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Review

Targeting Alzheimer's Disease Progression with Lignans from *Phyllanthus amarus*

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder marked by cognitive impairment and neuropathological hallmarks such as amyloid-beta plaque deposition and neurofibrillary tangle formation. Despite advances in understanding AD pathogenesis, effective disease-modifying therapies remain limited. Phyllanthus amarus, a medicinal plant known for its rich lignan content, has demonstrated notable neuroprotective, antioxidant, and anti-inflammatory properties. The present study aimed to evaluate the disease-modifying potential of lignans isolated from Phyllanthus amarus in experimental models of AD. Lignans were extracted and characterized, followed by comprehensive in vitro and in vivo assessments to determine their effects on AD-related pathological mechanisms. The results indicated that the isolated lignans significantly inhibited amyloid-beta aggregation, reduced neuroinflammatory responses, and enhanced neuronal survival. Additionally, administration of these lignans improved cognitive performance in AD-induced mouse models. Collectively, the findings highlight the promising therapeutic potential of Phyllanthus amarus-derived lignans as disease-modifying agents in Alzheimer's disease. This study supports the growing interest in plant-based bioactive compounds as viable candidates for the development of novel interventions for neurodegenerative disorders.

Keywords: Alzheimer's disease; Phyllanthus amarus; lignans; amyloid-beta; neuroprotection

INTRODUCTION

Today, deprived memory, sluggish recall, and lesser retention are widespread concerns worldwide. Alzheimer's disease (AD), the most prevalent neurodegenerative condition which impairs memory and the brain, hence is regarded as the main contributor to dementia in the aged population[1-2].AD is attributed to expedited accumulation of Aβ (beta-amyloid) plaque around neurons and neurofibrillary tangles (NFTs) comprised of tau proteins linked to hyper phosphorylated microtubules. The amyloidogenic proposition suggests Aβ as a crucial played in the AD pathologies[3]. The therapy/management of the CNS disorders is a problem due to the complexities of these disorders as well as the limitations of the available allopathic medications that are currently in the market. More than 100 years after the initial description of AD and the identification of Aβ as a key pathologic component, the search for effective anti-Aβ therapies continues[4].

Traditional medicine still contributes significantly to treatment regimen in developing nations. India has a rich history of ayurvedic medicines, which have provided effective treatment for numerous disease-like conditions using plants, plant parts, and plant-derived compounds[5,54]. *Phyllanthus amarus* Schumm and Thonn (Family Euphorbiaceae) generally referred as bhumi amla, is used since years to treat different disease-like conditions such as diabetes, dropsy, flu, and jaundice. It has been reported to exhibit hepatoprotective, diuretic, antiviral, anticancer, anti-inflammatory, and antioxidant activity. One of the *P. amarus* extract has demonstrated potential for the treatment of nervous debility and epilepsy[6]. Also, our laboratory has investigated pain modulatory potential and antifibromyalgic activity of different extracts of *P. amarus*[19-20]. This investigation was intended to assess the anti-Alzheimer-like potential of *P. amarus*. We have investigated probable mechanisms involved using molecular docking simulations.

MATERIAL AND METHODS

Animals used

In the current investigation, Swiss albino mice of any sex were employed. We used young mice, 2-3 months old, weighing about 20g, and old mice, 12-15 months old, weighing around 30g. Six mice were housed in each cage under the usual laboratory lighting (12-hour cycles of darkness and light) and temperature regimes. Water and food are available at all times. The studies took place between 9 am and 3 pm. Prior to the series of experimental experiments, animals were fasted for 12 hours (from food but not from water). Mice were given time to adjust to the laboratory setting.

The institutional animal ethical committee, which was established by the Ministry of Environment and Forests of the Government of India, New Delhi, to control and supervise the use of experimental animals, approved the experimental protocols, which were then carried out in accordance with the aforementioned guidelines. IAEC/ABCP/18/2018-19 and RCP/18 19/P-19 were the protocol numbers that were approved.

Drugs, Lignan-rich extract and chemical agents

The *P. amarus* leaf standardized methanolic extract, referred as *Phyllanthus amarus* lignan rich extract (PALRE), that has a greater than 2.5% of lignans (Ref.No.FP1112042PA/11LOT05) was obtained from Natural Remedies Pvt. Ltd., Bangalore. In this study, we employed Flumazenil (Flu) from Neon Laboratories Ltd, Mumbai, Scopolamine (Sco) from Alcon Inc., and sodium chloride (NaCl), potassium bromide (KBr), all from Loba chemicals. Diazepam of Ranbaxy laboratories and Piracetam (Pir) of Dr Reddy's Laboratories Ltd were purchased from a local medical shop.

Characterization of Lignan-rich extract by Fourier-Transform Infrared Spectrometry (FT-IR)- Shimadzu FT-IR-8400 was utilized to record the FT-IR spectrum. The diffusive reflectance gadget (DRS8000, Shimadzu Corp., Japan)-equipped FT-IR spectrometer and a data unit were utilized for recording the spectra. The specimens were made by combining a tiny amount of extracts—roughly 2-3 mg per extract—with 100 mg of dry potassium bromide. These samples were scanned at a resolution of 2

cm-1 for the wavelength range of 4000–400 cm–1. The FTIR-8000-SCS program was employed to record distinctive peaks.

Assessment of improvement in cognitive functions using Morris Water Maze test

The Morris Water Maze (MWM) is widely used test that evaluates the drug's potential in improving learning and memory. This model throws light on ability of the phytochemicals in improving cognitive functions impaired by Sco, thereby emphasizing its utility in cognitive disorders involving dementia. The MWM consists of a large water tank [48 x 28 x 18] cm filled with water, which is made opaque by adding milk. Water helps to eliminate olfactory obstructions if any and to provide an even unvarying environment within the maze. A [7x7] cm rectangular escape platform is constructed of water-resistant material (plexiglass in this study) that when submerged, allows experimental animals to stay on top. The platform is 10 cm in height and water is filled so that it is submerged 2 cm below the level of water surface. The water temperature is maintained at 26 ±1 degree Celsius.

