflammatory activity.

*Corresponding author: E-mail: mgrpharma@gmail.com;
Keywords: Alstonia scholaris; anti-inflammatory; docking studies; PASS (Prediction of Activity Spectra for Substances).

1. INTRODUCTION

A living, vascularized tissue's local response (reaction) to exogenous and endogenous stimuli is inflammation. The word comes from the Latin word "inflammare," which means to burn. Fundamentally, inflammation serves two purposes: to contain tissue damage and to localise and eradicate the cause. Inflammation can be divided into two categories: acute and chronic, depending on the host's ability to defend itself and the length of the response [1]. Carrageenan increases phospholipase A2 as cytotoxic effects cause inflammation to proceed. Cyclooxygenase pathway activation results from this model. Carrageenan-induced edema has a biphasic curve. Formalin causes a biphasic inflammatory response, with substance-P and bradykinin mediating the early neurogenic phase. While bradykinin, histamine, 5-HT, and prostaglandins are involved in the later stage. While medications like NSAIDs and corticosteroids inhibit the second phase, pharmaceuticals like opioids reduce both stages [2].

Alstonia scholaris, often known as the "devil's tree" or "blackboard tree" in English, is an evergreen tropical tree belonging to the Apocynaceae family. The plant is traditionally found to be useful for many ailments like anti-tuberculosis, antibacterial, anticancer, antitussive, and expectorant activities, bronchovasodilatory activity, anti-inflammatory, analgesic, wound healing, anti-diabetic, anti hyperlipidemic, antihypertension, anti-anxiety, antimalarial, hepatoprotective, antidiarrheal and spasmolytic activities etc [3]. The present study aimed to evaluate the anti-inflammatory activity of the ethanolic bark extract of Alstonia scholaris on carrageenan and formalin induced paw edema models and an attempt is made to establish the In silico studies of the active constituents of the extract using mcule, and PASS software.

2. MATERIALS AND METHODS

Plant Collection & Drying

The bark of Alstonia scholaris was collected from Hyderabad, Telangana in the month of November and was identified and authenticated. The bark is dried under shade for about six days and coarsely powdered in a mixer grinder. The powdered material was stored or taken up for extraction process.

Preparation of Plant Extraction

Soxhlet extraction is the process of continuous extraction in which the same solvent can be circulated through the extractor for several times. This process involves extraction followed by evaporation of the solvent. The vapours of the solvent are taken to a condenser and the condensed liquid is returned to the drug for continuous extraction.

Preliminary Phytochemical Analysis

The extract was subjected to preliminary phytochemical investigations to identify various phytoconstituents present in the ethanolic extract of Alstonia scholaris bark.

Acute Toxicity Studies

The acute toxicity studies were carried out using OECD 425 guidelines. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India (Reg. No. 1175/PO/ERe/S/08/CPCSEA).

Experimental Protocol

Wistar albino rats (approx 200-250 gm) were procured from Jeeva Life Sciences, Hyderabad. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals.

In vivo Methods for Evaluation of Anti-Inflammatory Activity

Carrageenan induced paw edema model

Carrageenan-induced paw edema is a biphasic phenomenon in nature. The first phase (1– 2 h after carrageenan injection) is mediated by histamine, serotonin, and bradykinins which are released from mast cells into the surrounding damaged tissues. The second phase (3–6 h after carrageenan injection) of inflammatory reaction is associated with the release of arachidonate.
metabolites such as prostaglandins, leukotrienes, and various cytokines such as IL-1β, IL-6, IL-10, and TNFα. [4].

Thirty healthy Albino rats of either sex weighing 200-250 gm were selected for the study. They were divided into 5 groups, each containing 6 animals (n=6). In this method, albino rats were fasted in individual cages for 24 hours. Group I received (Control) normal saline. Group II received (Disease control) Carrageenan, Group III received test drug EEAS at dose of 200 mg/kg, p.o. Group IV received test drug EEAS at dose of 400 mg/kg, p.o. Group V received (standard) Meloxicam (1 mg/kg, bd.wt, s.c). Group III, IV and V received carrageenan 1 h prior to the administration of their respective drugs. Paw edema was induced by injecting 0.1 ml of 1% w/v carrageenan into sub-plantar tissues of the left hind paw of each rat. Paw thickness was measured immediately after carrageenan injection (time 0) and at 1, 2, 3 and 4h using vernier calliper to assess the degree of inflammation [5].

Percentage inhibition was calculated using the following formula:

\[
\%\text{inhibition} = \left( \frac{V_T - V_i}{V_T} \right) \times 100
\]

Formalin induced paw edema model

Thirty healthy Albino rats of either sex weighing 200-250 gm were selected for the study. They were divided into 5 groups, each containing 6 animals (n=6). In this method, albino rats were fasted in individual cages for 24 hours. Group I received (Control) normal saline. Group II received (Disease control) Carrageenan, Group III received test drug EEAS at dose of 200 mg/kg, p.o. Group IV received test drug EEAS at dose of 400 mg/kg, p.o. Group V received (standard) Indomethacin (5 mg/kg, bd.wt, i.p.). Group III, IV and V received formalin 1 h prior to the administration of their respective drugs. Paw edema was induced by injecting 0.2 ml of 2% w/v formalin into sub-plantar tissues of the left hind paw of each rat. Paw thickness was measured immediately after formalin injection (time 0) and at 1, 2, 3 and 4h using vernier calliper to assess the degree of inflammation [6].

