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EVALUATION OF *IN-VIVO* ANTI-AGGRESSIVE ACTIVITY OF EXTRACTS OF *ALSTONIA SCHOLARIS* LEAVES AGAINST ANIMAL MODELS OF AGGRESSION

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Keywords:

Alstonia scholaris, Anti-aggressive activity, *In-vivo*, Stress, Foot shock

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ABSTRACT: Aggressive behavior is an important prerequisite for acquiring and maintaining feeding resources, territory and mating partners and, therefore, for the survival of an individual and the species. However, dysregulation of aggression among conspecifics can lead to severe injury and death. Aggression is prominently seen when a disturbance occurs in the fine balance of neurotransmitters such as 5-hydroxytryptamine, gamma-aminobutyric acid, dopamine, and receptor subtypes. The current study evaluated the *in-vivo* anti-aggressive potential of a standardized extract of *Alstonia scholaris* leaves. The extract of *Alstonia scholaris* was evaluated for its potential effects against defensive and offensive aggressive behavior models of rodents by oral administration at 250, 500 mg/kg BW dose levels once daily for 14 consecutive days as a suspension in a carboxymethylcellulose CMC, diazepam 1 mg/kg, p.o. was used as a standard anti-aggressive agent. Control group animals were given an equal volume of vehicle 10%, v/v, CMC suspension. The anti-aggressive activity was evaluated using the following validated models of aggression, *viz.:* Isolation-Induced Aggression, Resident-Intruder Aggression, and Water Competition Test, in Swiss albino mice. As a result, methanolic extract of *Alstonia scholaris* MAS showed dose dependant anti-aggressive activity in the aforementioned, validated models of aggression. This suggests that the MAS has a promising anti-aggressive activity qualitatively comparable to that of diazepam.

INTRODUCTION: Anxiety disorders are highly prevalent psychiatric illnesses that affect millions of people worldwide [1]. Anxiety is categorized under neuropsychiatric disorders, which encompass aberrant expressions of defensive and offensive behavior. An exaggerated or fearful response to an appropriate or inappropriate condition may be observed during anxiety [2].

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Anxiety is linked to fear and manifests as a future-oriented mood state that consists of a complex cognitive, affective, physiological, and behavioral response system associated with preparation for the anticipated events or circumstances perceived as threatening [3, 4, 5]. Therefore aggression is an "overt behavior with the intention of inflicting physical damage on the opponent [6].

Aggression generally ensues due to conflicting interests associated with restricted territory, electrical, sensory, chemical stimulation, or with the removal of positive re-enforcements [7]. Nature has bestowed our planet with an enormous wealth of medicinal plants that are highly esteemed

worldwide as a rich source of therapeutic agents for the prevention and cure of diseases and ailments. There are many herbs on earth that lie unexplored in medicine or science [8, 9]. *Alstonia scholaris*, commonly known as devil's tree, has been used for the treatment of many human ailments [10]. Literature suggests that *Alstonia scholaris* is useful in treating malaria, abdominal disorders, dyspepsia, leprosy, skin diseases, tumors, chronic and foul ulcers, asthma, bronchitis, helminthiasis, agalactia, and debility. Preclinical studies have shown that it possesses anti-microbial, anti-diarrhoeal, anti-plasmodial, anti-oxidant, anti-inflammatory hepatoprotective, nootrophic, anti-stress, anti-fertility, immunomodulatory, analgesic, anti-ulcer, wound healing, anti-cancer, chemopreventive, radiation protection, radiation sensitization, antitussive and chemosensitization activities [11, 12, 13]. The diverse pharmacological observations are supposed to be due to the presence of alkaloids, flavonoids, and phenolic acids [14, 15, 16]. The bark contains the alkaloids ditamine, echitenine, echitamine and strictamine [17].

MATERIAL AND METHODS:

Collection and Authentication of Crude Drug: The fresh *Alstonia scholaris* leaves were collected in November 2011 from the local area of Kanpur, Uttar Pradesh, India. The Taxonomic division authenticated the plant, National Botanical Research Institute NBRI, Lucknow, India Ref. no. NBRI/CIF/259/2011 and a voucher specimen were deposited for future references.

