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Cognitive and antioxidant potential of *Tabernaemontana divaricata* in Diazpem induced amnesia

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Abstract
The objective of the present study was to investigate and establish the antiamnesic/memory enhancing effect as well as the antioxidant capability of the ethanol extract of the leaves of *Tabernaemontana divaricata*. The extract was found to contain alkaloids, phenols, tannins, flavonoids and triterpenoids. The total phenolic content was determined and the total phenolics present in the ethanolic extract was found to be 44.86±2.689 GAE mg/g. The cognition enhancement potential of the ethanolic extract was determined by diazepam induced amesia model at two doses (200 mg/kg and 400 mg/kg) using actophotometer (locomotion) and pole climbing test (passive avoidance time). The percent improvement in locomotion compared to the disease control was found to be 24.49 and 29.31% respectively at the low and high dose of TLEE on the 8th day and 29.31 and 70.93% respectively at the low and high dose of TLEE on the 9th day suggesting a significant improvement in memory. On the other hand mice treated with TLEE were able to reduce the passive avoidance time by 13.29% at 200 mg/kg dose and 23.42% at 400 mg/kg dose on the 8th day whereas on the 9th day the time was further reduced by 27.75% at 200 mg/kg dose and 41.89% at 400 mg/kg dose on the 9th day as compared to the disease control suggesting a significant improvement in memory. The antioxidant activity of TLEE was studied by assessing the total reducing potential by reduction of potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}). The extract was able to produce time and dose dependent reduction in the substrate suggesting antioxidant activity.

Keywords: Cognition, amnesia, antioxidant, *Tabernaemontana divaricata*, diazepam

Introduction
Dementia has been a key factor in several syndromes like Alzheimer's disease and Parkinson's disease. Aging, stressful conditions, reduced brain metabolism, high oxidative stress levels, inflammation or reduced plasticity has been hypothesized to be involved in cognitive dysfunction associated with neurodegenerative disorders such as Alzheimer's (AD) or Parkinson's disease (PD) [1].

Plant-derived natural products are significant as sources of medicinal agents and models for the design of new remedies. Lately, research has been directed to traditional folk medicines as they are generally characterized by high acceptability and good toleration. Several reports mention the role of oxidative stress in dementia [2, 3].

Tabernaemontana divaricata is a flowering plant widely found throughout India. The plant has been extensively studies and is found to contain 66 alkaloids in 11 main classes: Vincosan, Corynanthean, Vallesiachotaman, Strychnan, Aspidospermatan, Plumeran, Eburan, Ibogan, Tacaman, Bis-indole and Miscellaneous. Several non-alkaloidal components have also been reported from the plant. Some of them include Anthranilate synthase, Prenyl transferase, Squalene synthetase, Tryptophan decarboxylase, Strictosidine synthase, Ascorbate peroxidase, Phenolic peroxidase, α–amyrin acetate, a-amyrin lupeol, β-sitosterol. The most common medicinal use of crude *Tabernaemontana divaricata* extract involves its antimicrobial action against infectious diseases such as syphilis, leprosy, and gonorrhoea, as well as its antiparasitic action against worms, dysentery, diarrhoea, and malaria [4-6]. The antioxidant activity in various models for *Tabernaemontana divaricata* has been reported in literature, but no hepatoprotective and action against oxidative stress has been reported till date. The objective of the present investigation is to evaluate the nootropic capability of the ethanolic extracts of *Tabernaemontana divaricata* leaf in diazepam induced dementia model.

Material
Chemicals and reagents used for the preparation of buffers, analytical solutions and other experimental purposes were of analytical grade and purchased from various sources.

Animals
Albino mice of either sex were chosen for the study. The animals were used after an acclimatization period of 7 days to the laboratory environment with access to food and water *Ad libitum*.

Collection and identification of plant material
The leaves of *Tabernaemontana divaricata* were collected from the local surrounding of Bhopal, Madhya Pradesh in the month of January. The authenticated plant leaves were washed with distilled water and were dried under shade and powdered using a blender at low speed.

