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Insilico Studies and Screening of Antiepileptic Activity of Leaf Extracts of Plumeria Pudica

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ABSTRACT
Epilepsy is a neurological disorder, affects people of all age groups. There are number of antiepileptic drugs are available, but most of them are highly susceptible to drug interactions and severe adverse effects. As a result, the use of herbal medicine is increasing becoming popular. So, aim of the study is to provide relevant information of antiepileptic potential of phytoconstituents present in Plumeria pudica. Itis a flowering plant belongs to the family Apocynaceae. The pulverized powder of leaves of Plumeria pudicawas extracted with six solvents. Extracts were analysed by insilico study, acute toxicity and invivoantiepileptic activity.GC-MS analysis revealed the presence of 63 phytoconstituents in different extracts.10 phytochemicals were docked towards the MAPK13 complex with inhibitor with the PDB ID 5EKO , which showed that beta-sitosterol(-5.462)had better docking score when compared to the standard diazepam (-4.738). There are no reports of acute toxicity in leaf extracts, hence toxicity study was carried out and found to be safe up to 2000 mg/kg. This is followed by the screening of antiepileptic activity using the pentylenetetrazole model and the maximal electric shock model.In the pentylenetetrazole model, The medium (200mg/kg) and high doses (400mg/kg) of ethanolic extract showed increasing latency to convulsions (114.13±1.36) and (125.52±1.18*) and reduced the duration of convulsions.

KEYWORDS: Plumeria pudica, antiepileptic, acute toxicity, docking, beta-sitosterol

I. INTRODUCTION

Epilepsy is a neurological disorder characterized by recurrent seizures, and it affects millions of people worldwide. It can cause changes in behavior, movements, feelings and levels of consciousness. It includes spasms and violent and uncontrolled spasmodic contraction and relaxations of the voluntary muscles[1].While there are existing antiepileptic drugs available, they may cause adverse effects and not be effective for all individuals. Therefore, there is a continuous need to explore natural sources, such as plants, for potential antiepileptic compounds. In the present study, the leaves of Plumeria pudicawere taken for extraction of compounds.Plumeria pudica is an ornamental flowering plant that has secondary metabolites with a variety of biological functions. This plant is also known as Nagchampa , wild Plumeria, white frangipani, lei flowers. Plumeria pudica is an evergreen, fast growing shrub containing one or two slender trunks. It can grow up to 5-8 feet height. The leaves are alternate and dark green in colour and they are fiddle shaped or spoon shaped. The plant contain large clusters of bright white flowers upto 3 inches (7.5 cm) with a small yellow center have five petals cover this plant as a beautiful bouquet. So, plant is called as "bridalbouquet"[2]. It belongs to the family Apocynaceae[3]. It possesses different pharmacological actions such as laxative, carminative, antiallergic, antimicrobial, cytotoxic, antiinflammatory, antileprosy, antiulcer, diuretic and anti-ascites properties[4]. The AChEinhibitoryactivity of leaves of Plumeria pudica onzebrafishbrainwascarriedoutbyPrasad et.al.,(2016). Both in-vitro and in-vivo assessments showed thatmethanolicextract of Plumeria pudicareduces the activity of AChE.Based onthis study, the presentwork was carried out[5]. AChE inhibitors can influence the cholinergic system and modulate neurotransmitter balance in the brain, which may result in antiepileptic effects. Their ability to enhance cholinergic transmission, exert neuroprotective effects, modulate GABAergic neurotransmission, and influence excitatory neurotransmitters contributes to their potential antiepileptic activity.

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II. MATERIALS AND METHODS

Collection of plant leaves and preparation of extracts

The leaves of Plumeria pudica were collected in the month of June-July from Manjeshwar, Kasaragod district. The extraction of coarsely powdered leaves was done by cold maceration process using chloroform, ethanol, methanol, n-hexane and petroleum ether and water. These extracts were investigated for phytochemical analysis followed by GC-MS analysis, insilicostudy, acute oral toxicity and invivo antiepileptic studies.

Figure 1: Plumeria pudica tree

Figure 2: Leaf

IN-SILICO STUDIES:

Different phytoconstituents present in Plumeria pudicawere screened for their anti-epileptic properties using in-silico methodologies in Schrödinger software.

A. Data collection:

10 different phytoconstituents from the plant P.pudicawere collected from the GC-MS report. Smiles of these ligands were obtained from the Pub-Chem.