Preparation of Extracts: The shaded air-dried leaves were crushed, pulverized, and sieved through 80# mesh size. The powdered drug 500 g was extracted successively by using a Soxhlet extractor with nonpolar to polar menstruum of increasing polarity beginning with methanol 65°C-75°C followed by water 80°C-100°C by continuous hot extraction method for 6 h. The menstruum was distilled off and concentrated under the reduced pressure in the methanolic extract to form a dark green mass.

Physicochemical Standardization: The evaluation of the ZME leaves was performed as per the WHO guidelines and Pharmacopeia. The detailed analysis of physicochemical parameters, including extractive values of nonpolar to polar solvents, total ash, water-soluble ash value, acid insoluble ash value, loss on drying LOD, fluorescence analysis, and pH of 1% and 10% solutions, were carried out [18, 19].

Preliminary Phytochemical Screening: The preliminary phytochemical study of all extracts of *Alstonia scholaris* leaves was carried out as per the WHO guidelines to find out the wide range of plant metabolites such as carbohydrates, phenolic compounds, alkaloids, glycosides, flavonoids, saponins, lipids, steroids, proteins, and tannins, *etc*.

HPTLC Fingerprint Profiling of Methanolic Extract of ZME:

Preparation of Standard and Sample Solution: The stock solutions of standard quercetin were freshly prepared by dissolving 1 mg of a compound in 1 ml of HPLC grade methanol and stored at 4°C until analysis. Working dilutions of standard solution and samples were duly filtered through a 0.45 mm Millipore ultra-membrane filter Pall, USA, for HPTLC analysis [20]. The sample solution was prepared as per the standard methods. Percentage extractive yield was calculated on a dried mass weight basis. The dried mass was dissolved in 98% methanol to prepare a stock solution of 10 mg/mL used to apply spots on HPTLC plates [21].

Instrumentation and Chromatographic Conditions: An automated TLC sampler ATS-V CAMAG, Switzerland sample applicator was used to dispense the aliquots of the standard stock solution and the prepared` samples. The plates were developed in the CAMAG automatic developing chamber ADC-2 twin trough chamber 20 cm × 20 cm. The satisfactory resolution for the separation of compounds in the MAS leaves was obtained in the toluene: ethyl acetate: formic acid 5:4:1 solvent system. HPTLC chromatograms were recorded at the wavelength of UV-254 nm [22].

Animals: Swiss albino mice 20 ± 2 g of either sex were obtained from the Institutional Animal Ethics Committee. Animals were randomly housed in groups of six in polypropylene cages at an ambient temperature of 25 ± 1°C and 45-55% relative humidity, with a 12 h light/dark cycle lights on at 7 am. The animals had free access to standard pellets and water *ad libitum*.

Experiments were conducted between 8:00 and 14:00. The experiments were conducted according to the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals CPCSEA, India, Approval No. IAEC/Col./11. Prior permission was obtained from the Institutional Animal Ethics Committee IAEC to carry out the experiments [23].

Acute Toxicity Studies: Acute toxicity study was carried out for the MAS following OECD guidelines, 2001. The Extract fraction suspended in water with 1% CMC in the dose of 2000 mg/kg body weight was orally administered to overnight-fasted, healthy mice n=3.

The animals were observed individually after dosing at least once during the first 30 min, periodically during the first 4h and daily thereafter for a total of 14 days [24].

Drug Treatments: Based on our earlier studies, the standardized MAS 1.15%, w/w of marsiline, HPLC was administered orally, as a polyethylene glycol PEG suspension in doses of 100, 200, and 400 mg/kg of body weight, once daily for 14 consecutive days. Experiments were conducted on day 14, 1 h after the last oral treatment. Diazepam 2.5 mg/kg, *p.o.* was used as the standard anti-aggressive agent for comparison. Control animals were treated with an equal volume of vehicle 10%, v/v, PEG suspension.