To assess spatial memory, young mouse with head pointing towards side of the pool, was released and the time taken (escape latency [EL]) to reach the submerged platform was noted. With previous exposure to this set up, the time the mouse is taking to find a hidden platform using only available external cues utilized to quantify the spatial memory. For acclimatization, the mice permitted to swim for 90s before beginning of the hidden platform training. Then the platform positioned in the middle of target quadrant of the pool and the animals released in the pool from opposite quadrant. Each mouse was given 90s to reach the platform. If the animal fails to locate the platform in 90s, then the animal was guided to the platform by the researcher. Then the mouse allowed remaining on platform for 20s to rest. Again, the mouse released from same place and time for reaching the submerged platform was recorded. Likewise, totally 4 trials were conducted in a row, in a day, and average time to reach the submerged platform was recorded, keeping similar experimental conditions.

Standard drug (Pir 200mg/kg) and PALRE in three doses, i.e. 100, 200, and 400 mg/kg) were administered orally and after 60 minutes all the groups were exposed to the training schedule. This procedure was repeated at 24 hour interval for three more days until each subject acquired minimum time interval to reach the submerged platform in the pool. On fourth day, all groups were administered Sco (1mg/kg, i.p.) 30 minutes later they were treated with test drugs and after 60 minutes they were tested for spatial memory. Latency to reach the platform in seconds (mean values) was calculated on days 1, 2, 3, 4, and 5. Day 2 is the day from which animals were treated with the drug. The mice were assessed again on the fifth day for spatial memory to check the ability of drug to restore Sco-induced amnesia (retention trial)[40-45].

Assessment of anti-Alzheimer activity in young versus aged mice using Elevated plus maze apparatus and with scopolamine-induced amnesia

Both young and aged Swiss albino mice utilized in this behavioral model. The 3-4 month old mice (young mice) weighing approximately 20g and 12-15 month old mice (aged mice) weighing approximately 30g were utilized. The PALRE in three doses, ie 100, 200, and 400 mg/kg) was administered orally for eight consecutive days to mice of both age groups. On eighth day, Sco 1mg/kg, was given intraperitoneally post 60 minutes of the last dose of test drugs (PALRE in three doses) to induce amnesia in young mice. Post 45 minutes of Sco treatment, animals were permitted to the training session on elevated plus maze (EPM) apparatus. The transfer latency (TL), i.e., movement between open and closed arms of EPM apparatus was recorded. The TLs recoded on eighth day are presented as results of acquisition trail. On the ninth day (i.e. after 24 hours) the mice were assessed again on EPM to record retention of memory (Retention trail). Piracetam (200mg/kg, i.p.) was used as reference standard and was injected for 8 consecutive days and procedures outlined in the above paragraph are followed. Similarly, animals in the control group received normal saline for 8 consecutive days.

In this investigation, the EPM equipment as stated for mice by Lister RG and Pellow et al. was used. The EPM test apparatus comprised of enclosed arms sized [37X5X12] cm and open arms sized [37X5] cm and a 12cm high wall linked to 2 closed and 2 open arms, and the wall is placed so that the alike arms were opposite to each other and all four arms are linked to each other by a [5X5] cm of central square. The wooden apparatus was elevated to a height of 25cm above the floor. Each mouse was placed individually in the central square with head pointing towards open arm and TL was recorded for 5 minutes. Each mouse was utilized only once and every test was conducted during scheduled time (and other conditions as specified above in Section 7.2.1). After each test is carried out, the EPM apparatus was cleaned using ethanol. The rationale behind utilizing EPM apparatus included the fear-provoking nature of the open arms and feeling of relative safety towards closed arms and assessing the retention of memory of animals to prefer closed arms over open arms. The EPM test is a widely utilized behavioral animal model for assessing memory and learning in rodents[7,45].

Assessment of Impact of Lignan-rich extract (PALRE) on Motor Coordination Activity of Mice

The complex system of motor coordination involves specific pattern of walking, balancing, and strength of muscle. It is well-established fact that sedatives (such as benzodiazepines, barbiturates, etc.) and other molecules/drugs that interfere with balancing or ambulatory activities or that weaken muscles have demonstrated impaired performance in the tests conducted using Rota-rod apparatus. Hence, Rota-rod apparatus is used popularly to estimate potential impact of test drugs on the motor coordination of rodents. The Rota-rod instrument (from Inco, Ambala,model no. K19616-2) comprised of a central bar (with a constant speed of 22 rpm) subdivided into 3 compartments by disks. A day (24 hours) before actual testing, mice were selected. The animals that failed to remain on the central bar for a period of 150s in two consecutive trials were excluded. Selected mice were treated with test drug (PALRE in three doses, ie 100, 200, and 400 mg/kg) or standard drug (Diazepam 2mg/kg) or vehicle as per the group and tests were carried out 30 minutes post treatment. The outcome measure included the time for which mice remained on the revolving bar. The cut-off time for each test was 150s.

Estimation of Acetylcholine [ACh] Levels in Brain by Quantifying Cholinesterase Inhibition

After the completion of Morris Water maze test, mouse from each group was euthanized via cervical detachment. The entire brain was taken right away and chilled in ice-cold phosphate buffer. After washing in ice-cold phosphate buffer it was homogenized in 5ml of phosphate buffer in Glass Teflon homogenizer. The brain homogenate then evaluated for enzyme activity.

