IJBPAS, September, Special Issue, 2021, 10(9): 709-722
ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)
'A Bridge Between Laboratory and Reader'
www.ijbpas.com

PHYTOCHEMICAL INVESTIGATION, ANTIOXIDANT, AND ANTI-INFLAMMATORY STUDIES OF *Atylosia goensis* Dalzell

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Received 25th June 2021; Revised 28th July 2021; Accepted 29th Aug. 2021; Available online 25th Sept. 2021

https://doi.org/10.31032/IJBPAS/2021/10.9.1055

ABSTRACT

Plant-based medicine gained importance due to its safety margin and multiple benefits. Regular intake of antioxidants is an alternative to avoid serious illness. The current investigation aims to measure the phenolic, flavonoid content, the antioxidant activity of *Atylosia goensis* Dalzell, and its anti-inflammatory profile. Total phenolic content was estimated by standard colorimetric assays and antioxidant activity was screened using DPPH and Nitric Oxide free radical scavenging assay. The extracts were screened for *In vitro* COX enzyme inhibition assay with the two isoenzymes (COX-1&COX-2) and ethanol extract was screened for *In vivo* anti-inflammatory activity using carrageenan induced rat paw edema model. Results disclosed that total phenolic and flavonoid content for the ethanol extract (38.24±0.72 &97.12±1.63) was found to be higher and is more effective in inhibiting the free radicals in DPPH assay (IC_{50}= 49.53 µg/ml) and Nitric Oxide free radical scavenging assay (IC_{50}=41.26 µg/ml). It is also a potent inhibitor for COX enzyme and a more selective inhibitor for COX-2 (IC_{50}= 65.14µg/ml). The ethanol extract was also reported to be safe at 2000 mg/ kg body weight and exhibited significant anti-inflammatory activity in the selected model in a dose-dependent manner at a dose of 200 and 400 mg/kg body weight. The presence of various secondary metabolites may be responsible either alone or in combination for the observed pharmacological activities. The quantity of the phenolic compounds and flavonoids can be directly correlated to the exhibited antioxidant and anti-inflammatory activities.

Keywords: *Atylosia goensis* Dalzell, Carrageenan induced rat paw edema model, COX inhibition, Free radical scavenging, Total flavonoid content, Total phenolic content

INTRODUCTION

Plants have been the source of medicine forages to cure several ailments. Plant-based medicine is chosen over synthetics for chronic diseases because of its safety and

diverse pharmacological aspects **[1]**. Since plants reserve immeasurable secondary metabolites with multifaceted biological targets, the researchers are fascinated to explore their biological profile towards drug discovery **[2, 3]**.

The production of free radicals is a defense mechanism to combat the pathogenic entry into the cellular environment. The restoration of this stress load has contended with scavenging enzymes in the body **[4]**. The disruption of symmetry between the production and the lavation of these reactive species creates cellular stress. It causes chronic and progressive neurodegenerative disorders such as diabetes, cardiovascular diseases, cancer, arthritis, etc. **[5-7]**. Antioxidant agents support scavengers to combat oxidative stress, improve body health, and delay aging. Naturally derived antioxidants such as flavonoids, tocopherol and tannins are renowned for their potent protective effect and health benefits with minimum risks over the synthetic antioxidants **[8]**.

Atylosia goensis Dalzell (syn: *Cajanus goensis* Dalzell) of the Fabaceae family is an evergreen vine that grows in peninsular India. This climber has broadly obovate leaves, pod villous, bracts 1.5 x 1 cm, glabrous keel, many yellow flowers, and the flowering season is September to February. Pods are densely hairy, 4 x 1 cm in size, seeds are with impression. Traditionally, in Western Ghats, it is used to treat rheumatic pains, fever, gastric problems. No pharmacological or phytochemical reports were documented for this species **[9-11]**. In this regard, the current research was planned to explore the total phenolic content, flavonoid content and estimate the antioxidant, anti-inflammatory potential of *Atylosia goensis* Dalzell using *In vitro* and *In vivo* models. The extracts were also subjected to preliminary phytochemical screening to determine the presence of secondary metabolites.

MATERIALS AND METHODS

Plant material

Atylosia goensis Dalzell was collected from forest areas of Chettukuzhy, Kerala. The plant material was authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati, and the voucher specimen was (0528) preserved in the herbarium.

