Original Research Article

IN VIVO AND *IN SILICO* ANTI INFLAMMATORY STUDIES OF *ALSTONIA SCHOLARIS* BARK EXTRACT

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 at a dose of 200 mg/kg bd.wt., 400 mg/kg bd.wt., and standard drug meloxicam at 1 mg/kg bd.wt. and indomethacin at 5 mg/kg bd.wt, showed significant inhibition in both the animal models. To understand the ligand-binding affinity of the active constituents of the extract, docking studies were performed for natural compounds against PDB ID: 2AZ5, PDB ID: 1IBC, PDB ID: 6COX and PDB ID: 4NOS. The docking results showed that the phyto constituents from the extract and standard drugs meloxicam and indometacin had shown highest glide scores with all the selected proteins which indicate a stronger receptor-ligand binding affinity. 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 Interleukin 1 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 as direct targets and indirect 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.

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. Depending upon the defense capacity of the host and duration of response, inflammation can be classified as acute and chronic [1]. Carrageenan stimulates phospholipase A2 while the progress of inflammation occurs due to cytotoxic effects. This model causes activation of cyclo-oxygenase pathway. The edema produced by carrageenan shows biphasic curve. Formalin induces biphasic inflammation where the early phase is neurogenic phase conciliated by substance-P and bradykinin. While the later phase shows involvement of prostaglandins, 5-HT, histamine and bradykinin. The drugs like opioids suppress both phases while the drugs like NSAIDs and corticosteroids inhibit the second phase [2].

Alstonia scholaris, commonly called blackboard tree or devil's tree in English, is an evergreen tropical tree in the family Apocynaceae. 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

2.1 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.

2.2 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.

2.3 Preliminary Phytochemical Analysis

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

2.4 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).

2.5 Experimental Protocol

Wistar albino rats (approx 200-250 gms) 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.

2.6 *In vivo* methods for evaluation of anti-inflammatory activity:

2.6.1 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, leukotriene's, and various cytokines such as IL-1β, IL-6, IL-10, and TNFα. [4].

Thirty healthy Albino rats of either sex weighing 200-250 gms 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:

$$\%\text{inhibition} = \frac{(V_C - V_t) \times 100}{V_C}$$

2.6.2 Formalin induced paw edema model:

Thirty healthy Albino rats of either sex weighing 200-250 gms 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].

2.7 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- nonsignificant.

2.8 *In silico* analysis

2.8.1 Molecular Docking Studies

2.8.1.1 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 (Ganga Raju M et al., 2021). The selected phytochemical constituents of the extract were docked against protein ID: 2AZ5, 1IBC, 6COX and 4NOS.

2.8.1.2 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).

2.8.1.3 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 [7].

2.8.2. Pass Software:

2.8.2.1 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: Pa - the probability of the compound being active and Pi - the probability of being inactive. Being probabilities, the Pa and Pi values vary from 0.000 to 1.000 (with three relevant decimals being calculated) and in general Pa + Pi < 1, since these probabilities are calculated independently. Pa and Pi 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[8].

3. RESULTS

Ethanolic bark extract of *Alstonia scholaris* bark were explored for its anti inflammatory activity. All the results in this study are included below.

3.1 Preparation and ethanolic extract of *Alstonia scholaris* bark:

Ethanolic bark extract of *Alstonia scholaris* bark was prepared by soxhlation technique. The percentage yield of the extract was calculated by using the following formula.

$$\% \text{ yield of extract} = \frac{\text{Amount of extract obtained}}{\text{Amount of powder used}} \times 100$$

$$= 60/310 \times 100$$

$$= 19.35\% \text{ w/w.}$$

3.2 Preliminary phytochemical analysis

The preliminary phytochemical investigation for ethanolic extract of *Alstonia scholaris* bark showed the presence of flavonoids, tannins, steroids, glycosides, alkaloids, saponins, and terpenoids.

3.3 Acute toxicity studies

Ethanolic bark extract of *A. scholaris* did not exhibit any signs of toxicity and mortality even up to 2000 mg/kg. bd.wt. All animals were safe even after 14 days of observation. The pharmacological evaluations were done at 200 and 400 mg/kg bd.wt.