MAPK13 complex with inhibitor with the PDB ID 5EKO was selected as the target protein which was obtained from RCSB PDB with a resolution of 2.00 Å[6].

B. Ligand preparation: Ligprep:-

A new project was created in a selected working directory, and the SMILES characters were moved into the workspace of Schrodinger Maestro. These ligands are subjected to the Schrodinger suit 2020-4 LigPrep function. The OPLS-3 force field was used to optimize the energy.

C. Protein preparation:

MAPK13 complex with inhibitor with the PDB ID 5EKO was imported from the RCSB PDB and visualized in the Maestro interface. The residues or water molecules beyond 5Å were eliminated.Finally, the protein energy was optimized using the OPLS_2005 force field.

D. Receptor grid generation:

Receptor grid was generated for co-crystal ligands and Diazepam using the GLIDE grid generation wizard. The binding site of the standard drug was chosen and active site grid was generated for molecular docking.

E. Molecular docking:

The energy-minimized ligands are docked with the optimised crystal structure of the protein in this stage. This aids in determining the affinity with which the ligands interact with the protein's active site (Lock - key affinity). Docking is a method for predicting the preferred orientation of one molecule to another when they are linked together to form a stable complex with the lowest overall energy. The level of association or binding affinity between two molecules may be predicted using knowledge about the preferred orientation. The ligands were docked into the protein's glide grid using the standard precision (SP) technique, followed by the extra precision (XP) algorithm in the GLIDE module of the Schrodinger 2020-4 suite device Maestro - 11.7.012. The docked posture of ligands and their

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interactions were studied once molecular docking was completed.

F. ADME prediction:

ADME calculations of ligands were carried out using the QikProp module of the Schrödinger suite 2020-4, that predicts a molecule's pharmacokinetic properties relating to its physicochemical properties. The parameters like H bond donor and acceptors, molecular volume, log P value, PSA are expected to be within acceptable range when compared to standard values.

G. MM/GBSA simulations:

Schrodinger Prime software and molecular mechanics/generalized Boltzmann Surface area methodology (MM/GBSA) were used to calculate the binding Free energy for the protein and group of ligands.

ACUTE ORAL TOXICITY STUDY

Acute toxicity studies were carried out using healthy female swiss albino mice weighing 25-30g of age 8-12 weeks older. Animals were obtained from central animal house, Nitte Centre for Animal Research and Experimentation (NUCARE), Paneer, Mangalore after getting approval from the IAEC (Reg.No: 1781/PQ/ERe Bi/S/2014/CPCSEA).The animals were housed in different cages in the animal room with a maintained temperature of 22^{O}C (±3^{O}C).Animal room was provided with artificial lighting with 12 hours of light and 12 hours dark cycle. Animals were provided with the standard dry pellet, purified water with ad libitum.

The acute oral toxicity study of plant extract was carried out by using Swiss albino mice by up and down method as per OECD 425 guidelines[7]. Animals were fasted prior to the dosing (food but not water withheld for 3-4 hours).The fasted body weight of each animal was determined and the dose was calculated according to the body weight. After the substance has been administered, food was withheld for 3-4 hours. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours and daily thereafter for a total 14 days[8].If the animal survives, the second animal receives a higher dose. If the first animal dies, the second animal receives a lower dose. In this present study, initially a dose of 1000mg/kg of ethanolic extract was administered to the mice. There was no behavioural changes or symptoms of toxicity were seen in this dose level. So, the dose was increased to 1500mg/kg and up to 2000mg/kg. Animals were observed for the following parameters :

a) Behavioural profile

Awareness : alertness, visual placing, stereotypy, passivity Mood : grooming, restlessness, irritability, fearfulness

b) Neurological profile

Motor activity, spontaneous activity, reactivity, touch response, pain response, startle response, tremor, gait, grip strength. Pinna, corneal reflex.

c) Autonomic profile :

Writhing, defecation, urination, piloerection, heart rate, respiratory rate

IN-VIVO ANTIEPIPLEPTIC SCREENING

Selection of doses

The doses for the antiepileptic study were selected based on the acute toxicity study. From the study it was found that ethanolic extract of plant leaves was found safe at the concentration of 2000mg/kg and not toxic effects were seen in Swiss albino mice.Therefore,1/5th (400mg/kg), 1/10th(200mg/kg),1/20th(100mg/kg) of the LD50 dose were selected for the study.