Standard Curve of ACh: Aliquots of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 ml of ACh in buffer added to different test tubes. Phosphate buffer added to each tube to give a volume of 1 ml. 2ml of Alkali added to each tube by shaking vigorously. After not less than a minute, 1 ml of HCL solution (pH of 1.2± 0.2) and 1 ml of $FeCl_3$ solution added. The absorbance of the color in each tube read at 540 nm. The control tube (with 0 ml of Ach) used to adjust the zero reading of the instrument. Plot of milimoles of Ach vs. absorbance obtained.

Determination of Cholinesterase Inhibition: Three types of test tubes were prepared: Tube 1: served as control that contained 1 ml of buffer (instead of ACh solution) and other reagents. Tube 2: served as sample or S that contained 1 ml of ACh solution, 0.1 ml of homogenate and was kept for incubation at 37°C ± 1°C for 1hr. Tube 3: served as STD or S60 that contained 1 ml of ACh solution and 0.1 ml of homogenate was added after the addition of Alk hyd which itself was added after incubation at 37 ºC±1ºC for 1 hr. After the incubation period, 2ml of Alk hyd was added by shaking vigorously to tubes 1and 2. After not less than a minute, 1 ml of HCL solution (pH of 1.2 ± 0.2) and 1 ml of FeCl3 added to all three test tubes. The resultant mixtures centrifuged and the absorbance of the supernatant read at 540 nm. Note: S60 used to correct the determination of non-enzymatic

hydrolysis of ACh since the homogenate added after incubation. The control tube (with 1 ml of buffer) used to adjust the zero of the instrument.

Assessment of involvement of GABA receptor

Flumazenil (Flu), a benzodiazepine antagonist, was used in experimental procedures to evaluate if this receptor may conceivably contribute to the test drugs effects PALRE at highest dose, ie, 400 mg/kg). Flu 2.5mg/kg was administered to a small group of mice (4 mice per treatment group), along with the test groups and the standard Diazepam (DZP) group, in order to assess the effects using the EPM model equipment in accordance with the above-described methods.

HPTLC analysis

Advanced phytochemical investigations performed on HPTLC for identification and characterization of bioactive extracts, with the help and supervision of experts from Anchrom lab Mumbai. The details of the instrumentation parameters and procedure for finger print analysis are outlined below.

Instrumentation and chromatographic conditions utilized for HPTLC analysis:

- Instrument-CAMAG Linomat 5 "Linomat5_080222" S/N 080222 (1.00.12)
- Spotting device - Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland).
- Syringe - 100 µl (Hamilton, Bonaduz, Switzerland).
- TLC chamber - Glass twin-trough chamber (20 x10 x 4 cm; Camag).
- Densitometer - TLC Scanner 3 linked to winCATS software (Camag).
- HPTLC plates - 20 x 10 cm, 0.2 mm layer thickness, precoated with silica gel 60 F254, Cat. No. 1.05548, E. Merck KgaA, Darmstadt, Germany.

Linomat 5 application parameters: Spray gas: Inert gas; Sample solvent type: Methanol; Dosage speed: 150 nl/s; Predosage volume: 0.2 ul; Syringe size: 100 µl; Number of tracks: 4-8; Application position: 8.0 mm; Band length: 8.0 mm; Solvent front position: 80.0 mm.

Preparation of Phyllanthus Sample Solutions:

Dried powdered extracts of *P. amarus* (200mg) re-extracted exhaustively with methanol using a sonicator for 1 h on a water bath. The methanol soluble portion filtered used for the further HPTLC analysis. The stock solution of the sample, having concentration of 0.4 mg/ml (0.4 µg/µl) was prepared.

Mobile Phases for General Finger Print Analysis: For the creation of typical chromatograms, other mobile phases, such as toluene: ethyl acetate (80:25) and toluene: ethyl acetate: formic acid (60:20:20), were explored. Out of the various mobile phases tried, Toluene:Chloroform: Ethanol (4:4:1, v/v) gave the best resolution for development of commonchromatogram for the analysis of the components of the extracts under study from each other.

General Fingerprint Analysis: HPTLC aluminum plates pre-coated with silica gel were asthe stationary phase. The plates not pre-washed with any solvent prior to chromatography.The samples spotted in the form of bands, with the help of a Camag 100 micro liter syringeusing a Camag Linomat V (Switzerland) sample applicator. A constant application rate 150nL/s employed. The slit dimension was kept at 6 mm × 0.45 mm, with a scanning speed of 20mm/second, and a data resolution of 100 µm/step was employed.

The composition of the mobile phase was toluene: chloroform: ethanol (4:4:1). The linear ascending development carried out in twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 minutes at room temperature (25 ± 2°C). The length of the chromatogram run was 80 mm. Subsequently, the plate allowed dry at room temperature. The separated bands on the HPTLC plates scanned over the wavelength of 200 – 540 nm. The source of radiation utilized was the deuterium illumination (D2 lamp) for 254 nm, Mercury (Hg) for 366 nm and for 540 nm. The images captured on Camagreprostar 3 with win-CATS software 4.05.

Statistical analysis

The mean and standard error were used to express every result. One-way ANNOVA was utilized for data analysis and then Dunnett's test to compare the outcomes of the test groups with those of the control group. As six mice were utilized per group, data generated has had 6 values per testing arm, ie, n=6.

FT-IR results for Lignan-rich extracts (PALRE)

Infrared spectroscopy is one of the most powerful yet simple and reliable analytical techniques that offer rapid identification of molecular structures and information on their chemical classification. The IR spectroscopy also helps in structural elucidation of novel phytochemicals derived from medicinal plants.