Reagents and chemicals

All the chemicals and reagents were procured from Sigma Aldrich (laboratory grade).

Preparation of extracts

The stems of *Atylosia goensis* Dalzell were collected and dried under shade. The dried stems were powdered and subjected to

exhaustive extraction using Soxhlet apparatus with n-hexane, ethyl acetate, ethanol, and water. The solvents were evaporated to dryness to get solid extract, and percentage yield was calculated **[12]**.

Phytochemical screening

The preliminary phytochemical investigation of all the extracts of *Atylosia goensis* Dalzell was carried out by employing standard protocols **[13]**.

Estimation of Total phenolic content

The total phenolic content of extracts of *Atylosia goensis* Dalzell was estimated by Folin-Ciocalteu (FC) method. 200µL of the extract solutions were mixed with 2.5ml of FC reagent (diluted to 10 times) and 2 ml of Na_2CO_3 solution (7.5% w/v) followed by proper mixing and incubation at 30°C for 90 min. Absorbance for all the samples was recorded at 765 nm and expressed in terms of mg equivalents of gallic acid. The calibration curve was constructed by plotting the absorbance against concentration, and the values are calculated in triplicates **[14]**.

Estimation of total flavonoid content

The total flavonoid content of the extracts was estimated by Zhishen colorimetric method. A volume of 125µL of the extracts is added to 75 µL of a 5% Sodium nitrite ($NaNO_2$) solution. After 6 min, 150 µL of $AlCl_3$ solution (10%) was added, followed by 750 µL of NaOH (1M). The final volume of the solution was made to 2500 µL with distilled water. After 15 min of incubation, the mixture turned pink, and the absorbance was measured at 510 nm. The total flavonoids content was expressed as gram equivalents of Rutin per gram dry weight **[15]**.

***In vitro* Antioxidant assay**

DPPH radical scavenging assay

DPPH assay was performed by preparing a solution of 25mg/L DPPH using ethanol and different concentrations of plant extracts with DMSO. In 96-well plate, 5µL of the extract solution followed by 195 µL of DPPH solution was added and incubated for 20 min at room temperature. The absorbance was measured at 515 nm for individual extracts, and the free radical scavenging activity was recorded by comparing the absorbance values with the blank. The above procedures were repeated using ascorbic acid as positive controls in triplicates. The antioxidant activity was calculated using the formula given below **[16]**.

% Free radical scavenging activity = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$

Nitric oxide radical scavenging assay

Sodium nitroprusside was used to screen NO free radical scavenging activity. 2 mL of sodium nitroprusside (10 mM) and 0.5 mL of phosphate buffer (pH-7.4) mixed with 0.5 mL of the test solution and

incubated for 150 min at 25 °C. Ascorbic acid solution and DMSO were used as standard and control, respectively. Sulfanilic acid reagent (1mL 0.33% of sulfanilic acid in 2% glacial acetic acid) was added to 0.5 mL of nitrite and incubated for 5 min. Naphthyl ethylene diamine dihydrochloride (NEDD, 1 mL of 1%) was added and incubated for 30 min at 25 °C **[17]**. The absorbance was recorded at 540 nm, and the percentage of nitric oxide inhibition was calculated as:

Percentage of nitric oxide radical scavenging assay = $[(A_{blank} - A_{sample})/A_{blank}] \times 100$

***In vitro* COX-1 and COX-2 inhibitory assay**

All the extracts were screened for COX-1 and COX-2 inhibitory activity (Cayman Chemical Co.) using the Cayman Cox enzyme kit and performed according to the manufacturer's guidelines. The degree of Wurster's blue oxidation by the peroxidase activity exerted by the COX enzymes was colorimetrically determined using a spectrophotometer. The experiment was done with n-hexane, ethyl acetate, ethanol, and aqueous extracts dissolved in DMSO and compared with standard celecoxib. Absorbance was measured at 590nm, and IC_{50} values were calculated in µM **[18]**.