Experimental Methods: The three most widely used rodent models, often used to detect potential effects of a therapeutically used anxiolytic drug on aggression were chosen to evaluate the effect of MAS on aggressive behavior, *viz.* Isolation-Induced Aggression and Resident-Intruder Aggression.

Isolation-Induced Aggression: Male mice of Swiss albino strain weighing 20-30 g were used. Mice were kept isolated in small cages for a period of 6 weeks. Prior to the administration of the test drug, the aggressive behavior of the isolated mouse was assessed against a male mouse similar in weight to that of the isolated mouse and accustomed to live in a group into the cage of an isolated mouse for 5 min. immediately, the isolated mouse started to attack the "intruder". The aggressive behavior of the isolated mouse was characterized by hitting the tail on the bottom of the cage, screaming, and biting. Isolated mice not exhibiting aggressive behavior were excluded from the test. One day after the initial trial, isolated animals were distributed into three groups 6 in each. They were treated with vehicle 1% CMC, *Alstonia scholaris* extract 250 & 500mg/kg body weight, and diazepam 1mg/kg body weight for three consecutive days. One hour after the last dose, the aggressive behavior of an isolated mouse against a male mouse was evaluated again for 5 min [25]. Aggressive behavior-related parameters assessed during this test were latency to first attack, screaming, pursuit frequency, tail rattling, aggressive posture, and the total number of fighting bouts.

Resident-Intruder Aggression: Resident male mice 400 ± 20 g were tested in their home cages for aggression against a smaller 200 ± 20 g male intruder. Before starting the experiments, each resident male rat was kept in pair with one female mice in a polypropylene cage for 15 days and they were randomly divided into four groups 6 pair in each. Drug treatment was started 16th day onward, and only male mice of each pair were administered with vehicle 1% CMC, *Alstonia scholaris* extract 250 & 500 mg/kg body weight or diazepam 1 mg/kg body weight for three consecutive days. A resident female was removed from the cage 30 min prior to the start of the test. One hour after the last treatment, a male intruder ~200 g was placed in the territorial cage of the resident male, and behavior of the resident male was observed for the next 15 min. During this period, the time until the first attack in seconds, number of attacks, and duration of each attack in seconds were recorded by a blind observer [26].

Statistical Analysis: Results are expressed as mean±SEM, and data analysis was performed using the SPSS statistics by using an unpaired student‘t’ test. The data were analyzed by one-way ANOVA, followed by Student’s t-test. P values of less than 0.05 were considered statistically significant.

RESULTS:

Physicochemical Evaluation Parameters: The calculated physicochemical parameters such as ash values, and extractive values of the leaves of *Alstonia scholaris* are presented in **Table 1.**

TABLE 1: PHYSICOCHEMICAL PARAMETER OF THE LEAVES OF *ALSTONIA SCHOLARIS*

S. no.	Physicochemical parameter	Results %w/w
1	Moisture content	1.85
2	Methanol soluble extractive value	5.25
3	Water soluble extractive value	2.44
4	Ash value	10.006
5	Acid insoluble ash value	1.0833
6	Water soluble ash value	5.582

Phytochemical Investigation: The results of phytochemical screening of the various extracts of *Alstonia scholaris* leaves revealed the presence of alkaloids, carbohydrates, steroids and sterol, glycoside, saponins, flavonoids, phenolic compounds, and triterpenoids shown in **Table 2.** These secondary metabolites possess various pharmacological effects and may be responsible for various actions of *Alstonia scholaris.*

TABLE 2: PRELIMINARY PHYTOCHEMICAL SCREENING OF VARIOUS EXTRACTS OF *ALSTONIA SCHOLARIS* LEAVES

S. no.	Name of phytoconstituents	Methanolic extract	Water extract
1	Carbohydrates	-	+ +
2	Alkaloids	+ +	+
3	Glycosides	+ +	-
4	Flavonoids	+ +	-
5	Saponins	+	+ +
6	Tannins	+	+
7	Proteins and amino acids	-	+ +
8	Triterpenoid	-	+
9	Steroids	+	-