RESULTS

The FT-IR results confirmed as PALRE as lignan-rich extract (See Figure 1).

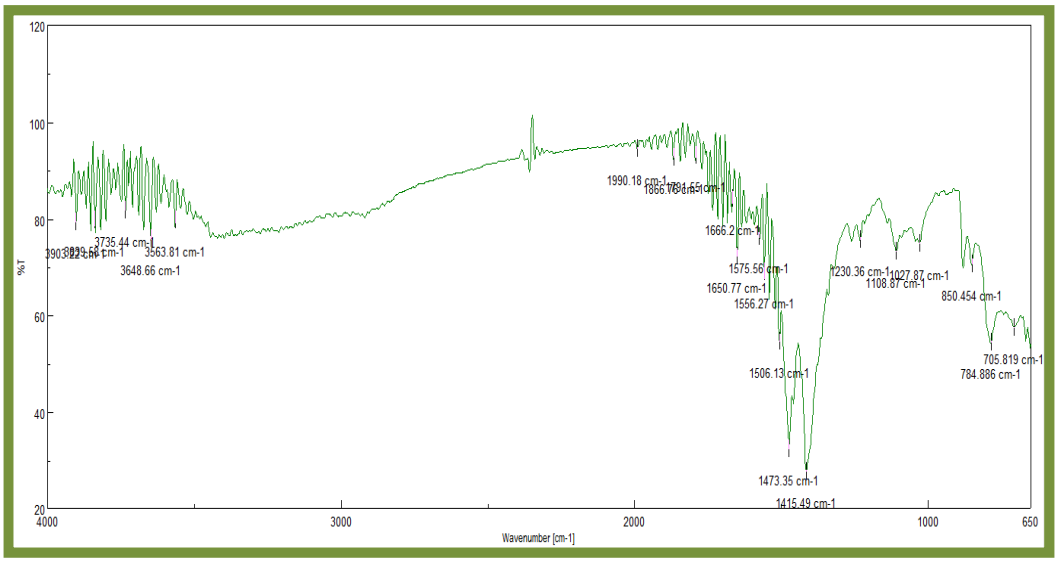

Figure 1: FT-IR spectra of Lignan-rich extract (PALRE)

Improvement in cognitive functions using Morris Water Maze test

Pre-treatment with PALRE extract at doses 200 and 400 mg/kg showed moderately substantial ($p<0.05$) fall in the EL to reach the platform on both 4th and 5th day as shown in Figure 2. However, animals in 100mg/kg dose group did not show significant decrease in EL. The decreased EL on 5th day signifies retention of memory. The results of higher doses (400mg/kg) reflected marked decrease in EL on 4th and 5th day which was comparable to that of piracetam group. Lastly, pre-treatment with PALRE extract showed more decrease in EL compared at all dosage points, i.e., 100/200/400 mg/kg.

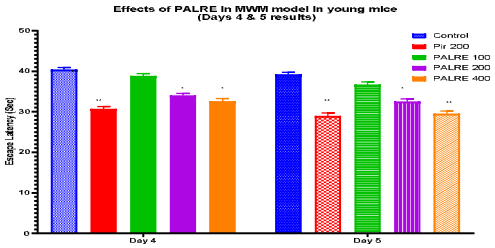

Figure 2:Effects of PALRE on escape latency in Morris Water Maze model

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set, by utilizing one-way ANNOVA + Dunnett's test, *P< 0.05, **P < 0.01, were deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard wasPiracetam 200mg/kg, orally given Anti-Alzheimer like activity in young and aging animals using Elevated Plus Maze test with

scopolamine-induced amnesia Lowing of TL is an indication of improvement in memory. The PALRE treated young and old mice noted remarkable reduction in TL on 9th day, signifying retention memory. Pre-treatment with standard drug, i.e. Pir 200mg/kg for 8 consecutive days resulted in decreased TL on 8th Day and on 9th Day as compared to control, which validates our model. The PALRE in doses 100, 200, and 400 mg/kg administered orally for eight consecutive days have resulted in remarkably decreased ($p<0.05$ for 100mg/kg dose groups and $p<0.01$ for 200 & 400 mg/kg dose groups) TL on 8th and 9th day in both aged and young, compared to control groups on EPM test apparatus. And, the results of higher doses of test extract (400mg/kg) reflected major decrease in TL on 9th day which were comparable to the results of Piracetam (Figures 3- 6). Treatment with Scopolamine (1mg/kg) resulted in significant increase in TL in young mice on 8th and 9th day as compared to control, signifies memory impairment. Also, it was observed that PALRE 400 mg/kg orally successfully reversed memory deficits induced by Sco in young mice (Figures 3 and 4).

The results of aged mice noted higher TL on 8th Day and on 9th Day when compared with TL values of young mice, which signifies impairment of memory and learning abilities in aged mice. The results also noted improved memory and the learning of aged animals compared to Sco-treated young mice as demonstrated by noteworthy decrease in TL when subjected to EPM tests. Lastly, pre-treatment with PALRE extract showed more decrease in TL.

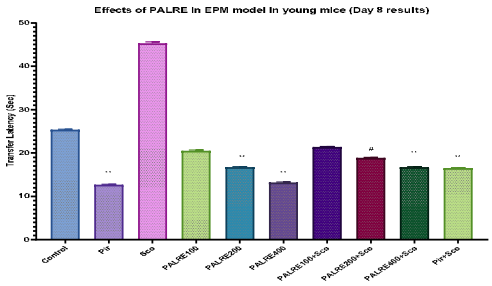

Figure 3: Effect of PALRE of *P. amarus* on transfer latency in EPM test in young mice on day 8

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test, **P < 0.01was deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard was Pir, ie, Piracetam 200mg/kg, orally given **Note** - Scopolamine 1 mg/kg was administered on Day 8 only, intraperitoneal.