Statistical Analysis

Values are expressed as Mean ± SEM, (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett’s test. All the groups were compared with control, disease control and standard groups. Significant values are expressed as control group (*=p<0.001, a=p<0.0001 b = p<0.001), and standard (A=p<0.0001, ns- non significant).

In silico Analysis

Molecular docking studies

Structure based drug design

Molecular docking is a kind of bioinformatics modelling which involves the interaction of two or more molecules to give the stable adduct. Depending upon binding properties of ligand and target, it predicts the three-dimensional structure of any complex. Molecular docking generates different possible adduct structures that are ranked and grouped together using scoring function in the software. MCULE is an online drug discovery platform offers a unique solution for pharma and biotech companies by providing molecular models. Docking is done by initially identifying protein from PDB homepage and ligand structures are drawn in MCULE [7]. The selected phytochemical constituents of the extract were docked against protein ID: 2AZ5, 1IBC, 6COX and 4NOS.

MCULE docking results

MCULE docking indicates that some of our compounds have good binding ability with TNF-α inhibitor (PDB ID: 2AZ5), Interleukin 1β converting enzyme inhibitor (PDB ID: 1IBC), COX inhibitor (PDB ID: 6COX) and nitric oxide synthase (PDB ID: 4NOS).

Ramachandran plot

Ramachandran plot has been generated from PROCHECK validation server which was used to access the quality of the model by looking into the allowed and disallowed regions of the plot [8].

Pass software

Input and output of pass: PASS uses as input data a MOL- or SD-file representing the structural information about the molecules under study. On the basis of these data, MNA descriptors (Multilevel Neighborhoods of Atoms) are generated automatically. Based on the statistics of MNA descriptors for active and inactive compounds from the training set, two
probabilities are calculated for each activity: \( P_a \) - the probability of the compound being active and \( P_i \) - the probability of being inactive. Being probabilities, the \( P_a \) and \( P_i \) values vary from 0.000 to 1.000 (with three relevant decimals being calculated) and in general \( P_a + P_i < 1 \), since these probabilities are calculated independently. \( P_a \) and \( P_i \) can be considered to be measures of the compound under study belonging to the classes of active and inactive compounds respectively, or can be seen as estimates for the first and second kinds of errors in the prediction [9].

3. RESULTS

The anti-inflammatory effects of Alstonia scholaris bark extract were studied. Below are listed all of the study's findings.

**Preparation and Ethanolic Extract of Alstonia scholaris bark**

The *Alstonia scholaris* bark was produced as an ethanolic extract using the soxhlation method. The following formula was used to obtain the extract's % yield.

\[
\% \text{ yield of extract} = \left( \frac{\text{Amount of extract obtained}}{\text{Amount of powder used}} \right) \times 100
\]

\[
= \frac{60}{310} \times 100 = 19.35\% \text{ w/w.}
\]

**Preliminary Phytochemical Analysis**

The *Alstonia scholaris* bark ethanolic extract underwent a preliminary phytochemical screening that indicated the presence of flavonoids, tannins, steroids, glycosides, alkaloids, saponins, and terpenoids.

**Acute Toxicity Studies**

Even at 2000 mg/kg bd. wt., the ethanolic bark extract of *A. scholaris* did not reveal any signs of toxicity or mortality. Even after 14 days of surveillance, all animals were secure. Pharmacological tests were conducted at doses of 200 and 400 mg/kg bd. wt.

**In vivo Anti-Inflammatory Activity**

**Carrageenan paw induced model**

Carrageenan administration results in oedema and a change in paw thickness. The normal medication and the appropriate test extract were given to the animals. The animals received 0.1 ml of a 1% carrageenan solution in the sub plantar area of the left hind paw after an hour. Paw thickness is measured hourly from 0-4 hours.

The percentage inhibition in paw volume at the fourth hour was estimated after measuring the paw thickness at 1h, 2h, 3h and 4 h. When compared to the control group, ethanolic extracts of *Alstonia scholaris* bark at doses of 200 mg/kg bd.wt, 400 mg/kg bd.wt, and standard meloxicam (1 mg/kg bd.wt.) all significantly reduced paw thickness (\( p<0.001 \)). At doses of 200 mg/kg body weight, 400 mg/kg body weight, and the common medication meloxicam at 1 mg/kg body weight, the extract significantly reduced inflammation by 20.96%, 25.80%, and 79.03%, respectively.

![Fig. 1. Paw of carrageenan induced rat](image)

**Formalin induced paw edema model**

The animals were injected with 0.1 ml of a 2% formalin solution in the sub plantar area of the left hind paw. Paw thickness was measured from 0 to 4 hours, on hourly basis. This is countered by the administration of the test extract and standard drug.