Pentylenetetrazole induced convulsion in mice[9,10,11] :

Experimental design: The animals were divided into 5 groups (n=6).The control group was administered with 0.6%w/v of CMC orally. The standard group receives diazepam at the dose of 5mg/kg administered intraperitoneally. The test extract was screened at three dose levels viz. low (100mg/kg), medium (200mg/kg),and high (400mg/kg). The extract was administered orally. After 1 hour, PTZ was injected intraperitoneally at the dosage of 80mg/kg and observed up to 30 minutes. The latency to onset of seizure, tonic convulsions, and status of each animal was observed.

Maximal electroshock induced convulsions in mice[9,10,11,12] :

Experimental design: Animals were divided into 5 groups (n=6). Animals were checked for sensitivity to electric shock 24hr before administration of test

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compounds and those animals which fail to show hind limb tonic extension were rejected. The control group received 0.6%w/v of CMC orally and the standard group received phenytoin (25mg/kg) intraperitoneally. The test extract was screened at three dose levels viz. low (100mg/kg), medium (200mg/kg) and high (400mg/kg). The extract was administered orally. After 60 minutes of administration of test extract, 60Hz alternating current of 150mA intensity for 0.2 sec was given using corneal electrodes. The animals were observed for the various phases of convulsions like tonic, flexion, extension, stupor and mortality due to convulsions. The onset of time of seizures, duration of tonic hind limb extension and mortality for each animal wasobserved. Decrease in duration of hind limb extension was considered as protective action. Abolition of the extensor phase was considered as anti-epileptic effect.

Statistical analysis

Graphpad Prism 8.0.2 software was used to calculate all of the data statistically. One-way analysis of variance(ANOVA) was performed on data from six animals per group. The experimental values were expressed as the mean ± Standard Error of Mean (SEM). Significance was determined by p values below 0.05,denoted by asterisks (*p < 0.05, **p<0.01, and ***p< 0.001).

III. RESULTS AND DISCUSSION

IN-SILICO STUDIES:

Table 1: Docking studies of phytoconstituents with 5EKO

Ligand	Docking score	MMGBSA dG Bind	HPI	PI	HB	PC
Diazepam (standard)	-4.738	-45.58	ALA52 ILE85 LEU167 MET107 PRO108 PHE109 MET110 VAL31 ALA157 VAL39		MET110	LYS116
N-hexadecanoic acid	-3.343	-90.49	LEU75 LEU76 VAL39 VAL31 ALA157 MET110 PHE109 MET107 ALA52 LEU167 ILE85 PHE169 LEU171	THR112 GLN111	ASP113	
Phytol	-4.802	-92.81	LEU75 LEU76 LEU171 PHE169 LEU167 ILE85 VAL31 ALA157 MET110 PRO108	THR112 GLN111	ASP113	

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			MET107 ALA52 VAL39			
Beta-sitosterol	-5.462	-63.13	LEU171 PHE169 LEU167 LEU75 LEU76 ILE85 ALA52 ALA157 LEU105 MET107 PRO108 PHE109 MET110 VAL31 VAL39	THR112 GLN111	MET110	
Oleic acid	-3.283	-102.55	LEU167 PHE169 LEU171 LEU75 LEU76 MET107 PRO108 PHE109 MET110 ILE85 ALA52 VAL31 VAL39 ALA157	THR112	ASP113 LYS116	
Squalene	-4.801	-104.81	MET107 PRO108 PHE109 MET110 ALA157 LEU75 LEU76 LEU171 PHE169 LEU167 VAL31 ALA52 ILE85 ALA35 VAL39	THR112 GLN111 SER33 SER38		
Octadecanoic acid,ethyl ester	-0.943	-93.01	LEU171 PHE169 LEU167 LEU76 LEU75 ALA52			