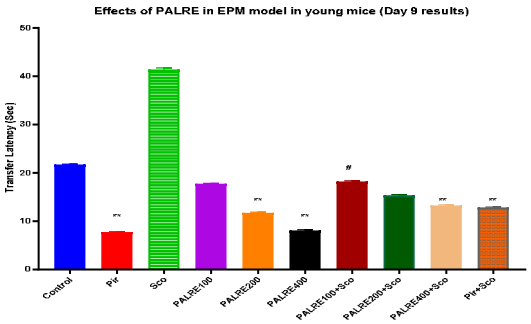

Figure 4:Effect of PALRE of *P. amarus* on transfer latency in EPM test in young mice on Day 9

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test, **P < 0.01was deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard wasPir, ie, Piracetam 200mg/kg, orally given **Note** - Scopolamine 1 mg/kg was administered on Day 8 only, intraperitoneal.

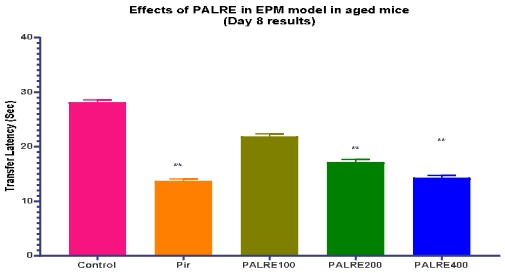

Figure 5:Effect of PALRE of *P. amarus* on transfer latency in EPM test in aged mice (age-induced amnesia model) on Day 8

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test, **P < 0.01was deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard wasPir, ie, Piracetam 200mg/kg, orally given

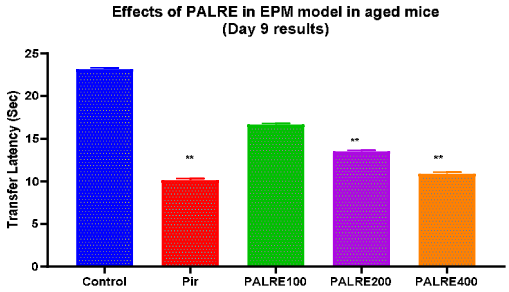

Figure 6: Effect of PALRE on EPM model in aged mice

Values showedabove are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test, **P < 0.01was deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard wasPir, ie, Piracetam 200mg/kg, orally given

Effect of Lignan rich extract on motor activity

The results of animals treated with PALRE 100mg/kg, and PALRE 200mg/kg does not show significant change in motor coordination activity when compared with the results for control group.

However, at a higher dose (PALRE 400mg/kg) the findings of both the extracts seems to be sedative (p<0.01). Also, extract showed more impact on motor coordination compared with PALRE extract as showed in the Figure 7.

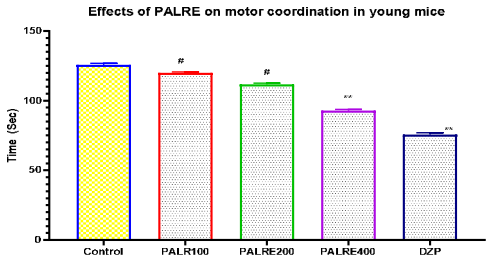

Figure 7: Effect of PALRE on motor coordination

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test, **P < 0.01was deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard wasDiazepam 2mg/kg.

Effect of PALRE on Acetylcholine [ACh] levels in mouse brain

Pre-treatment with PALRE in three doses, ie 100/200/400 mg/kg exhibited significant protective effect against ACh breakdown as indicated by increase in amount of unhydrolysed ACh and decrease in the amount of hydrolysed ACh. Increased levels of unhydrolysed acetylcholine in brain homogenate of treated animals, gives an indication of acetyl cholinesterase inhibitory activity of PALRE in three doses, ie 100, 200, and 400 mg/kg). Also, results presented more rise in unhydrolysed acetylcholine levels in brain homogenate of mice treated with PALRE extract at all dose levels, i.e., 100, 200, and 400 mg/kg; which signifies effectiveness of lignan-rich extract (Figure 8).

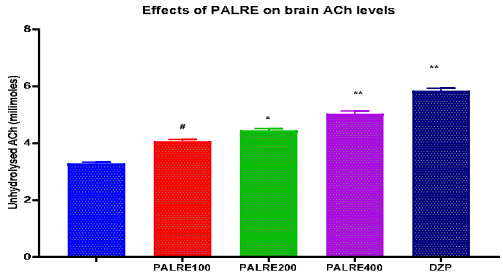

Figure 8: Effect of *P. amarus* extracts (and PALRE) on Acetlycholinestarase levels in mice brain.

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control set by utilizing one-way ANNOVA + Dunnett's test,* denotes p<0.05 and**P < 0.01,were deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard utilized wasDiazepam 2mg/kg.

Blockade of the anti-amnesic effect of Lignan-rich extracts by Scopolamine

In the EPM test, Flumazenil 2.5 mg/kg inverted the impact of diazepam 2 mg/kg and PALRE extract at the dose of 400 mg/kg on the timeframe spent and amount of entrancesin the open wing of EPM device, suggestive of probable method of action of PALRE extract via GABA-A receptor as indicative in Figure 9.

One of the widely utilized tests used to find novel benzodiazepine-like anxiolytic drugs is the EPM testand flumazenil is most-widely used experimental tool to study benzodiazepine antagonism. In this context, the activity of the lignan-rich (PALRE) extract in relieving anxiety-like effects in the EPM model may indicate that the GABA-A/benzodiazepine receptor complex has been positively modulated.