Whereas ++= strongly present; += partially present; - = absent

High-performance Thin Layer Chromatography: In this study, several solvent systems were used for the estimation of this triterpenoid and were investigated to evaluate the combinatorial separation of these compounds in a single solvent system and between different components of the extract. Among the different solvents systems investigated, a mobile phase consisting of toluene: ethyl acetate: formic acid in the ratio of 8: 2: 1 v/v/v demonstrated good resolution between other peaks of the extract. The procedure for separating and determining different compounds in the methanolic fraction of *Alstonia scholaris* leaves using HPTLC densitometry is reported at seven-point calibration curves in which amount of kaempherol in the leaves of *Alstonia scholaris* was found to be 0.024%.

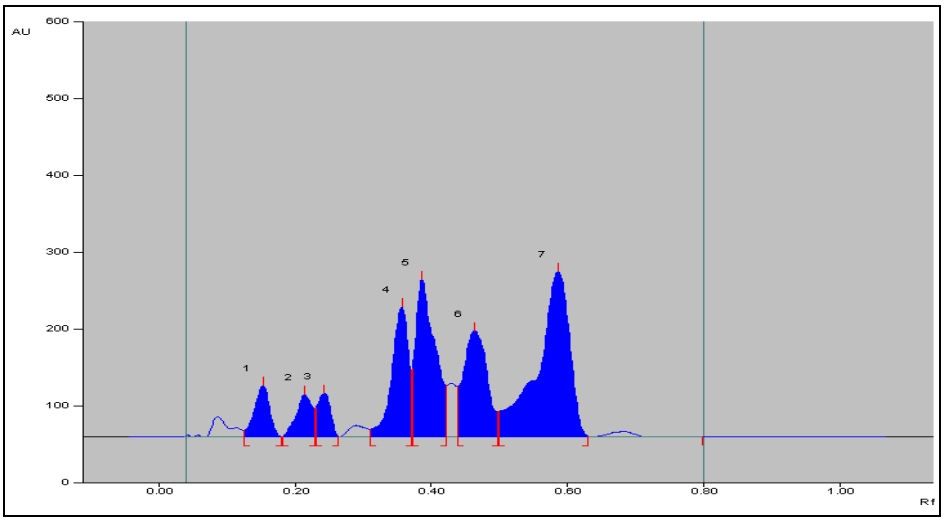

FIG. 1: HPTLC CHROMATOGRAM OF LEAVES EXTRACT HAS BEEN SHOWN UNDER UV-254nm WAVELENGTH

Alstonia scholaris methanol extract showed seven compounds having an R_f value of 0.11, 0.19, 0.35, 0.42, 0.49, 0.56 and 0.62 at λmax 254nm. HPTLC chromatogram and densitograms were obtained from standard compounds and methanolic fractions **Fig. 1-2**, separation of all bands of plant samples and standard is Kaempherol.

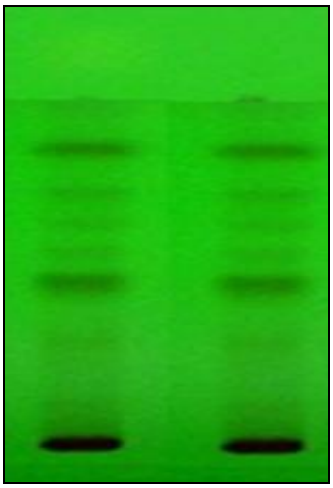

FIG. 2: HPTLC PHOTOGRAPH OF METHANOLIC EXTRACT OF *P. NIGRUM* AT 254 nm

Isolation-Induced Aggression: The extract extended the latency period to the first attack and the number of fighting episodes. The extract also significantly reduced several aggressive postures, number of screamings, and tail rattle frequency.

Qualitatively, these effects of extract were identical to that of the diazepam as shown in **Table 3** and **Fig. 3.** n = 6, *p< 0.001, **p< 0.05, ***p< 0.01, #p> 0.05Non significant as compared to control.