Acute toxicity studies

For acute toxicity studies of *Atylosia goensis* Dalzell, OECD-425 guidelines were followed. The first rat was administered with 2000 mg/kg body weight of the extract. Every hour the clinical symptoms were observed, initially for three and continued for two days. If the animal is dead, the dose is adjusted to a lower dose by a factor of 1.3. If the animal survives, four more animals are administered with the same dose and observed for clinical symptoms for the next two days. The long-term toxicity was screened by following the procedure for 14 days. Based on the animals that survived, the lethal dose (LD_{50}) value was drawn **[19]**.

***In vivo* Anti-inflammatory activity**

Carrageenean-induced rat paw edema model was used for the anti-inflammatory activity of ethanol extract of *Atylosia goensis* Dalzell. Inflammation was induced with 1% saline suspension of carrageenan (0.1 ml) to the sub plantar region of the right hind paw of each rat before induction of inflammation to the groups as mentioned below:

Group	Treatment
Normal control	**Saline**
Disease control	**Saline+ carrageenan**
Positive control	**Diclofenac(20mg/kg)+ carrageenan**
Low dose	***A. goensis* ethanol extract (200mg/Kg body weight)+ Carrageenan**
High dose	***A. goensis* ethanol extract (400mg/Kg body weight)+ Carrageenan**

The rat paw edema was determined by measuring paw volume using a plethysmograph at regular intervals (0, 30, 60, 120, and180 minutes) **[20-21]**. The anti-inflammatory potential of the extracts was calculated from the reduction in the paw volume calculated as-

Percentage of inhibition = $[(\Delta A_{control} - \Delta A_{treatment})/ \Delta A_{control}] \times 100$

$\Delta A_{control}$= Difference in the paw volume of normal control and disease control

$\Delta A_{treatment}$= Difference in the paw volume of normal control and test groups (positive, low, and high dose)

Statistical Analysis

Statistical analysis was performed using GraphPad Prism TM software, version 6.0 (GraphPad Software, Inc., San Diego, CA, USA), and the values are represented as mean ± SEM. ANOVA (one-way) followed by Dunnet's t-test for multiple comparisons. The level of significance (p value) was considered as less than 0.05.

RESULTS

Preliminary phytochemical screening

The percentage yield of the plant was calculated **(Table 1)**. The preliminary phytochemical study of all the extracts of *Atylosia goensis* Dalzell exposed secondary metabolites such as alkaloids, carbohydrates, flavonoids, phenols, steroids, terpenoids, glycosides, tannins, saponins **(Table 2)**.

Total phenolic content

The phenolic compounds can give protection against the cellular stress in the body and are directly proportional. Total phenolic contents of all extracts of *Atylosia goensis* Dalzell were evaluated by Folin–Ciocalteu method taking gallic acid as the standard. A calibration curve was plotted against the absorbance values versus different concentrations of gallic acid **(Figure 1)**. The total phenolic content of the extracts was calculated from the regression equation of calibration curve ($Y = 0.0049x + 0.0271$; $R^2 = 0.9987$) and expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g) **(Table 3)**. Total phenolic content values were observed high in ethanol extract of *Atylosia goensis* Dalzell (38.24±0.72) followed by aqueous extract (31.36±1.25) and ethyl acetate extract (24.35±0.47).

The flavonoid content

Flavonoids are an important class of phenolic type plant secondary metabolites with significant adaptogenic properties. Previous reports witnessed the beneficial effects of flavonoids either in free form or glycosidic form in chronic diseases. The flavonoid content of *Atylosia goensis* Dalzell was determined by a colorimetric

method using the Zhishen technique and is found to be 97.12±1.63 of gram equivalence of Rutin at 510 nm for ethanol extract **(Table 4 and Figure 2)** that was high among the others. The calibration curve was made by linear regression, and the results represented the average of triplicates. The total phenolic content of the extracts was calculated from the regression equation of the calibration curve ($Y = 3.5343x-0.0687$; $R^2 = 0.9956$) and expressed as mg Rutin equivalents (RE) per gram of sample in dry weight (mg/g). The results showed that the ethanol extract was rich in flavonoids, since the polarity of the ethanol is sufficient to solubilize majority of the flavonoids in the plant material.