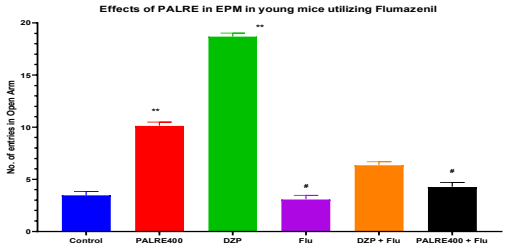

Figure 9: Elevated Plus Maze task effects of Flumazenil on PALRE pretreament

Values showed above are in Mean ± SEM; N was 6.Comparatively to the control setof young mice by utilizing one-way ANNOVA + Dunnett's test,* denotes p<0.05 and **P < 0.01, were deemed noteworthy; while # denotes P>0.05, i.e., non-significant results.Positive standard utilized was Diazepam 2mg/kg.

HPTLC Finger prints of Lignan-rich PALRE extract

HPTLC fingerprinting analyses of the methanolic extract of *P. amarus* (PALRE) was conducted for detection class of compounds i.e., for lignans. The peaks observed in the graphs clearly indicate significant content of lignans in PALRE extract (see Figures 10 to 12).

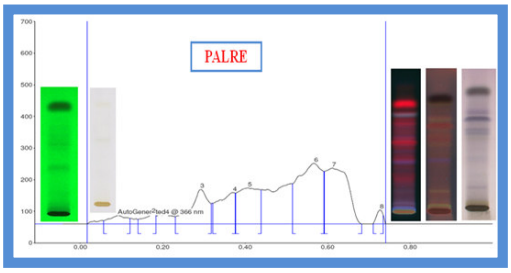

Figure 10: HPTLC Finger prints of *P. amarus* standardized extracts PALRE and

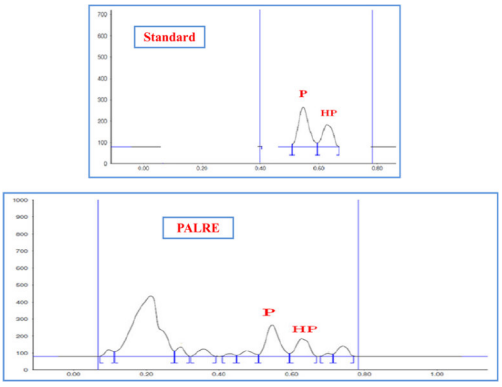

Figure 11: HPTLC chromatogram of PALRE for lignans detection, utilizing markers -phyllanthin (P) and hypophyllanthin (H)], mobile phase composed of chloroform: methanol: water (7:3:0.4, v/v).

Detection of Lignans

For detection of Lignans mobile phase used was chloroform: methanol: water (7:3:0.4, v/v) and the derivatization was carried with the help of Vanillin sulphuric acid.

Chromatographic finger print analysis of the phyllanthus extracts taken at 254 nm wavelength for detection of lignans showed unique Rf loci at 0.61. The extract showed presences of compounds of lignan class. HPTLC finger print profile and 3d spectra for detection of lignans in phyllanthus extracts taken at 254 nm wavelength is depicted in Figure 10 -12. The maximum height peak of 354.2 was observed with extract. The number of auto generated was

15. The Rf range for was 0.13 - 0.74 and for PALRE was 0.12 - 0.74. The details of Rf range, number of Peaks, number of auto-generated tracks and maximum height of peaks is summarized in Table 1.

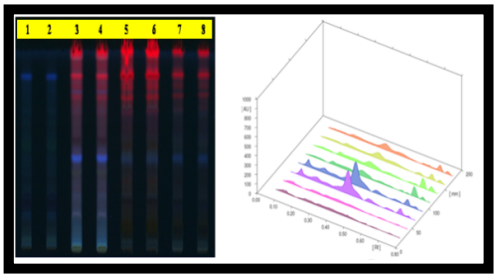

Figure 12: Phytochemical finger print and 3d spectra of phyllanthus extract taken at 254 nm wavelength for detection of class of compounds [Lignans

Table 1: Chromatographic finger print analysis of phyllanthus extract taken at various wavelength for detection of class of compounds [Lignans]

Details at specific wavelength	Parameters					
	Rf range	Rf of Unique loci	No of Peaks	No of Auto Generated tracks	Max Height	Area % range
254 nm wavelength	0.08 - 0.74	0.63	11	15	20.1 - 177.4	0.86 - 26.63
366 nm wavelength	0.12 - 0.74	0.39	9	12	11.4 - 101.7	2.41 - 44.58
Derivatives 366 nm wavelength	0.19 - 0.71	0.30	3	8	17.5 - 24.1	8.53 - 51.87
540 nm wavelength	0.45 - 0.70	0.45	3	6	19.6 - 30.0	19.76 - 50.69

Chromatographic finger print analysis of the phyllanthus extracts taken at 366 nm wavelength for detection of lignans shows unique Rf loci at 0.40. extract showed presences of 9 compounds of lignan class, with maximum % of area covered by extract. HPTLC finger print profile and 3d spectra for detection of lignans in phyllanthus extracts taken at 366 nm wavelength is depicted in Figure 10. The maximum height peak of 245.5 was observed with extract. The number of auto generated peaks for extracts were 12 each. The Rf range for was 0.19 - 0.71. The details of Rf range, number of Peaks, number of auto-generated tracks, and maximum height of peaks is summarized in Table 1.