The percentage inhibition in paw volume at 4 hours was estimated after measuring the paw thickness at 1 h, 2 h, 3 h and 4 h. When compared to the control group, ethanolic extract of *Alstonia scholaris* bark at doses of 200 mg/kg bd.wt, 400 mg/kg bd.wt, and conventional indomethacin (5 mg/kg bd.wt) resulted in a significant decrease in paw volume (\( p<0.0001 \)). The extract inhibited inflammation significantly by 53.62%, 63.76%, and 60.86% at doses of 200 mg/kg bd.wt, 400 mg/kg bd.wt, and 5 mg/kg bd.wt of indomethacin, respectively.
### Table 1. Effect of EEAS Carrageenan induced paw edema model

|                      | Change in paw thickness (mm) at different hours | % inhibition |
|----------------------|-----------------------------------------------|--------------|
|                      | 1hr | 2hr   | 3hr   | 4hr   |             |             |
| Control              | 0.62±0.028 | 0.62±0.028 | 0.62±0.02 | 0.62±0.02 | -           |             |
| Disease control      | 1.39±0.011 | 1.81±0.012 | 2.17±0.022 | 2.41±0.057 | -           |             |
| EEAS (200 mg/kg)     | 1.29±0.035 | 1.60±0.025 | 1.85±0.024 | 1.78±0.01  | 20.96%      |             |
| EEAS (400 mg/kg)     | 1.14±0.03 | 1.37±0.02 | 1.61±0.023 | 1.60±0.01  | 25.80%      |             |
| Meloxicam 1 mg/kg    | 0.79±0.027 | 1.08±0.022 | 1.27±0.03  | 0.92±0.048 | 79.03%      |             |

Values are expressed as Mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett’s test. Results were expressed as when compared to control group (* = p<0.0001, ** = p<0.001) disease control (a=p<0.0001), standard (A= p<0.0001) ns (non-significant).

### Table 2. Formalin induced paw edema model

|                      | Change in paw thickness (mm) | % inhibition |
|----------------------|-------------------------------|--------------|
|                      | 1hr | 2hr   | 3hr   | 4hr   |             |             |
| Control              | 0.69±0.02 | 0.69±0.02 | 0.69±0.02 | 0.69±0.02 | -           |             |
| Disease control      | 1.42±0.015 | 1.81±0.014 | 2.19±0.01 | 2.4±0.01 | -           |             |
| EEAS (200 mg/kg)     | 1.37±0.14 | 1.57±0.016 | 1.76±0.014 | 1.69±0.02 | 53.62%      |             |
| EEAS (400 mg/kg)     | 1.31±0.016 | 1.41±0.012 | 1.64±0.01 | 1.56±0.012 | 63.76%      |             |
| Indomethacin in 5mg/kg | 0.90±0.02 | 1.13±0.018 | 1.25±0.014 | 1.17±0.02 | 60.86%      |             |

Values are expressed as Mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnett’s test. Results were expressed as when compared to control group (* = p<0.0001). When compared to disease group (a = p<0.0001) and when compared to standard group (A= p<0.0001, B= p<0.001) ns = (non-significant).
In silico Analysis

Molecular docking

Table 3. MCULE docking scores

| Sl. no | Compounds               | 2AZ5  | 1IBC  | 6COX  | 4NOS  |
|--------|-------------------------|-------|-------|-------|-------|
| 1      | Vanillic acid           | -5.3  | -5.0  | -4.5  | -6.4  |
| 2      | Venoterpine             | -5.3  | -4.4  | -4.4  | -6.5  |
| 3      | Loganetin               | -6.1  | -5.0  | -4.6  | -5.8  |
| 4      | Dibutyl phthalate       | -6.4  | -5.0  | -4.1  | -8.0  |
| 5      | Guaia-3,9-diene         | -6.8  | -4.9  | -5.1  | -7.6  |
| 6      | 3,6-Bis[2-methylphenyl]-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione | -9.0  | -7.3  | -6.5  | -9.9  |
| 7      | Stigmasterol            | --    | -6.5  | -5.5  | -9.3  |
| 8      | Diospyrolide            | -6.8  | -6.2  | -4.4  | -7.3  |
| 9      | Pentanoic acid          | -3.9  | -3.7  | -3.1  | -4.6  |
| 10     | n-Hexadecanoic acid     | -5.0  | -3.8  | -3.7  | -5.7  |
| 11     | Meloxicam               | -7.2  | -6.3  | -5.3  | -10.0 |
| 12     | Indomethacin            | -7.7  | -6.4  | -4.9  | -9.0  |

G score = glide score, the more negative the Glide score, the more favorable the binding

Ramachandran plot analysis

Protein 2AZ5, 1IBC, 6COX and 4NOS were analyzed for Ramachandran plot to know amino acid presence in different regions of respective protein tabulated in table 4 and pictorial representation in Fig. 2.