DPPH radical scavenging assay

A standard curve was plotted **(Figure 3)** using various concentrations of ascorbic acid. At a higher concentration (100 µg/mL), *Atylosia goensis* Dalzell ethanol extract with 71.22% of inhibition (IC_{50}= 49.53 µg/ml) stood high among the extracts next to ascorbic acid (82.11%; IC_{50}= 36.66 µg/ml) followed by aqueous extract (61.77; IC_{50}= 69.23 µg/ml) and ethyl acetate extract (51.78; IC_{50}= 98.8 µg/ml) **(Table 5 and Figure 4)**. Ethanol extracts were inferred to be potent antioxidants among all extracts **(Figure 5)**.

Nitric oxide radical scavenging activity

Similarly, At 100 µg/mL of concentration, the ethanol extract showed 72.95 % of inhibition with IC_{50} values 41.26 µg/ml followed by ethyl acetate extract 53.67% with IC_{50} values 84.01µg/ml **(Table 6)**. Whereas for ascorbic acid, it was found to be 85.92% with IC_{50} values 34.34µg/ml **(Figure 7)**.

***In vitro* COX-1 and COX-2 inhibitory assay**

All the extracts of *Atylosia goensis* Dalzell were screened for their ability to inhibit the COX enzyme (Table 7). The ethanol extract was showing potent and selective towards COX-2 (IC_{50}= 65.14µg/ml) than COX-1(IC_{50}= 74.15 µg/ml). At 100 µg/mL of concentration, ethanol extract is showing potent COX inhibition that is comparable with standard celecoxib (IC_{50}= 66.29 & 34.57 µg/ml for COX-1&2). At this higher concentration, ethanol extract showed 59.78% COX-1inhibition and 65.41% COX-2 inhibition, followed by aqueous extract (42.7%&39.78%) **(Figure 8)**.

Acute toxicity studies

The results showed that the administration of *Atylosia goensis* Dalzell was harmless at the upper limit dose (2000 mg/kg). Clinical symptoms such as tremors, aggressiveness etc. were not observed at this dose.

Invivo anti-inflammatory activity

The plant extracts' acute anti-inflammatory activity was screened using the

carrageenan-induced rat paw edema model **(Table 8)**. Carrageenan developed edema in the rat paw after 30 min and was swollen to a maximum volume of (1.82±0.018). Treating the animals with ethanol extract of *Atylosia goensis* Dalzell in oral doses of 200 and 400 mg/kg exhibited a significant reduction in the edema volume compared to standard Diclofenac **(Figure 9)**. Interestingly, ethanol extract started showing anti-inflammatory activity after 30 minutes of administration (1.098mm) and progressed with the time up to 180 minutes (1.104mm) compared to the standard Diclofenac (1.296±0.019 to 1.82±0.018).

Table 1: Percentage yield

Percentage yield	n-hexane extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
	2.38	7.14	10.37	8.92

Table 2: Phytochemical screening of *Atylosia goensis* Dalzell

Phytochemicals	n-hexane extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
Alkaloids	+	+	+	-
Glycosides	-	+	+	+
Flavonoids	-	+	+	-
Terpenoids	+	+	+	-
Steroids	+	+	-	-
Tannins	-	-	+	+
Proteins	-	-	+	+
Carbohydrates	-	-	+	+
Amino acids	-	-	+	+
Saponins	-	+	+	+

+ indicates the presence and – indicates the absence of phytochemicals

Table 3: Total phenolic content of *Atylosia goensis* Dalzell

Extract	Total phenolic content mg/ml
AGH (*A. goensis* n-hexane extract)	1.15±0.31
AGEA (*A. goensis* ethyl acetate extract)	24.35±0.47
AGE (*A. goensis* ethanol extract)	38.24±0.72
AGAQ (*A. goensis* aqueous extract)	31.36±1.25

***All values are expressed as mean±SD for three determinations**

Table 4: Total flavonoid content of *Atylosia goensis* Dalzell

Extract	Total flavonoid content mg/ml
AGH (*A. goensis* n-hexane extract)	2±0.64
AGEA (*A. goensis* ethyl acetate extract)	17.16±0.94
AGE (*A. goensis* ethanol extract)	97.12±1.63
AGAQ (*A. goensis* aqueous extract)	48±1.37

***All values are expressed as mean±SD for three determinations**

Table 5: Percentage inhibition and IC_{50} values of extracts and ascorbic acid at different concentrations in DPPH assay