3.4 *In vivo* anti-inflammatory activity:

3.4.1 Carrageenan paw induced model

Administration of carrageenan into the paw causes edema and change in paw thickness. The animals were administrated with their respective test extract and the standard drug. After 1 hour the animals were administrated with 0.1 ml of 1% solution of carrageenan in to the sub plantar region of left hind paw. Paw thickness is measured hourly from 0-4 hours.

Table 1: Effect of EEAS Carrageenan induced paw edema model

	Change in paw thickness (mm) at different hours				
	1hr	**2hr**	**3hr**	**4hr**	**% inhibition**
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* ns A	1.60±0.025* aA	1.85±0.024* aA	1.78±0.01^{*n} sA	20.96%
EEAS (400 mg/kg)	1.14±0.03^{*a} A	1.37±0.02^{*a} A	1.61±0.023* aA	1.60±0.01^{*a} A	25.80%
Meloxicam 1 mg /kg	0.79±0.027* ^{*a}	1.08±0.022* a	1.27±0.03^{*a}	0.92±0.048* a	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<0.001) ns (non significant).

The paw thickness at 1h, 2h, 3h, and 4h were measured and the percentage inhibition in paw volume at the 4th hour was calculated. A significant reduction in paw thickness of

ethanolic extract of *Alstonia scholaris* bark at the dose of 200 mg/kg bd.wt ($p<0.001$), 400 mg/kg bd.wt ($p<0.001$) and standard meloxicam (1 mg/kg bd.wt.) ($p<0.05$) was observed when compared to the control group. The extract at a dose of 200 mg/kg bd.wt., 400 mg/kg bd.wt., and standard drug meloxicam at 1 mg/kg bd.wt., showed a significant inhibition of inflammation by 20.96%, 25.80% and 79.03% respectively.

Figure 1: Paw of carrageenan induced rat

3.4.2 Formalin induced paw edema model:

Administration of formalin into the paw causes edema and change in paw thickness. The animals were administrated with their respective test extract and the standard drug. After 1 hour the animals were administrated with 0.1 ml of 2% solution of formalin in to the sub plantar region of left hind paw. Paw thickness is measured hourly from 0-4 hours.

Table 2: Formalin induced paw edema model

	Change in paw thickness (mm)				
	1hr	**2hr**	**3hr**	**4hr**	**% inhibition**
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^{*nsA}	1.57±0.016^{*aA}	1.76±0.014^{*aA}	1.69±0.02^{*aA}	53.62%
EEAS (400 mg/kg)	1.31±0.016^{*nsB}	1.41±0.012^{*aA}	1.64±0.01^{*aA}	1.56±0.012^{*aA}	63.76%
Indomethacin in 5mg/kg	0.90±0.02^{nsa}	1.13±0.018^{*a}	1.25±0.014^{*a}	1.17±0.02^{*a}	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).

The paw thickness at 1h, 2hr, 3hr, and 4hr were measured and the percentage inhibition in paw volume at the 4 hrs was calculated. A significant reduction in paw volume of ethanolic extract of *Alstonia scholaris* bark at the dose of 200 mg/kg bd.wt (p<0.0001), 400 mg/kg bd.wt (p<0.0001) and standard indomethacin (5 mg/kg bd.wt) (p<0.001) was observed when compared to the control group. The extract at dose of 200 mg/kg bd.wt, 400 mg/kg bd.wt, and standard drug indomethacin at 5 mg/kg bd.wt, showed a significant inhibition of inflammation by 53.62%, 63.76% and 60.86% respectively.

3.5 *In silico* analysis

3.5.1 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

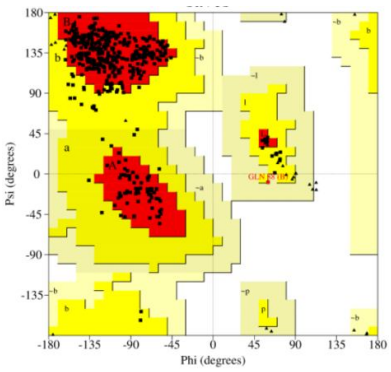
G score = glide score, the more negative the Glide score, the more favorable the binding.