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			VAL31 MET110 PHE109 PRO108 MET107 VAL39 ILE85 TYR36			
Hexadecenoic acid,ethyl ester	-4.521	-110.49	LEU171 PHE169 LEU167 LEU76 LEU75 ALA52 ILE85 MET107 PRO108 PHE109 MET110 VAL31 VAL39 TYR36		MET110	
Ergost-5,8(14)-dien-3-ol	-4.308	-62.35	LEU167 LEU76 MET107 ILE85 PHE109 MET110 ALA52 ALA157 VAL31 VAL39	GLN111 THR112 HID30	ASP168	
Pentanoic acid,10-undecenyl ester	-2.655	-85.23	LEU75 LEU76 LEU171 PHE169 LEU167 MET107 PRO108 PHE109 MET110 ALA52 ILE85 ALA157 VAL31 VAL39			
9,12 octadecadienoic acid(z,z)-methyl ester	-2.598	-88.19	ILE85 VAL31 LEU167 PHE169 LEU171 LEU76 LEU75	THR112	LYS116	

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			LEU105 ALA52 MET107 PRO108 PHE109 MET110 VAL39 VAL158 ALA157			

*HPI- Hydrophobic interaction, PI - Polar interaction with ligand, HB - Hydrogen bonding, PPS - Pi-pi stacking, PC - Pi cations

Table 2: Physicochemical properties of phytoconstituents

Ligand	**Molecular weight**	**Molecular volume**	**PSA**	**QPlogPw**	**H-acceptor**	**H-donor**
Acceptable range	130.0 –725.0	500.0 – 2000.0	7.0 – 200.0	4.0 – 45.0	2.0 – 20.0	0.0 – 6.0
Diazepam (standard)	284.744	896.098	46.901	7.188	4	0
9,12 octadecadienoic acid(z,z)-methyl ester(P10)	294.476	1281.425	35.783	1.044	2	0
Pentanoic acid,10-undecenyl ester (P9)	254.412	1135.442	36.224	1.122	2	0
Ergost-5,8(14)-dien-3-ol (P8)	398.671	1409.695	21.042	3.789	1.7	1
Hexadecenoic acid,ethyl ester (P7)	284.481	1270.961	35.034	0.557	2	0
Octadecanoic acid,ethyl ester (P6)	312.535	1389.721	36.645	0.296	2	0
Squalene(P5)	410.725	1758.771	0.000	-3.083	0	0
Oleic acid(P4)	282.465	1224.029	51.734	2.463	2	1
Beta-sitosterol(P3)	414.713	1457.614	22.279	3.670	1.7	1
Phytol (P2)	296.535	1294.708	23.019	2.040	1.7	1
N-hexadecanoic acid (P1)	256.428	1116.919	50.333	2.363	2	1

Table 3: Lipinski's Rule of five

Ligand	**Molecular weight**	**HB Donor**	**HB Acceptor**	**QPlog p o/w**	**RO5**
Acceptable range	≤500	≤ 5	≤ 10	< 5	≤ 5
Diazepam (standard)	284.744	0	4	2.992	0
9,12 octadecadienoic acid(z,z)-methyl	294.476	0	2	6.346	1

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ester(P10)					
Pentanoic acid,10-undecenyl ester (P9)	254.412	0	2	5.436	1
Ergost-5,8(14)-dien-3-ol (P8)	398.671	1	1.7	7.214	1
Hexadecenoic acid,ethyl ester (P7)	284.481	0	2	6.248	1
Octadecanoic acid,ethyl ester (P6)	312.535	0	2	7.003	1
Squalene(P5)	410.725	0	0	13.918	1
Oleic acid(P4)	282.465	1	2	5.905	1
Beta-sitosterol(P3)	414.713	1	1.7	7.473	1
Phytol (P2)	296.535	1	1.7	6.334	1
N-hexadecanoic acid (P1)	256.428	1	2	5.247	1

Table 4:ADMET properties of the compounds

Ligand	**QPPCaco**	**% Human Oral Absorption**	**CNS**	**BBB**	**hERG**
Acceptable range	<25 poor, >500 great	1-low 2-medium 3-high	–2 (inactive), +2 (active)	–3.0 – 1.2	concern below –5
Diazepam (standard)	2684.726	3	1	0.199	-5.097
9,12 octadecadienoic acid(z,z)-methyl ester(P10)	2998.954	1	-1	-0.920	-5.670
Pentanoic acid,10-undecenyl ester (P9)	3322.530	3	-1	-0.786	-5.509
Ergost-5,8(14)-dien-3-ol (P8)	4026.510	1	0	-0.205	-4.547
Hexadecenoic acid,ethyl ester (P7)	3131.098	1	-1	-0.964	-5.559
Octadecanoic acid,ethyl ester (P6)	2941.715	1	-2	-1.152	-5.863
Squalene(P5)	9906.038	1	2	2.038	-6.224
Oleic acid(P4)	227.332	1	-2	-1.599	-3.644
Beta-sitosterol(P3)	3404.598	1	0	-0.340	-4.545
Phytol (P2)	2844.938	1	-1	-0.923	-5.318