Concentration µg/mL	Ascorbic acid	n-hexane extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
100	82.11	25.71	51.78	71.22	61.77
75	67.16	18.92	36.47	59.74	53.66
50	58.67	12.67	24.61	52.67	42.35
25	43.48	8.05	18.34	38.31	31.08
IC_{50} µg/ml	36.66	100.83	98.8	49.53	69.23

Table 6: Percentage inhibition and IC_{50} values of extracts and ascorbic acid at different concentrations in NO free radical scavenging assay

Concentration µg/mL	Ascorbic acid	n-hexane extract	Ethyl acetate extract	Ethanol extract	Aqueous extract
100	82.11	25.71	51.78	71.22	61.77
75	67.16	18.92	36.47	59.74	53.66
50	58.67	12.67	24.61	52.67	42.35
25	43.48	8.05	18.34	38.31	31.08
IC_{50} µg/ml	36.66	100.83	98.8	49.53	69.23

Table 7: IC_{50} values of extracts for COX enzyme

Treatment	IC_{50} values (µg/ml)	
	COX-1	COX-2
Celecoxib	66.29	34.57
AGH	213.72	291.69
AGEA	139.28	194.52
AGE	74.15	65.14
AGAQ	124.82	131.19

Table 8: Effect of *Atylosia goensis* Dalzell on paw edema volume in carrageenan-induced rat paw edema model

Concentration µg/mL	0 min	30 min	60 min	120 min	180 min
Disease control	1.104±0.015	1.296±0.019	1.326±0.054	1.644±0.025	1.82±0.018
Positive control	1.106±0.016	0.984±0.029*	0.918±0.025*	0.864±0.015*	0.816±0.015*
Low dose	1.11±0.015	1.098±0.024*	0.972±0.025*	0.924±0.019*	0.888±0.025*
High dose	1.098±0.014	1.104±0.011*	0.942±0.014*	0.942±0.014*	0.866±0.02*

Values are in mean ± SD; (n =6), p<0.05 was considered as statistically sgnificance