Table 4. Ramachandran plot status with protein with 2AZ5, 1IBC, 6COX and 4NOS

| Residues                        | 2AZ5     | 1IBC     | 6COX     | 4NOS     |
|---------------------------------|----------|----------|----------|----------|
| Most favorable region (%)       | 90.2     | 87.8     | 74.1     | 89.4     |
| Additional allowed regions (%)  | 9.6      | 11.4     | 22.5     | 10.4     |
| Generously allowed regions (%)  | 0.2      | 0.9      | 2.5      | 0.2      |
| Disallowed regions (%)          | 0.0      | 0.0      | 0.8      | 0.0      |

![Ramachandran plot](image1.jpg)  
![Ramachandran plot](image2.jpg) 

a) 2AZ5  

b) 1IBC
Fig. 2. Ramachandran Plot showing different regions

PASS studies

Table 5. Anti-inflammatory activity predicted for the active constituents of *Alstonia scholaris* using PASS

| Sl. No | Compound                      | Probable Activity (Pa) | Probable Activity (Pi) | Biological Activity                                      |
|--------|-------------------------------|------------------------|------------------------|----------------------------------------------------------|
| 1      | Vanillic acid                 | 0.720                  | 0.002                  | Anti-inflammatory, intestinal                             |
|        |                               | 0.702                  | 0.016                  | Prostaglandin-E2 9-reductase inhibitor                    |
|        |                               | 0.670                  | 0.008                  | TNF expression inhibitor                                  |
|        |                               | 0.485                  | 0.010                  | NOS2 expression inhibitor                                 |
|        |                               | 0.395                  | 0.022                  | Non-steroidal anti-inflammatory agent                     |
| 2      | Venoterpine                   | 0.428                  | 0.048                  | TNF expression inhibitor                                  |
|        |                               | 0.372                  | 0.111                  | Anti-inflammatory                                         |
|        |                               | 0.235                  | 0.049                  | Interleukin 6 antagonist                                   |
|        |                               | 0.106                  | 0.100                  | Interleukin 1 antagonist                                   |
| 3      | Loganetin                     | 0.822                  | 0.005                  | Anti-inflammatory                                         |
| 4      | Dibutyl phthalate             | 0.497                  | 0.058                  | Anti-inflammatory                                         |
| 5      | 2H-1-Benzopyran-2-one,7-acetyl-8-[acetyloxy]-4-methyl | 0.460 | 0.069 | Anti-inflammatory |
|        |                               | 0.327                  | 0.086                  | TNF expression inhibitor                                  |
| 6      | Stigmasterol                  | 0.913                  | 0.004                  | Prostaglandin-E2 9-reductase inhibitor                    |
|        |                               | 0.344                  | 0.078                  | TNF expression inhibitor                                  |
| 7      | Diospyrolide                  | 0.640                  | 0.024                  | Anti-inflammatory                                         |
|        |                               | 0.326                  | 0.070                  | Prostaglandin-E2 9-reductase inhibitor                    |
| 8      | D-Friedoolean-14-en-3-one     | 0.306                  | 0.099                  | TNF expression inhibitor                                  |
|        |                               | 0.842                  | 0.055                  | Anti-inflammatory                                         |
|        |                               | 0.354                  | 0.064                  | Prostaglandin-E2 9-reductase inhibitor                    |
| 9      | Lupeol acetate                | 0.331                  | 0.084                  | TNF expression inhibitor                                  |
|        |                               | 0.737                  | 0.012                  | Anti-inflammatory                                         |
|        |                               | 0.494                  | 0.040                  | Prostaglandin-E2 9-reductase inhibitor                    |
| 10     | Pentanoic acid                | 0.646                  | 0.009                  | TNF expression inhibitor                                  |
| 11     | n-Hexadecanoic acid           | 0.646                  | 0.009                  | TNF expression inhibitor                                  |
| 12     | Betulin                       | 0.629                  | 0.026                  | Anti-inflammatory                                         |
| Sl. No | Compound | Probable Activity (Pa) | Probable Activity (Pi) | Biological Activity |
|-------|----------|------------------------|------------------------|---------------------|
| 13    | Meloxicam | 0.849                  | 0.005                  | Anti-inflammatory   |
|       |          | 0.465                  | 0.004                  | COX2 inhibitor      |
|       |          | 0.374                  | 0.025                  | Non-steroidal anti-inflammatory agent |
| 14    | Indomethacin | 0.755                | 0.004                  | Anti-inflammatory   |
|       |          | 0.440                  | 0.014                  | NOS2 expression inhibitor |
|       |          | 0.422                  | 0.004                  | Cyclooxygenase inhibitor |
|       |          | 0.311                  | 0.005                  | Cyclooxygenase 2 inhibitor |

Table 6. Adverse effects predicted for the active constituents of *Alstonia scholaris* using PASS (Prediction of Activity Spectra for Substances)