3.5.2 Ramachandran plot Analysis

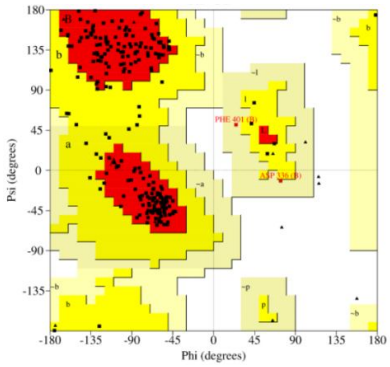
Protein 2AZ5, 1IBC, 6COX and 4NOS were analyzed for Ramachandran plot to know amino acid presence in different regions of respective protein tabulated in table 4 and pictorial representation in figure 2.

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

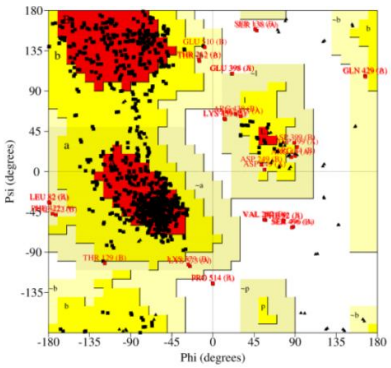
Residues	2AZ5	1IBC	6COX	4NOS
Most favorable region (%)	90.2	87.8	74.1	89.4
Additional allowed regions (%)	9.6	11.4	22.5	10.4
Generously allowed regions (%)	0.2	0.9	2.5	0.2
Disallowed regions (%)	0.0	0.0	0.8	0.0



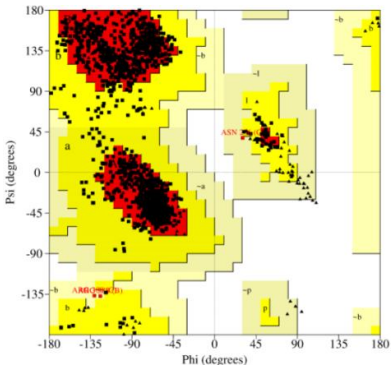
a) 2AZ5



b) 1IBC



c) 6COX



d) 4NOS

Figure 2: Ramachandran Plot showing different regions

3.5.3 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
		0,368	0,068	TNF expression inhibitor
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
		0,306	0,099	TNF expression inhibitor
8	D-Friedoolean-14-en-3-one	0,842	0,005	Anti inflammatory
		0,354	0,064	Prostaglandin-E2 9-reductase inhibitor
		0,331	0,084	TNF expression inhibitor

9	Lupeol acetate	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
13	Meloxicam	0,849	0,005	Anti inflammatory
		0,465	0,004	Cyclooxygenase inhibitor
		0,374	0,025	Non-steroidal anti inflammatory agent
14	Indomethacin	0,755	0,004	Non-steroidal anti inflammatory agent
		0,440	0,014	NOS2 expression inhibitor
		0,422	0,004	Cyclo oxygenase inhibitor
		0,311	0,005	Cyclo oxygenase 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	Guaia-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	Diospyrolide	-	-	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
		0.768	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
		Cyclooxygenase-2	0.0628	Histamine H2 receptor	0.1568