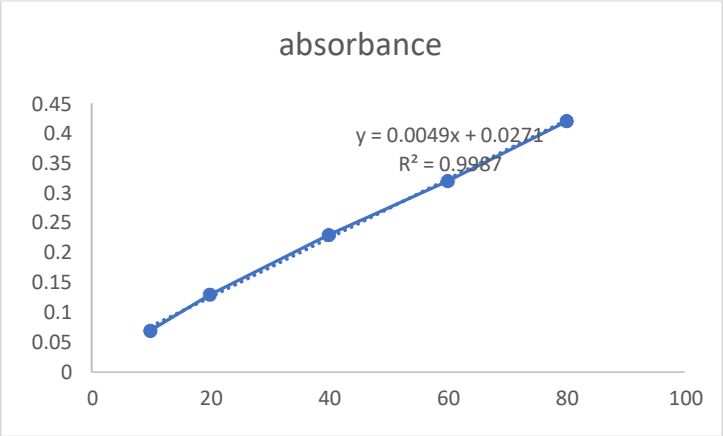

Figure 1: Calibration curve for Gallic acid

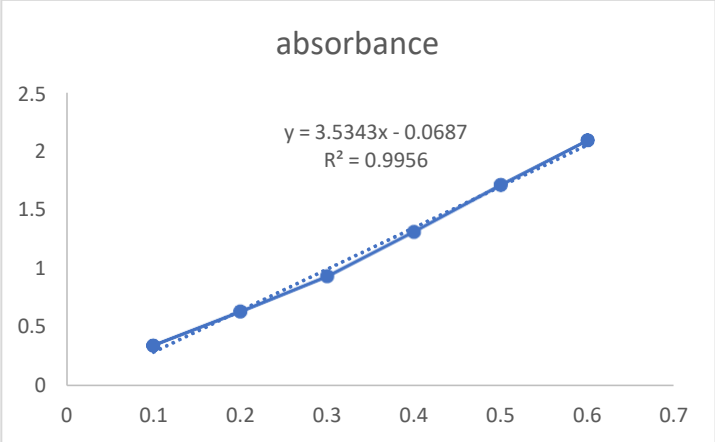

Figure 2: Calibration curve for Rutin

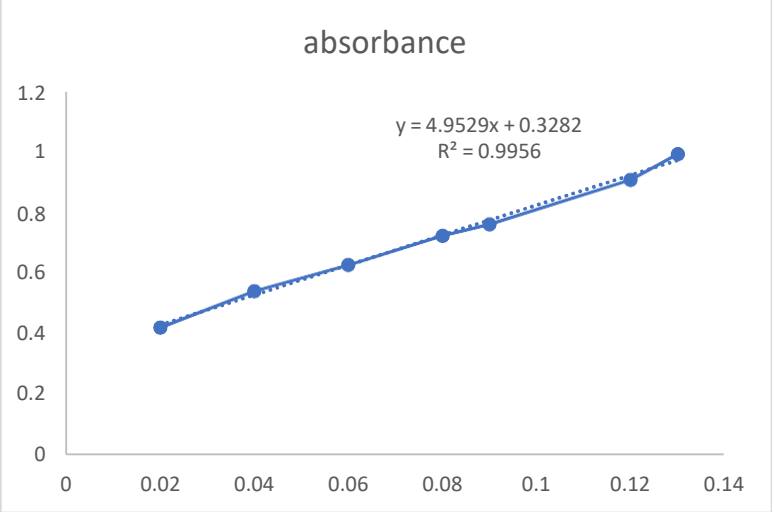

Figure 3: Calibration curve for Ascorbic acid

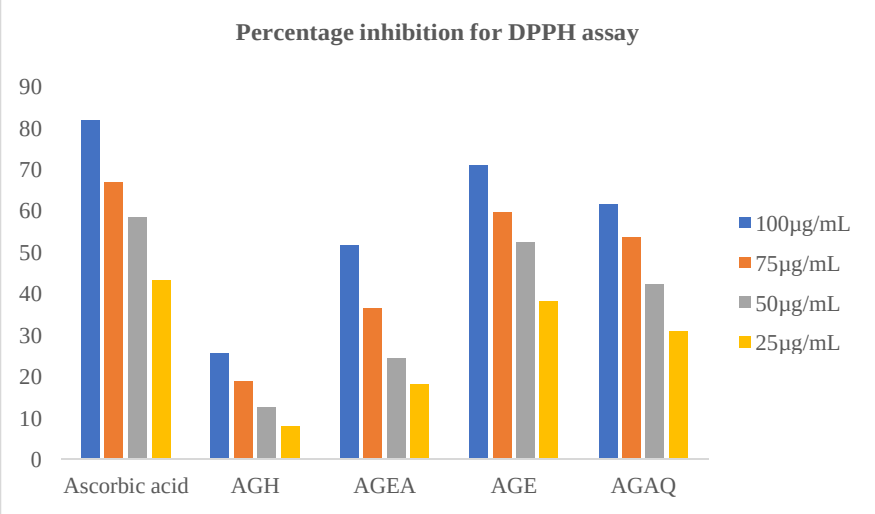

Figure 4: Percentage inhibition of extracts at different concentrations for DPPH assay