| Sl. No | Compound | Pa    | Pi    | Adverse effect |
|-------|----------|-------|-------|----------------|
| 1     | Vanillic acid | 0.424 | 0.241 | Hepatotoxicity |
|       |          | 0.329 | 0.129 | Nephrotoxicity |
|       |          | 0.303 | 0.300 | Arrhythmia     |
|       |          | 0.272 | 0.301 | Cardiac failure|
| 2     | Venoterpine | 0.338 | 0.138 | Cardiac failure|
|       |          | 0.315 | 0.281 | Arrhythmia     |
| 3     | Loganetin | 0.379 | 0.097 | Nephrotoxicity |
| 4     | Dibutyl phthalate | 0.418 | 0.064 | Myocardial infarction |
|       |          | 0.363 | 0.219 | Arrhythmia     |
|       |          | 0.329 | 0.129 | Nephrotoxicity |
| 5     | Guai-3,9-diene | 0.430 | 0.237 | Hepatotoxicity |
| 6     | 3,6-Bis[2-methylphenyl]-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione | 0.482 | 0.205 | Hepatotoxicity |
| 7     | 2H-1-Benzopyran-2-one,7-acetyl-8-[acetyloxy]-4-methyl | 0.558 | 0.032 | Cardiac failure |
|       |          | 0.494 | 0.198 | Hepatotoxicity |
|       |          | 0.384 | 0.198 | Arrhythmia     |
| 8     | Stigmasterol | -    | -     | No Adverse effects predicted. |
| 9     | Diospyroside | -    | -     | No Adverse effects predicted |
| 10    | D-Friedoolean-14-en-3-one | -    | -     | No Adverse effects predicted |
| 11    | Lupeol acetate | -    | -     | No Adverse effects predicted |
| 12    | Pentanoic acid | 0.500 | 0.050 | Nephrotoxicity |
|       |          | 0.474 | 0.029 | Hepatotoxicity |
| 13    | n-Hexadecanoic acid | 0.500 | 0.050 | Nephrotoxicity |
|       |          | 0.474 | 0.209 | Hepatotoxicity |
| 14    | Betulin | -    | -     | No Adverse effects predicted |
| 15    | Meloxicam | 0.969 | 0.003 | Cardiac failure |
|       |          | 0.955 | 0.004 | Myocardial infarction |
|       |          | 0.786 | 0.065 | Hepatotoxicity |
|       |          | 0.600 | 0.026 | Nephrotoxicity |
| 16    | Indomethacin | 0.902 | 0.026 | Hepatotoxicity |
|       |          | 0.802 | 0.007 | Myocardial infarction |
|       |          | 0.788 | 0.005 | Cardiac failure |
Table 7. Direct and possible target Prediction for the active constituents of *Alstonia scholaris* using PASS

| S. No | Compound | Direct Target | Confidence | Possible Target | Confidence |
|-------|----------|---------------|------------|----------------|------------|
| 1     | Vanillic acid | Prostanoid EP4 receptor | 0.1110 | Cyclooxygenase-1 | 0.2499 |
|       |          | Prostanoid EP1 receptor | 0.0628 | Histamine H2 receptor | 0.1568 |
|       |          | Prostanoid IP receptor | 0.0413 | Prostanoid EP2 receptor | 0.1018 |
|       |          | Interleukin-1 receptor-associated kinase 3 | 0.0284 | Prostaglandin E synthase | 0.0241 |
|       |          | Interleukin-1 receptor-associated kinase 3 | 0.0711 | Prostaglandin E synthase | 0.0241 |
| 2     | Venoterpine | TNF-alpha | 0.0211 | Prostanoid EP2 receptor | 0.0902 |
|       |          | Prostanoid IP receptor | 0.0970 | Prostanoid EP2 receptor | 0.0902 |
|       |          | Prostanoid IP receptor | 0.0296 | Prostanoid FP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Histamine H2 receptor | 0.1547 |
| 3     | Loganetin | TNF-alpha | 0.0970 | Prostanoid EP2 receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0296 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid EP4 receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid EP4 receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid EP4 receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
| 4     | Dibutyl phthalate | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
| 5     | 3,6-Bis[2-methylphenyl]-2,5-dihydropyrido[3,4-c]pyrrole-1,4-dione | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|      | 2H-1-Benzopyran-2-one,7-acetyl-8-[acetyloxy]-4-methyl | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0102 | Prostanoid IP receptor | 0.0507 |
| 7     | Stigmasterol | Arachidonate 15-lipoxygenase | 0.0918 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0888 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0888 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0888 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0888 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0888 | Prostanoid IP receptor | 0.0507 |
| 8     | Diospyrolide | TNF-alpha | 0.1476 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.1407 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.1583 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.1583 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.1583 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.1583 | Prostanoid IP receptor | 0.0507 |
| 9     | D-Friedoolean-14-en-3-one | Prostanoid IP receptor | 0.0517 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
| 10    | Lupeol acetate | Prostanoid IP receptor | 0.0517 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
|       |          | Prostanoid IP receptor | 0.0354 | Prostanoid IP receptor | 0.0507 |
| 11    | Pentanoic acid | Prostanoid EP2 receptor | 0.0311 | Prostanoid EP2 receptor | 0.2239 |
|       |          | Prostanoid EP2 receptor | 0.1355 | Prostanoid EP2 receptor | 0.2239 |
|       |          | Prostanoid EP2 receptor | 0.1355 | Prostanoid EP2 receptor | 0.2239 |
|       |          | Prostanoid EP2 receptor | 0.1355 | Prostanoid EP2 receptor | 0.2239 |
|       |          | Prostanoid EP2 receptor | 0.1355 | Prostanoid EP2 receptor | 0.2239 |
|       |          | Prostanoid EP2 receptor | 0.1355 | Prostanoid EP2 receptor | 0.2239 |