Chromatographic finger print analysis of the derivatised phyllanthus extracts taken at 366 nm wavelength for detection of lignans showed unique Rf loci at 0.20 and 0.37. The extracts showed presences of 3 compounds each, of lignan class, with maximum % of area covered by PALRE extract. HPTLC finger print profile and 3d spectra of the derivatised phyllanthus extracts for detection of lignans taken at 366 nm wavelength is depicted in Figure7-62. The Rf range for PALRE was 0.13 - 0.70 and for PALRE was 0.45 - 0.70. The maximum height peak of 30.6 was observed with PALRE extract. The number of auto generated peaks for extracts were 5 and 8, respectively. The details of R_f range, number of peaks, number of auto-generated tracks and maximum height of peaks is summarized in Table 1.

Chromatographic finger print analysis of the phyllanthus extracts taken at 540 nm wavelength for detection of lignans did not showed any unique Rf loci. extracts showed presences of 2 and 3 compounds of lignan class respectively, with maximum % of area covered by PALRE extract. HPTLC finger print profile and 3d spectra for detection of lignans in phyllanthus extracts taken at 540 nm wavelength is depicted in Figure 7-63. The maximum height peak of 30.0 was observed with PALRE extract. The number of auto generated peaks for extracts were 6 each. The Rf range for PALRE was 0.13 - 0.70 and for PALRE was 0.45 - 0.70. While the other details of Rf

range, number of Peaks, number of auto-generated tracks and maximum height of peaks is summarized in Table 1.

DISCUSSION

This investigation assessed in detail the anti-amnesic activity of standardized extract (PALRE) of the *P. amarus* in mice by utilizing exteroceptive and interoceptive behavioral rodent models viz. MWM test, EPM test, and Sco-induced amnesia. The HPTLC fingerprint analysis supported the investigational studies helped to see path forward.

Morris water maze

Further, anti-Alzheimer potential of both the plant extracts was evaluated using Morris water maze test. Application of scopolamine to block muscarinic acetylcholine receptors causing cognitive deficit is a representative model used to evaluate the anti-dementia activity of herbal extracts. Morris water maze model has been extensively utilized to investigate the neurological mechanisms underlying spatial navigation to influence special cognitive processes. The same model can also be used to test working memory by changing the hidden platform from one quadrant to another quadrant. Pre-treatment with the plant extract of *P. amarus* (PALRE) at doses 100mg/kg, 200 mg/kg and 400 mg/kg remarkably ($P<0.05$) reduced the time required reaching the platform post scopolamine treatment. It improved basal as well as scopolamine-impaired performance with respect to acquisition and retention of memory. These results signifying possible anti-AD like activity of PALRE extracts may have been mediating via cholinergic pathway.

Elevated Plus Maze

The EPM test is an established rodent model for evoking an approach-avoidance conflict and for assessing the retention of memory of animals to prefer closed arms over open arms. An animal is thought to be in a happy mood, be anxiety-free, have anti-amnesia, anti-Alzheimer's-like activity when it stays for longer time in open arms. In this study, two extracts of the *P. amarus* in three different dosage forms (100, 200, and 400 mg/kg), separately, produced significant effect in a dose dependent manner compared to Piracetam group. Also, results of 100 and 200 mg/kg groups indicated that at these dose levels PALRE extract did not affect the rodent's motor coordination abilities. The EPM test is well-known model to assist in the quest for novel benzodiazepine-like anti-anxiety drugs and to that point, we have communicated supportive results while demonstrating anxiolytic activity of *P. amarus* extracts. Also, in recent years a significant amount of research has been done that is throwing light on the relationship of GABAergic signaling system contributing to AD pathogenesis. In these contexts, the activity of the PALRE in doses 100, 200, and 400 mg/kg) in relieving dementia/Alzheimer-like behavior in EMP model possible via modulation of the GABA-A/benzodiazepine receptor complex [12,13,14,46].

Study of exteroceptive and interoceptive behavioral models (scopolamine, and ageing induced amnesia) using elevated plus Maze in mice reveals that P.amarus treated mice possess anti-dementia/ anti-Alzheimer-like and anti-amnesic activities. It was noted that the reduction of spontaneous motor activity could be related to the calmness/sedative effect. In this investigation, the motor activity results demonstrated dose-dependent sedative activity of PALRE extract of *P. amarus* [13,15].

Motor coordination test

In this study, *P. amarus* extract in three different dosage forms (100, 200, and 400 mg/kg), separately, produced distinct effects. Comparatively PALRE treatment showed more impact on motor co-ordination. PALRE at lower doses (100 and 200 mg/kg) did not show significant impact on the motor coordination.

It was evident that at a advanced dose (400mg/kg) as well as PALRE significantly impaired motor coordination of mice. It was also observed that PALRE showed more impact on motor coordination compared with treatment. The calming/sedative effects of *P. amarus* extracts may be responsible for the decrease in spontaneous motor coordination activity.

GABAnergic mechanisms of *P. amarus*

It has been demonstrated that the equilibrium amid excitation (glutamate) and inhibition (GABA) in the brain is impacted by the interactivity amidst Aβ, GABAergic signaling, and acetylcholine. Targeting different GABA receptor subtypes has the prospective to provide benefit to people with AD to overcome their memory problems, according to several preclinical interventions reported. The development of anti-AD medications may benefit from targeting GABAergic signaling, according to current research, as multiple preclinical and clinical studies have shown impaired GABAergic cell metabolism in AD. Also, in recent years a significant amount of research has been done that is throwing light on the relationship of GABAergic signaling system contributing to AD pathogenesis. [56-59]

To aid in the hunt for innovative benzodiazepine-like anxiolytic remedies, the EPM test is a well-known model and to that point, we have communicated supportive results while demonstrating anxiolytic activity of *P. amarus* extracts. The actions of *P. amarus* may interact with the GABA/benzodiazepine receptor complex in the brain, according to recent in vivo research in the EPM model and molecular docking studies. Flumazenil negated the effect of diazepam and *P. amarus* on the amount of entrances and timeframe spent in the open wing of the EPM device, as seen in the EPM test, suggesting a potential GABA-A receptor-based mode of action for *P. amarus*. Therefore, additional research was considered essential for determining the precise mechanism (s) by which *P. amarus* shows its disease modifying potential and detailed investigation of its efficacy in neurodegenerative diseases including AD.