Preparation of Extract
Powdered leaves (115 g) were evenly packed in the extractor of the soxhlet apparatus and defatted using petroleum ether using hot continuous extraction process for about 6-12 h. The defatted marc was dried, weighed and again placed in the extractor for ex. The extract was filtered while hot through Whatman filter paper to remove any impurity and concentration by evaporating the solvent using rotary vacuum evaporator. The oleo-resinous extract was collected and placed in desiccator to get rid of the excessive moisture**.** The dried/semidried extracts were stored in desiccator for further experimental procedures [7].

Qualitative Phytochemical Screening
The two extracts were evaluated by phytochemical qualitative reactions for identifying the presence or absence of usual plant secondary metabolites. The screening was performed for triterpenes/steroids, alkaloids, glycosides, flavonoids, saponins, tannins, and phenolic acids. The color intensity or the precipitate formation was used as analytical responses to these tests [8].

Total Phenolic Content
The extraction of phenolic compounds was based on a modification of the method reported 9 [9]. The extract (0.1 g each) were dissolved in DMSO and the solutions were stored at 4 °C in amber colored bottles and served as the stock solutions for subsequent analyses. For determining the total phenolic content 200 µL of extract sample was mixed with 1.4 mL purified water and 100 µL of Folin-Ciocalteu reagent was added to it. After 2 min, 300 µL of 20% Na_2CO_3 aqueous solution was added and the mixture was allowed to stand for 2 h. The absorbance was measured at 765 nm with a UV-Vis spectrophotometer. Standard solutions of gallic acid (10-100 ppm) were treated similarly for constructing the calibration curve. The control solution contained 200 µL of methanol and suitable reagents, and it was prepared and incubated under the same conditions as the rest of the samples. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of the dry sample.

Acute toxicity study
A total of three animals were used which received a single oral dose (2000 mg/kg) of ethanol extract of *Tabernaemontana divaricata*. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h and daily thereafter for a period of 14 days [61, 62]. Once daily observations were made for changes in skin and fur, eyes and mucous membrane (Nasal) and also respiratory rate, circulatory (Heart rate and blood pressure), autonomic (Salivation, perspiration, urinary incontinence, and defecation) and central nervous system (drowsiness, tremors and convulsion) changes. Mortality, if any, was also observed over the period of 2 weeks.

Pharmacological Activity
Treatment protocol
The anti-amnesic effect of the methanolic extract of *Tabernaemontana divaricata* leaf ethanol extract (TLEE) was assessed in an *in vivo* model of amnesia produced by diazepam. This evaluation was conducted utilizing an Actophotometer and Cook's pole climbing device.
The acclimatized animals were divided into 5 groups of each 6 animals as per the approved protocol. Each group served individual purpose.

- **Group 1:** Served as normal control and received normal diet and saline.
- **Group 2:** Disease control received 1 mg/kg of diazepam, i.p on 8th day.
- **Group 3:** Standard control received 1 mg/kg of Donepezil per oral for 1-8 days + 1 mg/kg of diazepam, i.p on 8th day
- **Group 4:** Received 200 mg/kg of TLEE per oral for 1-8 days + 1 mg/kg of diazepam, i.p on 8th day.
- **Group 5:** Received 400 mg/kg of TLEE per oral for 1-8 days + 1 mg/kg of diazepam, i.p on 8th day.

On 8th and 9th day the behavioral parameters like basal activity score by actophotometer and passive avoidance time by cook's pole climbing apparatus were evaluated [10].

Antioxidant Evaluation
The anti-oxidant activity [11] B Different concentrations of the test solution (50-250 µg/mL) in ethanol (1.0 mL) were diluted with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and mixed with 2.5 mL 1% potassium ferricyanide. After incubation at 50 °C for 20 minutes, 2.5 mL of 10% trichloroacetic acid (TCA) were added to the mixture. 2.5 mL of the reaction mixture was diluted with an equal amount of distilled water and absorbance was measured at 700 nm after treatment with 0.5 mL of 0.1% ferric chloride ($FeCl_3$). Increased absorbance of the reaction mixture indicates an increase in reduction capability.

Results and Discussion
Extraction and phytochemical screening
The extracts was dark brown to green and obtained as sticky or dry solids with a yield of 1.2 and 19.6% for petroleum ether and ethanol extracts. Sterols and terpenes were found to be present in the petroleum ether extract whereas the ethanol extract revealed the presence of alkaloids, phenolics, tannins, flavonoids and terepene.