50
| S. No | Compound       | Direct Target             | Confidence | Possible Target                | Confidence |
|-------|----------------|---------------------------|------------|-------------------------------|------------|
|       |                | receptor                  | 0.1349     | Histamine H2 receptor         | 0.1167     |
|       | Prostanoid EP4 | receptor                  | 0.1249     | Arachidonate 5- lipoxygenase  | 0.0286     |
|       | Prostaglandin E synthase | Arachidonate 5- lipoxygenase | 0.2762 | Serotonin 4 (5-HT4) receptor | 0.0250     |
|       | Cyclooxygenase-1 | Nitric oxide synthase, inducible | 0.0248 | Prostanoid EP4 receptor       | 0.0331     |
|       | Prostanoid EP3 receptor | Prostanoid EP3 receptor | 0.0038 | Prostanoid EP3 receptor       | 0.0200     |
|       | Prostanoid EP1 receptor | Prostanoid EP1 receptor | 0.0026 | -                             |            |
| 12    | n-Hexadecanoic acid | TNF-alpha             | 0.0311     | Prostanoid EP2 receptor       | 0.2239     |
|       |                | Prostanoid EP2 receptor | 0.1355     | -                             |            |
|       |                | Arachidonate 5- lipoxygenase | 0.2762 | Cyclooxygenase-1             | 0.1303     |
|       |                | Prostanoid EP4 receptor | 0.1349     | Histamine H2 receptor         | 0.1161     |
|       |                | Prostaglandin E synthase | 0.1249     | Prostaglandin E synthase      | 0.0706     |
|       |                | Nitric oxide synthase, inducible | 0.0248 | Serotonin 2 (5-HT2) receptor | 0.0268     |
|       |                | Cyclooxygenase-1         | 0.0779     | Prostanoid DP receptor        | 0.0377     |
|       |                | TNF-alpha                | 0.1760     | -                             |            |
|       | Prostanoid IP receptor | Nitric oxide synthase, inducible | 0.1391 | -                             |            |
|       |                | Prostanoid IP receptor   | 0.0343     | -                             |            |
| 13    | Betulin        | Arachidonate 5- lipoxygenase | 0.0529 | Cyclooxygenase-1             | 0.4676     |
|       |                | Prostaglandin E synthase | 0.1611     | Cyclooxygenase-2              | 0.1015     |
| 14    | Meloxicam      | Arachidonate 5- lipoxygenase | 0.4069 | Cyclooxygenase-1             | 0.2158     |
|       |                | Prostaglandin E synthase | 0.3388     | Arachidonate 5- lipoxygenase  | 0.0084     |
| 15    | Indomethacin   | Cyclooxygenase-1         | 0.0842     | -                             |            |
|       |                | Cyclooxygenase-2         | 0.0610     | -                             |            |

4. DISCUSSION

Anti-Inflammatory Activity

The immune system's reaction to adverse stimuli like pathogens, damaged cells, poisonous substances, or radiation is inflammation, which has the dual purpose of eliminating harmful stimuli and starting the healing process. Therefore, inflammation is a defence process that is essential for health [10]. A well-known test for evaluating the efficacy of anti-inflammatory drugs involves the induction of rat oedema by carrageenan. This phlogistic drug causes biphasic oedema, which is characterized by neutrophil infiltration and the production of prostaglandin E2, cytokines (mostly IL-1), and NO in the second phase, and histamine and serotonin release from mast cells in the first phase [11]. The formalin test produces a biphasic reaction, with the first phase being the immediate, painful neurogenic effect of formalin. Prostaglandin, serotonin, histamine, bradykinin, and cytokines such interleukin-1 beta, interleukin-6 tumour necrosis factor-alpha, and nitric oxide have a role in the second phase of the inflammatory reactions [12]. The various phytochemical active constituents identified in the ethanolic bark extract of *Alstonia scholaris* were saponins, steroids, alkaloids, flavonoids, phenols, fatty acids, carbohydrates, triterpenoids and tannins. Triterpenoid luteol acetate's (anti-inflammatory) properties are likely due to its capacity to stop the synthesis of pro-
inflammatory mediators like TNF- and IL-1 [13]. It's interesting to note that a number of in vitro studies have demonstrated that betulinic acid (BA) can suppress the generation of NO, primarily in macrophage cultures activated by bacterial lipopolysaccharide (LPS) and/or interferon gamma (IFN-γ) [14]. Additionally, COX-2 activity is inhibited by BA, which reduces the production of prostaglandin E2 (PGE2) [15]. Numerous studies have shown that palmitic acid and its derivatives decrease heat nociception whereas fatty acids (Palmitic acid) lower prostaglandin and leukotriene levels [16]. By blocking the mediators of acute inflammation, β-sitosterol (Steroid) demonstrated a considerable suppression of carrageenan-induced rat paw inflammation, showing its anti-inflammatory potential. Additionally, it was demonstrated that stigmasterol and β-sitosterol, whether in their free or ester forms, have strong anti-inflammatory properties [17]. Stigmasterol (Steroids) inhibit inflammatory mediators such as histamine, 5-hydroxytryptamine, bradykinin, serotonin and prostaglandins [18]. Vanillic acid (Phenol) shown anti-inflammatory effect by reducing hyperalgesia, leukocyte recruitment, oxidative stress, IL-33, TNF, and IL-1 production [19].