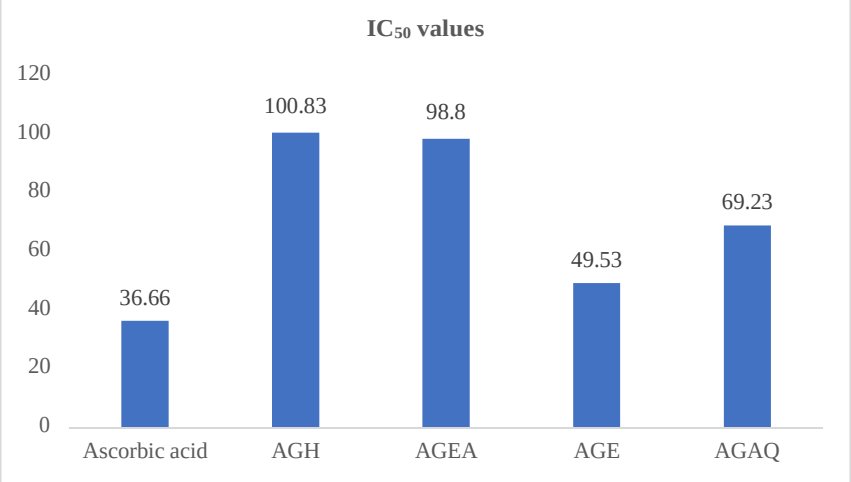

Figure 5: IC_{50} values of extracts for DPPH assay

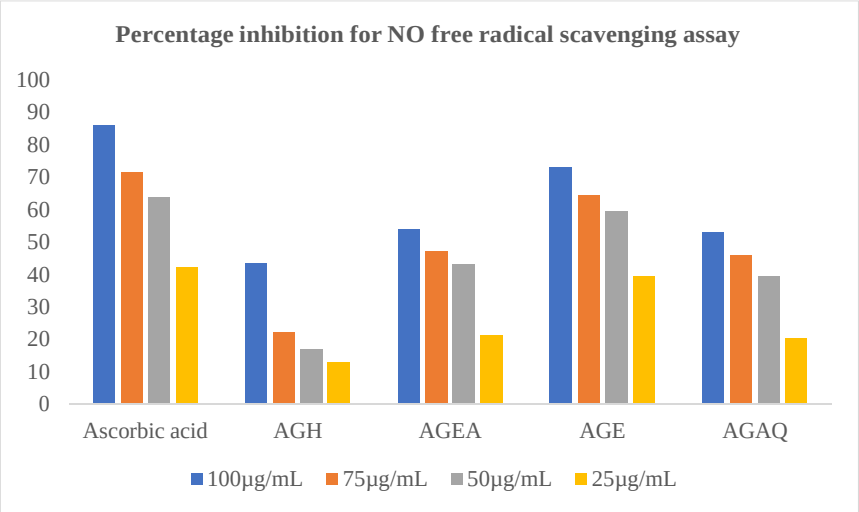

Figure 6: Percentage inhibition of extracts at different concentrations for NO free radical scavenging assay