		Prostanoid EP1 receptor	0.0413	Prostanoid EP2 receptor	0.1018
		Cyclooxygenase-1	0.0284	Prostaglandin E synthase	0.0241
		Interleukin-1 receptor-associated kinase 3	0.0711		
2	Venoterpine	TNF-alpha	0.0211	Prostanoid EP2 receptor	0.0902
		Interleukin-1 receptor-associated kinase 3	0.0051	Serotonin 1e (5-HT1e) receptor	0.0625
		Prostanoid EP4 receptor	0.0102	Prostanoid IP receptor	0.0138
3	Loganetin	TNF-alpha	0.0970	Prostanoid EP2 receptor	0.0556
		Prostanoid IP receptor	0.0296	Prostanoid FP receptor	0.0507
4	Dibutyl phthalate	Serotonin 3a (5-HT3a) receptor	0.1103	Histamine H2 receptor	0.1547
		Arachidonate 5-lipoxygenase	0.0694	Cyclooxygenase-1	0.0087
		Prostanoid EP4 receptor	0.1012		
		Prostanoid IP receptor	0.0733	Prostaglandin E synthase	0.0499
		Prostaglandin E synthase	0.0024		
5	3,6-Bis[2-methylphenyl]-2,5-dihydropyrrolo[3,4- c]pyrrole-1,4-dione	Interleukin-1 receptor-associated kinase 4	0.4273	Serotonin 1e (5-HT1e) receptor	0.2458
				Prostaglandin E synthase	0.0348
				Arachidonate 5-lipoxygenase	0.490
				Serotonin 5a (5-HT5a) receptor	0.0315
6	2H-1-Benzopyran-2-one,7-acetyl-8-[acetyloxy]-4-methyl	Arachidonate 15-lipoxygenase	0.0918	Serotonin 1e (5-HT1e) receptor	0.3903
7	Stigmasterol	TNF-alpha	0.1476	-	-
		Prostanoid IP receptor	0.1407		

8	Diospyrolide	TNF-alpha	0.1583	-	-
		Prostanoid IP receptor	0.0888		
		Nitric oxide synthase, inducible	0.0380		
9	D-Friedoolean-14-en-3-one	Prostanoid IP receptor	0.0517	Cyclooxygenase-2	0.0032
		TNF-alpha	0.3590		
		Nitric oxide synthase, inducible	0.2432		
		Arachidonate 15-lipoxygenase	0.0736		
10	Lupeol acetate	Prostanoid IP receptor	0.1050	-	-
		TNF-alpha	0.1610		
		Nitric oxide synthase, inducible	0.0354		
11	Pentanoic acid	TNF-alpha	0.0311	Prostanoid EP2 receptor	0.2239
		Prostanoid EP2 receptor	0.1355	Cyclooxygenase-1	0.1303
		Prostanoid EP4 receptor	0.1349	Histamine H2 receptor	0.1167
		Prostaglandin E synthase	0.1249	Arachidonate 5-lipoxygenase	0.0286
		Arachidonate 5-lipoxygenase	0.2762	Serotonin 4 (5-HT4) receptor	0.0250
		Cyclooxygenase-1	0.0779	Prostanoid EP4 receptor	0.0331
		Nitric oxide synthase, inducible	0.0248	Prostanoid IP receptor	0.0262
		Prostanoid EP3 receptor	0.0038	Prostanoid EP3 receptor	0.0200
		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	0.1349	Histamine H2	0.1161

		receptor		receptor	
		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
13	Betulin	TNF-alpha	0.1760	-	-
		Prostanoid IP receptor	0.1391		
		Nitric oxide synthase, inducible	0.0343		
14	Meloxicam	Arachidonate 5-lipoxygenase	0.0529	Cyclooxygenase-1	0.4676
				Cyclooxygenase-2	0.1015
		Prostaglandin E synthase	0.1611		
15	Indomethacin	Cyclooxygenase-1	0.4069	Cyclooxygenase-1	0.2158
		Cyclooxygenase-2	0.3388	Arachidonate 5-lipoxygenase	0.0084
		Arachidonate 5-lipoxygenase	0.0842		
		Histamine H1 receptor	0.0610		

4. DISCUSSION

4.1 Anti-inflammatory activity

Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation, and acts by removing injurious stimuli and initiating the healing process. Inflammation is therefore a defense mechanism that is vital to health [9]. Carrageenan-induced rat paw edema is a classic test for examining the effectiveness of anti-inflammatory agents. This phlogistic agent evokes biphasic edema, the first phase of which is mediated by the release of histamine and serotonin from mast cells and the second phase of which involves neutrophil infiltration and the release of prostaglandin E2, cytokines (mainly IL-1β) and NO [10]. Formalin test is a biphasic response where first phase is the direct effect of formalin, which involves neurogenic pain. The second phase is