**Molecular Docking Studies**

In the area of computer-based drug research, wherein small molecules are screened by positioning and scoring them in a protein's binding site, molecular docking still shows a lot of potential. Using MOCLE software, docking analyses of isolated chemicals from *Alstonia scholaris* ethanolic bark extract and common medications were performed. The various constituents identified in the plant extract are vanillic acid, venoterpine, loganetin, dibutyl phthalate, Guai-a-3,9-diene, 3,6-Bis[2-methylphenyl]-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione, 2H-1-Benzopyran-2-one,7-acetyl-8-[acyloxy]-4-methyl-, stigmasterol, diospyrolide, D-Friedoolean-14-en-3-one, lupeol acetate, pentanoic acid, n-Hexadecanoic acid, betulin, and standard drug meloxicam and indomethacin were subjected to docking against PDB ID: 2AZ5, 1IBC, 6COX and 4NOS [7]. The docking results shown that vanillic acid, venoterpine, loganetin, dibutyl phthalate, guai-a-3,9-diene, 3,6-Bis [2-methylphenyl]-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione, n-hexadecanoic acid, stigmasterol, diospyrolide, D-Friedoolean-14-en-3-one, pentanoic acid and standard drug meloxicam and indomethacin have shown highest glide scores with all the selected proteins which indicate a stronger receptor-ligand binding affinity.

Docking results made it abundantly evident that the phytochemical components stated above might have exhibited an anti-inflammatory mechanism similar to that of well-known medications like meloxicam and indomethacin. The discovered proteins, PDB ID: 2AZ5, 1IBC, 6COX, and 4NOS, are modelled. The PROCHECK programme and the Ramachandran plot were used to examine the 3D model's characteristics. The Ramachandran plot makes it clear that predicted models have the most advantageous regions, as well as additional allowed regions, usually allowed regions, and disallowed regions. A Ramachandran plot analysis of the percentage distribution of the protein residues reveals whether the anticipated models are accurate or not. A high-quality model should have over 90% of the most preferred region, according to the Ramachandran plot. Proteins such PDB ID: 2AZ5, 1IBC, 6COX, and 4NOS displayed favored regions in the range of 70-90%, which amply demonstrated the high calibre of the models chosen for the current investigation.

**PASS Software**

A useful method for identifying prospective molecules and the biological activity of certain phytoconstituents for their anti-inflammatory effect is the prediction of activity spectra of substances (PASS). Selected phytoconstituents' anti-inflammatory properties were predicted using the canonical simplified molecular-input line-entry system from PubChem.com and PASS online. It was anticipated that some phytoconstituents might exert their effects more effectively than commercially available medications. On the other hand, a number of new directions in which the in vitro and in vivo assessment of the phytoconstituents can be done based on the expected activities of PASS were predicted. The search process should be streamlined more effectively for the researchers. Prediction of activity spectra of substances (PASS) is a tool that can anticipate the pharmacological properties of a substance in advance and aids in the screening of potentially useful pharmacological leads for a specific ailment. It forecasts the range of potential organic actions for a molecule in terms of potential activity (Pa) and potential inactivity (Pi) [19]. Selected active phytochemical constituents of *Alstonia scholaris* and standard drugs were subjected to pass software for anti-inflammatory activity.
The results of these active constituents like probable activity (Pa) and probable inactiveness (Pi) and biological activity were given in table 5. The possible interventions of selected active constituents of *Alstonia scholaris* were found to be Anti-inflammatory, intestinal, Prostaglandin-E2 9-reductase inhibitor, TNF expression inhibitor, NOS2 expression inhibitor, Interleukin 6 antagonist, Interleukin 1 antagonist, and Cyclooxygenase inhibitor. Selected active phytochemical constituents of *Alstonia scholaris* were subjected to pass software for adverse effects and the results were tabulated in table 6. From the results, the constituent’s like stigmasterol, diospyrolide, D-Friedoolean-14-en-3-one, lupeol acetate, betulin, were found to be free from any adverse effects whereas the remaining constituents and standard drugs were predicted with hepatotoxicity, nephrotoxicity, cardiac failure and arrhythmia and myocardial infarction. Selected active phytochemical constituents of *Alstonia scholaris* were subjected to pass software for direct and possible targets and results were given in Table 7.

All the phytoconstituents and standard drugs were found to have interventions with Prostanoid EP4 receptor, Prostanoid EP2 receptor, Prostanoid EP1 receptor, Cyclooxygenase-1, Cyclooxygenase-2, Histamine H1 receptor, Histamine H2 receptor, TNF-alpha, Arachidonate 5-lipoxygenase and Nitric oxide synthase, inducible.

According to the aforementioned, PASS is a crucial mechanism for clearly displaying the compounds of interest for the targeted biological activities. This aids the researchers in justifying their work.

5. CONCLUSION

According to *in vivo* and *in silico* investigations the ethanolic bark extract of *Alstonia scholaris* clearly has anti-inflammatory efficacy in rodents models. More research is required to identify the specific phytochemical components of the extract and determine the precise mechanism underlying its anti-inflammatory effect.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

Animal Ethic committee approval has been collected and preserved by the author(s).