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N-hexadecanoic acid (P1)	267.129	3	-2	-1.396	-3.236

The docking results showed that beta-sitosterol and phytol had better dockingscores when compared to the standard diazepam. Beta-sitosterol was exhibited the best docking score which was -5.462 when compared to diazepam which was -4.738.The docking score of other compounds i.e.,N-hexadecanoic acid, oleic acid, squalene, octadecanoic acid, ethyl ester, hexadecenoic acid,ethyl ester,ergost-5,8(14)-dien-3-ol,pentanoic acid,10- undecenyl ester**,**9,12 octadecadienoic acid(z,z)-methyl ester was found to be -3.343,-3.283,-4.801,-0.943,-4.521,-4.308,-2.655,-2.598 respectively. All compounds except squalene possess required physicochemical properties. These compounds possessed molecular weight, hydrogen bond donor, hydrogen bond acceptors in the normal range.The one violation is due to increased QPlog p o/w than the recommended range which implies reduced permeability through biological membranes. Yet, due to only one violation from the rule of 5 the compounds are said to have oral bioavailability. As per the in-silico report, almost all the compounds were having similar to better affinity while interacting with the protein whereas phytol and beta-sitosterol were found to have the best affinity with lowest binding energy. In case of physicochemical, ADME properties and Lipinski Rule of five, Pentanoic acid,10-undecenyl esterwas found to be the best candidate with significant docking result similar to that of standard Diazepam along with good physicochemical and ADME properties