Phytochemical screening and HPTLC Fingerprint analysis for class of Lignans

Investigations on the phytochemical hunt of *P. amarus* extracts discovered the occurrence of lignans, flavonoids, saponins, glycosides, alkaloids, proteins, steroids, and phenolic compounds [13-18]. Lignans were noted to possess antioxidant, analgesic, anti-arthritic, antiinflammatory, and immunomodulatory activity. Bioactive phytochemicals in the class of lignans such as niranthin, nirtetralin, and phyltetralin have reported ant-iinflammatory potential. In addition, niranthin and lignan-rich portion were reported anti-inflammatory like activity [47-53]. The notably corilagin within P.amarus reported to display antihyperalgesic activity [54].

The phytochemical fingerprint analysis of the PALRE extract signifies that the anti-Alzheimer-like anti-amnesic potential noted in this investigation can be considered aligned to the lignans. The HPTLC fingerprint analysis of revealed significant peaks of PALRE extract. Additionally, the HPTLC chromatograms of PALRE demonstrated presence of lignans by utilizing phyllanthin and hypophyllanth as marker compounds. The HPTLC investigations on the classes of compounds clearly denoted that the PALRE extract has high level of lignans. Hence, the data from HPTLC chromatograms and assessment of class of compounds utilizing HPTLC as a basis supports the fact that the standardized extracts contain substantial amount of lignans in PALRE. So, we can propose that the presence of these phytochemicals might relate to the bioactivity in this investigation.

Docking analysis

Principally histopathologic lesions of AD included Aβplaques, NTFs, and ultimately depletion of neurons. NFTs comprised of hyperphosphorylated tau protein which when aggregated result in neuronal death [36]. Apolipoprotein E (ApoE) consisted of 299 amino acid proteins programmed by the APOE gene. The APOEε2, ε3, and ε4 polymorphisms were found to alter the likelihood of AD development in a dosage-dependent fashion. It was testified that APOE ε4 is linked with higher risk for AD. And the effects of APOE gene on AD were reportedly mediated through the effects of ApoE protein on Aβ accumulation in the brain and cerebrovasculature [35]. In one of key pathologic hypotheses of AD, loss of acetylcholine neurons in the basal forebrain results in loss of memory and worsening of other cognitive functions. To date, three cholinesterase inhibitors (CIs) are approved as first-line remedy for mild to moderate AD:

donepezil, rivastigmine, and galantamine. Available data on humans suggested these three CIs possesses cognitive benefits and they are useful in alleviating everyday activities and overall function in sufferers of mild to moderate AD. The molecular docking investigations have shown that these anti-dementia-like, anti-Alzheimer-like effects of the *P. amarus* extracts possibly attributable to the hydrogen/hydrophobic bonds and Vander wall interactions of these small molecules (flavanoids and lignans) with the Apolipoprotein Ɛ4, acetylcholinestarate, and/or tau protein at molecular level in the brain. Thus, the docking results of present study are suggesting significance of flavanoids such as Niruriflavone, Quercetin, Quercetol, Rutin, and Kaempferol; such as Ellagic acid, Gallic acid, Catechin, Gallocatechin, isoCorilagin, and Corilagin; and lignans such as Niranthin, Phyllanthin, lintetralin, hypoPhyllanthin, and Nirtetralin in treating AD. A lot of research has already been conducted to assess flavanoids as therapeutic targets for treating AD and it is noteworthy to mention that these compounds were unable to generate clinical evidence as promising therapies [37]. At the same time, have been reported to improve general well-being, possesses health- alleviating potential, and have neuroprotective and antioxidant effects versus oxidative stress-led neurological impairment, neural inflammation, and neurotoxins [38]. Lignans have also been found useful therapeutic agents that can improve the cognitive impairment induced by AD [39]. Beta-amyloid and tau protein are leading targets for Alzheimer's disease-modifying therapies. So, the correlation between tau, Aβ and other factors is a key to to develop effective AD-modifying regimen [36]. Recently, plants and plant-derived bioactive phyto-compounds considered key leads by researchers to develop safer and effective therapeutic regimens. Hence, further investigations are deemed necessary for elucidating the exact mechanism and detailed investigation of efficacy of these phytochemicals from the class of lignans [16].

Proposition of mechanism of action of *P. amarus*

The P. amarus extracts and its active constituents were thought to stimulate the brain's central Ach (Acetylcholine) functioning via inhibiting AChE (acetylcholinesterase), despite the fact that the processes behind their anti-amnesic activities continue to be revealed. Additionally, it has been proposed that compounds that improve cognition stimulate cholinergic transmission via having an agonistic or antagonistic impact on the GABAA/benzodiazepine receptor, and that this complex regulates the release of Ach. P. amarus extracts could successfully manage memory dearth in a rodent (mouse) process of Sco-led dementia by enhancing cholinergic system performance and perhaps activating GABA neurons.

CONCLUSION

Overall, the outcomes of interoceptive/exteroceptive behavioral rodent models put forward the smaller dosage of the lignan-rich extract (PALRE) showed slightly better anti-Alzheimer-like action, whereas at 400mg/kg dose, outcomes suggest sedative potential for both the extracts. Hence, this investigation hints the outcomes that the *P. amarus* lignan-rich extract could potentially have a role in the AD treatment plan. It also throws light on the need to further investigate role of lignans as potential disease-modifying therapies in AD.

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