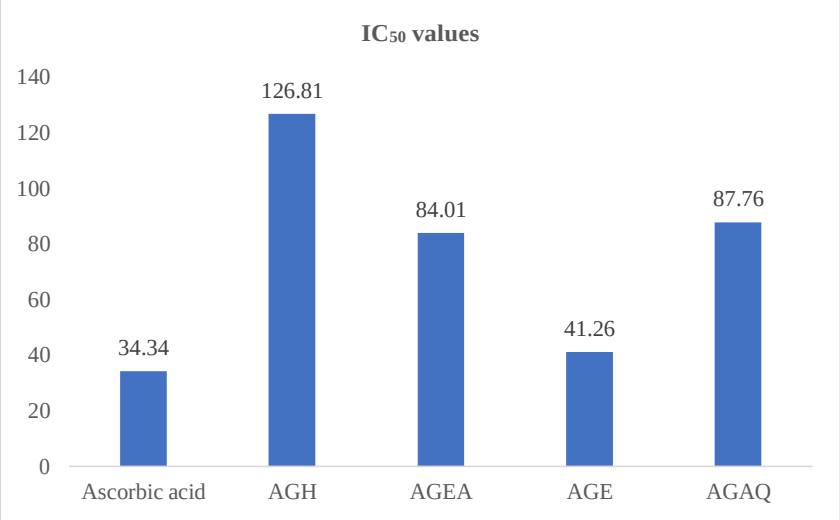

Figure 7: IC_{50} values of extracts for NO free radical scavenging assay

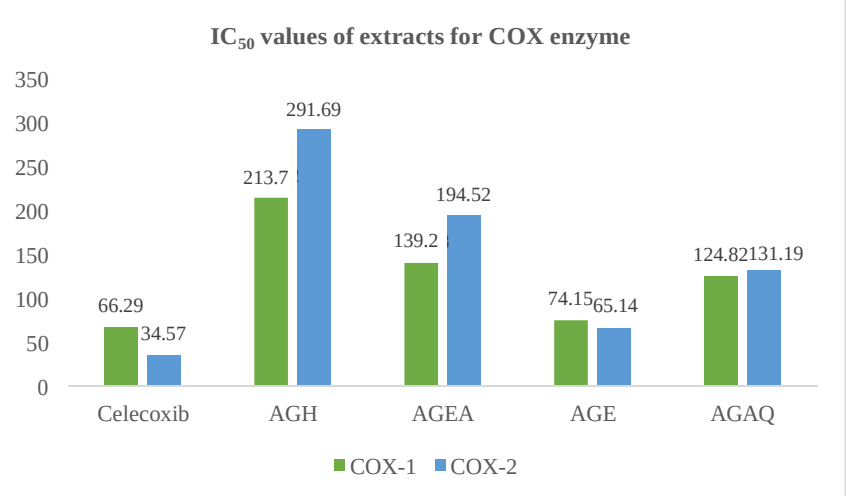

Figure 8: IC_{50} values of extracts for COX enzyme

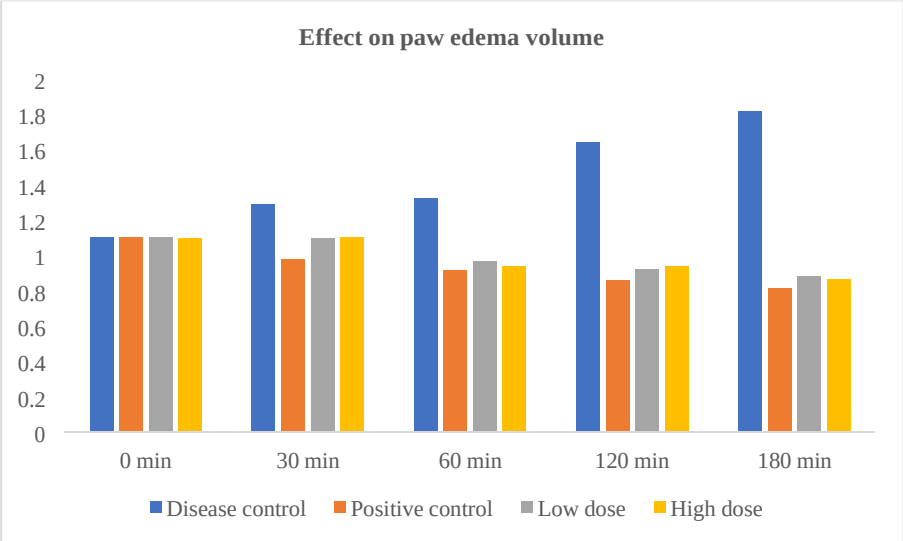

Figure 9: Effect of *Atylosia goensis* Dalzell on paw edema volume in carrageenan-induced rat paw edema model

DISCUSSIONS

Flavonoids and tannins are the major polyphenolic compounds produced as metabolic end products of plants that help the detoxification process. They are considered powerful agents to neutralize the free radicals due to their ability to react with them and are regarded as antioxidants, supporting the scavenging mechanism of the human body. Ample literature is available to support the protective effect of these polyphenolics to address various chronic and lifestyle disorders associated with stress. Since synthetic agents are associated with side effects, naturally occurring antioxidants are preferred in nutraceuticals for promoting health **[22]**.

It is essential to determine the antioxidant potential of the plants to understand their efficiency in protecting against cellular stress **[23]**. Total phenolic, total flavonoid content for n-hexane, ethyl acetate, ethanol, and aqueous extracts of *Atylosia goensis* Dalzell were evaluated using standard protocols. *In vitro* antioxidant screening in DPPH and NO free radical assay was performed for all extracts, and the ethanol extract was found to be rich in the phytochemicals and exhibited potent antioxidant and COX enzyme inhibition activity. Compared to the standard celecoxib, ethanol extract was found to be more selective towards COX-2 than COX-1. The extract was safe at 2000 mg/kg from the acute toxicity studies, and working doses were calculated (10^{th} and 5^{th} part of 2000 mg/kg) as 200 mg/kg and 400 mg/kg and further subjected to *In vivo* anti-inflammatory screening.

In the carrageenan-induced rat paw edema model, at both doses, the ethanol extract exhibited a statistically significant ($p<0.05$) reduction in the edema volume compared to the standard Diclofenac. Improvement in

the paw edema volume started from the 30 min of dosage and lasted even for 3 hours, indicating the potent anti- inflammatory activity.

Further investigation is in progress to evaluate complete phytochemical and pharmacological profiles to justify traditional applications, especially the anti-inflammatory activity of *Atylosia goensis* Dalzell.

ACKNOWLEDGEMENT

The authors are thankful to the Dept. of Pharmacognosy, Dayananda Sagar University, Bangalore, for supporting the current research work.

CONFLICTS OF INTEREST:

The authors declare that they have no conflict of interest.

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