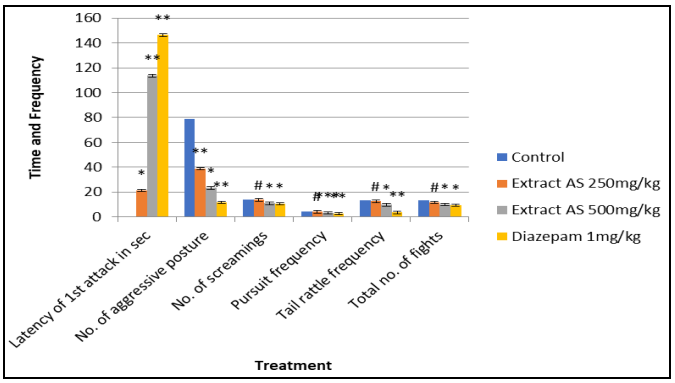

FIG. 3: EFFECT OF METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* ON ISOLATION-INDUCED AGGRESSION TEST

Resident-Intruder Aggression Test: The extract treatment prolonged the latency period of the first attack and reduced the total duration and mean a number of fights, as shown in **Table 4** and **Fig. 4.**

Mean numbers of lateral threats and aggressive grooming were also lowered in the extract-treated group as compared to the control group. The observed effects of diazepam in this model were qualitatively similar to those of extract.

TABLE 4: EFFECT OF METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* ON RESIDENT-INTRUDER AGGRESSION TEST

Treated groups	Dose	Latency of 1st attack in sec mean	No. of threats mean	No. of aggressive grooming mean	No. of fights mean	Duration of fights in sec mean
Control	CMC	0.47±0.02	8.67±1.34	4.33±0.32	6.67±0.13	41.67±1.73
Extract AS	250mg/kg	23.33±2.50**	5.33±0.24***	3.67±0.13**	6.67±0.19#	31.67±0.82*
Extract AS	500mg/kg	45.70±2.80**	3.00±0.30**	3.00±0.13*	4.70±0.54**	24.67±0.71*
Diazepam	1mg/kg	47.33±2.78**	2.67±0.19**	3.10±0.17***	2.33±0.19**	21.33±0.59*

n = 6, *p< 0.001, **p< 0.05, ***p< 0.01, #p> 0.05Non significant as compared to control.

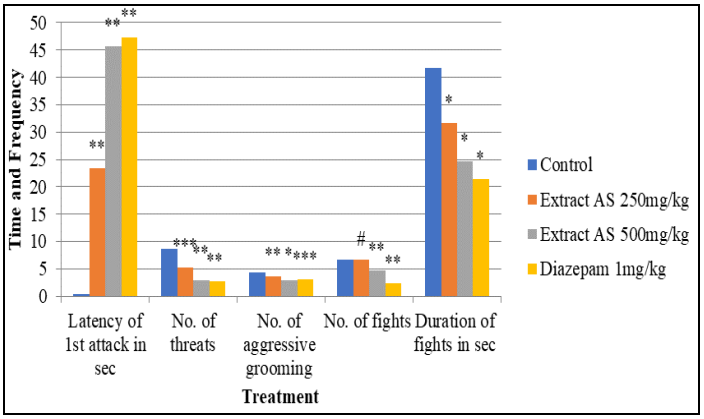

FIG. 4: EFFECT OF METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* ON RESIDENT-INTRUDER AGGRESSION TESTS n = 6, *p< 0.001, **p< 0.05, ***p< 0.01, #p> 0.05 Non significant as compared to control

Resident-Intruder Aggression Test: The extract treatment prolonged the latency period of the first attack and reduced the total duration and mean number of fights as shown in **Table 5** and **Fig. 5.** Mean numbers of lateral threats and aggressive grooming were also lowered in the extract-treated group as compared to the control group. The observed effects of diazepam in this model were qualitatively similar to those of extract.