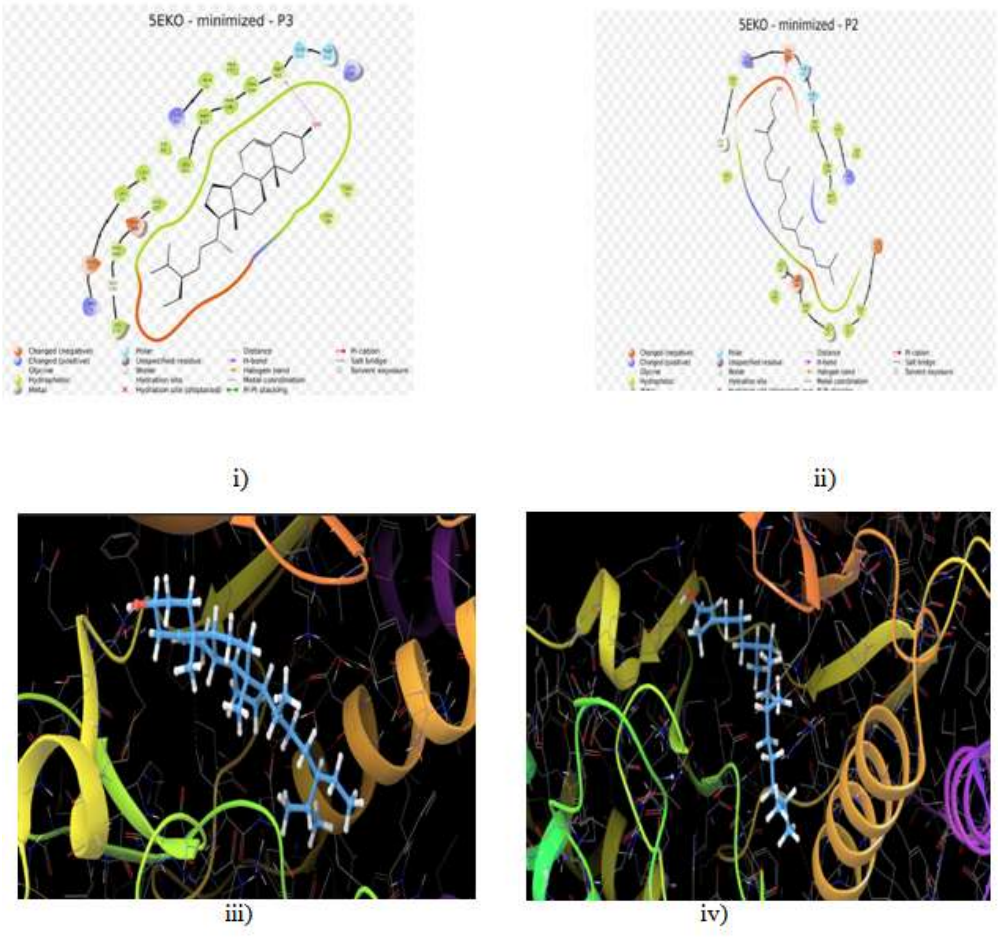

Figure 3 : 2D interaction of P3 and P2 (i,ii)) and 3D interaction of P3 and P2 (iii,iv) with PDB 5EKO

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ACUTE TOXICITY STUDY

The acute oral toxicity study was carried out on Swiss albino mice by using up and down method according to the OECD guidelines 425.The ethanoilc extract of the leaves of Plumeria pudica at the dose of 2000mg/kg, did not show any behavioral changes or symptoms of toxicity during the short-term (48 h) and long-term (14 days) observation period. Hence, the plant extract was found to be safe up to the tested dose of 2000mg/kg, p.o.

EVALUATION OF INVIVO ANTIEPILEPTIC ACTIVITY

Pentylenetetrazole induced convulsions:

Table 5 : Effect of ethanolic extract of P.pudica leaves in the PTZ model

Groups	Treatment	Latency (Sec/ 0.5hr)	Tonic convulsion (Sec/ 0.5hr)	Status of the animal after 0.5hrs		Status of animal after 24hrs	
				No of animals alive	% protection	No of animal alive	% Protection
I	Control	58.26± 0.87	99.52± 0.56	0/6	0	0/6	0
II	Diazepam (5mg/kg)	165.96± 0.57*	123.56± 0.24**	6/6	100	6/6	100
III	Low (100mg/kg)	65.05± 0.41**	112.58± 1.05*	5/6	83.33	5/6	83.33
IV	Medium (200mg/kg)	114.13± 1.36*	135.36± 0.76ns	6/6	100	6/6	100
IV	High (400mg/kg)	125.52± 1.18*	98.38± 0.57*	6/6	100	6/6	100

Values are expressed as mean ± SEM for 6 animals; ***p < 0.0001, *p < 0.001 and *p < 0.05 Vs control.

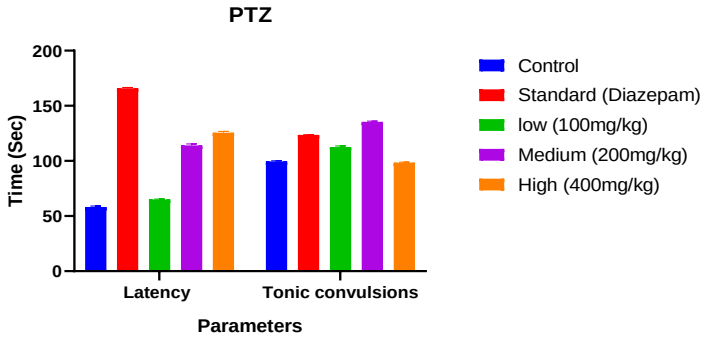

Figure 4: Effect of P.pudica leaf extract on latency and tonic convulsions in PTZ model

In PTZ model, ethanolic leaf extracts at the dose of 100mg/kg, 200mg/kg and 400mg/kg were administered to the mice and latency to onset of seizures and tonic convulsions were measured. The results indicated a significant decrease in duration of convulsions across the three doses of ethanolic extract as compare to control and standard values. The standard diazepam showed

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longer latency to tonic convulsions (165.96±0.57*seconds) and shorter duration of convulsions(123.56±0.24**) compare to control group.The medium and high dose of ethanolic extract showed similar efficacy in increasing latency to convulsions (114.13±1.36*) and (125.52±1.18*) and reducing the duration of convulsions (135.36±0.76^{ns}) and (98.38±0.57*) when compared to the control and standard group. This revealed that medium and high doses of ethanolic leaf extract showed significant antiepileptic activity.