ACKNOWLEDGEMENT

The authors are grateful to the management of the Gokaraju Rangaraju College of pharmacy, for the constant support and encouragement during the course of the work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mohan H. Text book of pathology. 7th Ed.[S.L]: Jaypee brothers medicals P, 2015; 533-537.
2. Shelar P, Mishra A. Animal Models of Inflammation for Assessment of Anti-Inflammatory Drugs. SGVU Journal of Pharmaceutical Research & Education, 2020;5(2): 490-508.
3. Kaushik P, Kaushik D, Sharma N, Rana A. *Alstonia scholaris* It’s Phytochemistry and pharmacology 2011;2(2): 361-379.
4. Karim N, Khan I, Khan W, Khan I, Khan A, Halim SA, Khan H, Hussain J, Al-Harrasi A. Anti-nociceptive and Anti-inflammatory Activities of Asparacosin A Involve Selective Cyclooxygenase 2 and Inflammatory Cytokines Inhibition An *in vitro*, *in vivo*, and *in-silico* Approach. Prime Archives in Immunology 2019; 2020:1-28.
5. Solanki HK, Shah DA, Maheriya PM, Patel CA. Evaluation of Anti Inflammatory Activity of Probiotic On Carrageenan-Induced Paw Edema In Wistar Rats. International Journal of Biological Macromolecules. 2015;72:1277–1282
6. Soyocak A, Kurt H, Cosan DT, Saydam F, Calis IU, Kolac UK, Koroglu ZO, Degirmenci I, Mutlu FS, Gunes HV. Tannic acid exhibits anti-inflammatory effects on formalin-induced paw edema model of inflammation in rats. Human & Experimental Toxicology. 2019:1296-1301.
7. Suvarchala Reddy VNVL, Anarthe SJ, Raju MG, Akhila M, Raj GBP. Molecular docking studies of isolated compounds from *Cassia fistula* on HMG-COA reductase. Asian Journal of Research in Chemistry. 2019;12(2):89-93.
8. Raju MG, Yadav EV, Reddy VNVLS, Nicholas M. Pharmacological and *In silico* Evaluation of Methanolic Flower Extract of *Tagetes patula* as Antidepressant and Anxiolytic. Bulletin of Environment,
Pharmacology and Life Sciences. 2021; 10(3):29-35.

9. Raju MG, Yadav V, Reddy VNVLS. Natural Compounds AS D2 Receptor Agonist, M4 Receptor Antagonist and ACHE Modulator: Mechanistic and in Silico Modelling Studies. Journal of Research in Medical and Dental Science. 2021; 9(6):26-35.

10. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017; 9(6):7204–7218.

11. Coura CS, Souza RB, Rodrigues JG, Vanderlei ES, Araújo IN, Ribeiro NA, Frota AF. Mechanisms Involved in the Anti-Inflammatory Action of a Polysulfated Fraction from Gracilaria cornea in Rats. Plosone 2015;10(3):0119319

12. Arzi A, Olapour S, Yaghooti H, Karampour NS. Effect of Royal Jelly on Formalin Induced-Inflammation in Rat Hind Paw. Jhundishapur Journal of pharmaceutical products. 2015; 10(1):22466.

13. Lucetti D, Lucett CP, Bandeira M, Veras NH, Silva AH and Leal LK. Anti-inflammatory effects and possible mechanism of action of lupeol acetate isolated from Himatanthus drasticus (Mart.) Plumel Journal of Inflammation. 2010;7 (60):1-11.

14. Dunstan C, Liu B, Welch CJ, Perera P, Bohlin L. Alphitol, a Phenolic Substance from Alphitonia zizyphoides Which Inhibits Prostaglandin Biosynthesis In Vitro. Phytochemistry 1998;48:495–497.

15. Yun Y, Han S, Park E, Yim D, Lee S, Lee CK, Cho K, Kim K. Immunomodulatory Activity of Betulinic Acid by Producing Pro-inflammatory Cytokines and Activation of Macrophages. Archives of Pharmacal Research. 2003;26:1087–1095.

16. Erdogan TF, Akkol EK, Suntar I, and Gonenc TM. Fatty Acid Compositions and Anti-inflammatory Activities of Tripleurospermum parviflorum (Willd.) Pobed and Tripleurospermum tenuifolium ( Kit.). Records of natural products 2015; 9(3):394-403.

17. Hassan EM, Matloub AA, Aboutabl ME, Ibrahim NA, Mohamed SM. Assessment of anti-inflammatory, antinociceptive, immunomodulatory, and antioxidant activities of Cajanus cajan L. seeds cultivated in Egypt and its phytochemical composition, Pharmaceutical Biology. 2016;54:8, 1380-1391.

18. Boddawar GD, Dhwale SC, Shaikh SS. Assessment of anti-inflammatory potential of Sesbania bispinosa Linn leaf extracts and fractions by acute and chronic models. Alexandria Journal of Medicine, 2016; 52:289-293.

19. Staurengo-Ferrari L, Badaro-Garcia S, Hohmann MSN, Manchope MF, Zaninelli TH, Casagrande R, Verri WA Jr. Contribution of Nrf2 Modulation to the Mechanism of Action of Analgesic and Anti-inflammatory Drugs in Preclinical and Clinical Stages. Front Pharmacol. 2019; 9:1536.