TABLE 5: EFFECT OF METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* ON RESIDENT-INTRUDER AGGRESSION TEST

Treated groups	Dose	Latency of 1st attack in sec mean	No. of threats mean	No. of aggressive grooming mean
Control	CMC	0.47±0.02	8.67±1.34	4.33±0.32
Extract AS	250mg/kg	23.33±2.50**	5.33±0.24***	3.67±0.13**
Extract AS	500mg/kg	45.70±2.80**	3.00±0.30**	3.00±0.13*
Diazepam	1mg/kg	47.33±2.78**	2.67±0.19**	3.10±0.17***

n = 6, *p< 0.001, **p< 0.05, ***p< 0.01, #p> 0.05Non significant as compared to control.

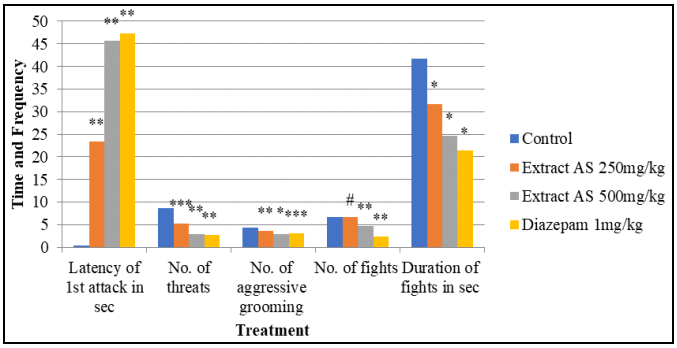

FIG. 5: EFFECT OF METHANOLIC EXTRACT OF *ALSTONIA SCHOLARIS* ON RESIDENT-INTRUDER AGGRESSION TEST

DISCUSSION: One of the difficult medical and social problems associated with neuropsychiatric disturbances is aggression. Analysis of aggression in different animal species could provide a firm understanding of human violence and the therapeutic measures to be taken to combat it [27]. Exposure of an animal to a threatening situation elicits a behavioral repertoire referred to as aggression. The behavioral profile may be manifested in the form of offensive and defensive aggression [28]. Offensive behavior is characterized by the initiative of the aggressor and devastation to the opponent [29]. On the other hand, defensive behavior lacks initiative, and the animal does not impose intentional damage [30].

The qualitative phytochemical studies carried out so far reveal the presence of alkaloids, coumarins, flavonoids, reducing sugars, simple phenolics, steroids, saponins and tannins. Of the various phytochemicals estimated, lipid and saponin were found in larger amounts than others. The above different chemical compounds detected in *A. scholaris* could make the plant helpful in treating different ailments and have potential for providing useful drugs for human use [31]. Various antidepressant drugs have been reported to be effective in the treatment of aggression [32, 33]. Alkaloids have been identified as one of the main components of *Alstonia scholaris* extract responsible for its antidepressant effects [34, 35, 36]. Therefore, the observed antiaggressive activity of alstonine and scholaricine adds a new potential use in the wide spectrum of *Alstonia scholaris* for the treatment of neurological disorders.

The reported HPTLC method was found to be rapid, simple and accurate for quantitative estimation of phytochemicals in methanolic extract of leaves of *Alstonia scholaris.* HPTLC analysis of the sample revealed wide variability in the methanolic extract showing the presence of seven spots with R_f value.11, 0.19, 0.35, 0.42, 0.49, 0.56 and 0.62 at λ max 254nm. In the Isolated-induced aggression test, the serotoninergic system is implicated in aggressive states, and it has been hypothesized that decreasing serotoninergic activity may encourage aggressive behavior [37]. In the Resident-intruder test, fighting is known to occur frequently in male mouse groups. In this study, the possible impact of individual aggressiveness on fighting in groups and on the social status of animals was studied. The effect of diazepam on the values of aggressive behavior shows the tranquilizer activity. This is because of its capacity as an anxyolitic agent that has an activity to the level of the GABAA receptors [38]. In conclusion, the methanolic extract of *Alstonia scholaris* was found to be efficacious in producing serenity and masking the constellation of behavioral changes encountered

during aggressive bouts making it a promising naturally derived product.

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