involved in the inflammatory reactions mediated by prostaglandin, serotonin, histamine, bradykinin and cytokines, such as interleukin-1 beta, interleukin-6 tumor necrosis factor-alpha eicosanoids, and Nitric Oxide [11]. 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. Lupeol acetate (Triterpenoid) possessed an anti-inflammatory activity which is probably related to its ability to prevent the production of pro-inflammatory mediators, such as TNF-α and IL-1β [12]. Interestingly, several in vitro studies have shown that Betulinic acid (BA) can inhibit the production of NO, mainly in macrophages cultures stimulated with bacterial lipopolysaccharide (LPS) and/or interferon gamma (IFN-ɣ) (Yun et al., 2003). BA also inhibits cyclooxygenase-2 (COX-2) activity and, therefore, decrease prostaglandin E2 (PGE2) synthesis (Dustan et al., 1998). Several studies have indicated that fatty acids (Palmitic acid) reduce the levels of prostaglandins and leukotriene's while Palmitic acid and its derivatives decreased thermal nociception [13]. β- sitosterol (Steroids) exhibited a marked inhibition of carrageenan-induced rat paw inflammation by inhibiting the mediators of acute inflammation; indicating its anti-inflammatory activity. Furthermore, proved that β-sitosterol and stigmasterol, either in free or ester forms have significant anti inflammatory activity [14]. Stigmasterol (Steroids) inhibit of inflammatory mediators such as histamine, 5-hydroxytryptamine, bradykinin, serotonin and prostaglandins [15]. Vanillic acid (Phenol) mitigated hyperalgesia, leukocyte recruitment, oxidative stress, IL-33, TNFα, and IL-1β production, indicating its anti-inflammatory activity [16].

4.2 Molecular docking studies

Molecular docking continues to holds great promise in the field of computer-based drug design which screens small molecules by orienting and scoring them in the binding site of a protein. Docking analysis of isolated compounds from ethanolic bark extract of *Alstonia scholaris* and standard drugs were carried out using MCULE software. The various constituents identified in the plant extract are vanillic acid, venoterpine, loganetin, dibutyl phthalate, Guaia-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-[acetyloxy]-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.

The docking results shown that vanillic acid, venoterpine, loganetin, dibutyl phthalate,

guaia-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 indometacin have shown highest glide scores with all the selected proteins which indicate a stronger receptor-ligand binding affinity.

Docking results clearly indicated that the chemical constituents mentioned above might have shown similar mechanism to that of the standard drugs like meloxicam and indomethacin as anti-inflammatory agents. The proteins identified namely PDB ID: 2AZ5, 1IBC, 6COX and 4NOS are modeled and the qualities of the 3D model were evaluated using the PROCHECK program and assessed using the Ramachandran plot. It is evident from the Ramachandran plot that predicted models have most favorable regions, additionally allowed regions, generally allowed regions and disallowed regions. Such a percentage distribution of the protein residues determined by Ramachandran plot shows that the predicted models are of good quality or not. According to Ramachandran plot a good quality model would be expected to have over greater than 90% in the most favored region. Proteins like PDB ID: 2AZ5, 1IBC, 6COX and 4NOS showed favored region in the range of 70-90% which clearly indicates that the selected models in the present study are of good quality.

4.3 PASS software:

Prediction of activity spectra of substances (PASS) is a valuable interface that should be adopted as an archetypal tool for predicting the potential molecules and to predict the biological activity of certain phytoconstituents for their anti-inflammatory activity. The anti-inflammatory activity of selected phytoconstituents were predicted by engaging the canonical simplified molecular-input line-entry system obtained from PubChem.com followed by using PASS online. Several phytoconstituents were predicted to have effects better than marketed drugs. On the other hand, several new paths were predicted in which the *in vitro* and *in vivo* evaluation of the phytoconstituents can be made on the basis of PASS predicted activities. It would allow the researchers to streamline the lookup more efficiently. Prediction of activity spectra of substances (PASS) is such a device which can predict the pharmacological homes beforehand and would help in screening pharmacological manageable leads for a particular condition. It predicts the spectra of organic things to do for a molecule in terms of probable activity (Pa) and probable inactiveness (Pi) [16]. 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.

From the above PASS is an important tool for effectively showing the compounds of interest for the biological actions of interest. This helps the researchers to rationalize the research.

5. CONCLUSION

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

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