Maximal electric shock induced convulsions:

Table 6 : Effect of ethanolic extract of P.pudica leaves

Groups	Treatment	Flexion	Extensor	Clonus	Stupor	HLE	% protection
I	Control	2.3± 0.36	22.09 ± 0.86	35.16± 1.05	85.26± 0.51	109.53± 1.43	0
II	Phenytoin	0.9± 0.11*	9.36± 0.36**	14.25± 1.32*	30.58± 0.55*	65.89± 0.65*	100
III	Low (100mg/kg)	2.1± 0.10**	15.25± 0.96*	23.56± 0.53^{ns}	41.25± 1.05*	89.58± 1.45*	66.6
IV	Medium (200mg/kg)	2.9± 0.40*	11.25± 1.43*	29.56± 1.05*	65.58± 0.52*	65.89± 1.05**	83.33
IV	High (400mg/kg)	1.9± 0.49*	16.58± 0.53*	20.58± 0.51**	42.58± 0.47*	78.85± 0.51^{ns}	83.33

Values are expressed as mean ± SEM for 6 animals; ***p < 0.001, *p < 0.001 and *p < 0.05 Vs control.

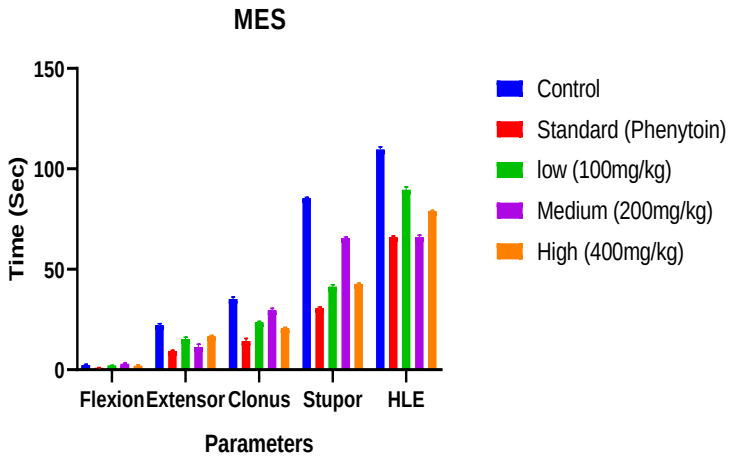

Figure 5: Effect of P.pudica leaf extract on duration of convulsions in MES model

In the MES model, the standard phenytoin showed a significant reduction in all seizure parameters compare to control group. It showed 100% of protection against seizure. All the doses of extract showed a significant reduction in seizure parameters as compared to the control group. The medium dose showed highest flexion (2.9±0.40*),clonus (29.56±1.05*) and stupor (65.58±0.52*) and high dose of extract showed highest extensor value (16.58± 0.53) as compared to the control group. HLE durations of low, medium and high doses are 89.58±3.56*,65.89±2.58** and 78.85±1.25^{ns} respectively. The medium and high doses of

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ethanolic extract of leaves exhibited lowest period of HLE of 65.89±1.05** and 78.85±0.51^{ns} seconds indicating highest % percentage protection against seizure. whereas, the low dose of extract was less effective against MES seizure.

Stupor

Extensor

Hindlimb extension

Flexion

Figure6 : Animals showing different phases of convulsions

IV. CONCLUSION

The study reports the successful extraction, insilico study, acute oral toxicity study and evaluation of antiepileptic activity of the leaf extracts of plumeria pudica. In the present study, 10 phytoconstituents present in leaf were docked towards the MAPK13 complex with inhibitor (5EKO), which showed that beta-sitosterol (-5.462) had better docking score when compared to the standard diazepam (-4.738). The acute oral toxicity study of ethanolic leaf extract on mice was revealed that,the extract was found to be safe up to the tested dose of 2000mg/kg. Anti-epileptic activity was evaluated using in-vivo methods by PTZ-induced convulsion and MES-induced convulsion. In PTZ model, the medium and high doses of ethanolic extract showed similar efficacy in increasing latency to convulsions (114.13±1.36) and (125.52±1.18*) and reducing the duration of convulsions (135.36±0.76^{ns}) and (98.38±0.57*) when compared to the standard group. In case of MES model, the medium and high doses of ethanolic extract of leaves exhibited lowest period of HLE of 65.89±1.05 and 78.85±0.51 seconds indicating highest % percentage protection against seizure. The phytochemicals present in the

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ethanolic extract of leaf responsible for the antiepileptic acitivity.so, it can be concluded that leaf extacts of Plumeia pudica possess antiepileptic activity, it can be used as herbal medicine to treat seizure.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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