Total Phenolic content
The plant flavonoids and polyphenols are greatly effective free radical scavenging and antioxidant activity [12]. The diseases associated with free radicals are healed by polyphenol and flavonoids. The phenolic compounds have been recognized as antioxidant and have been known to show medicinal activity as well as for exhibiting physiological functions [13-17]. The total phenolic content of the extract of *Tabernaemontana divaricata* was found to be 1.08±0.033

GAE mg/g and 44.86±2.689 GAE mg/g in the petroleum ether and ethanol extract respectively.

Acute toxicity

The acute toxicity test was performed by using the dried ethanol extract at concentration of 2000 mg/kg to the test animal, administered orally. As none of the tested animals died, the LD_{50} was considered to be more than 2000 mg/Kg and any dose less than 2000 mg/Kg would be considered for testing of pharmacological activity.

Effect on locomotor activity

The amnesia inducing drug Diazepam significantly diminshed the basal activity of the mice to by 39.33% on the 8th day and 42.69% on the 9th day. The reduction in basal activity represent the amnesia in the mice. Mice treated with TLEE were able to improvise the number of crossings (Basal score) to 196.2±1.0954 at 200 mg/kg dose and 249.6±1.1401 at 400 mg/kg dose on the 8th day whereas on the 9th day the basal score further improved to 203.8±1.3038 at 200 mg/kg dose and 258.8±0.8366 at 400 mg/kg dose on the 9th day. The percent improvement in locomotion compared to the disease control was found to be 24.49 and 29.31% respectively at the low and high dose of TLEE on the 8th day and 29.31 and 70.93% respectively at the low and high dose of TLEE on the 9th day suggesting a significant improvement in memory. The standard drug was able to produce 43.65 and 71.86% improvement in locomotion (Figure 1).

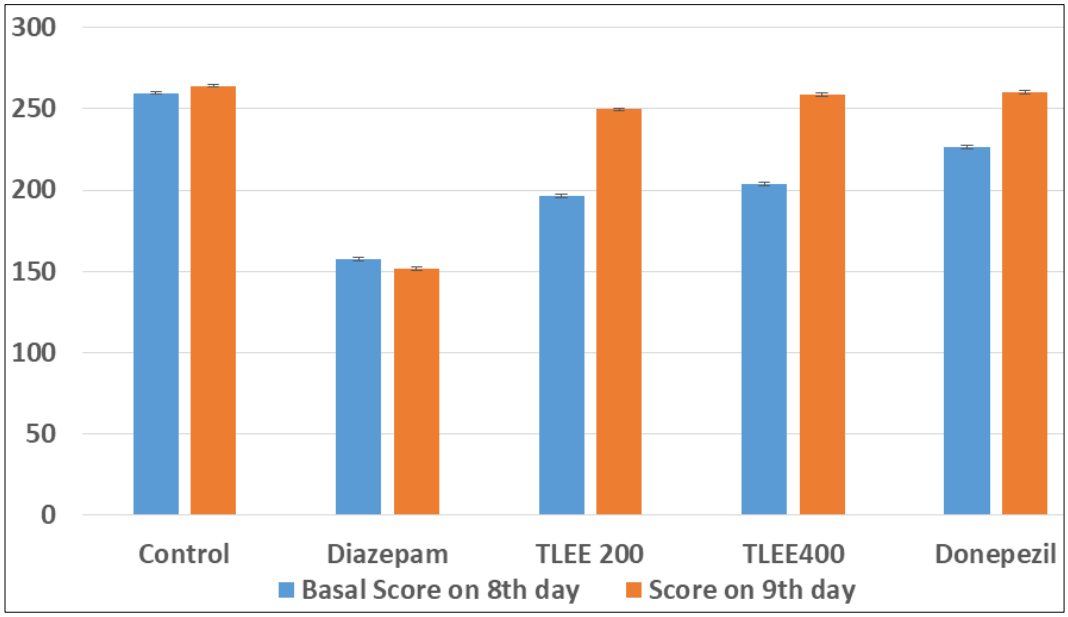

Fig 1: Effect of TLEE on locomotion of mice in actophotometer

The improvement in locomotion by TLEE exhibits the potential of the extract to cause a significant stimulation in the activity of the central nervous system. Actophotometer is used to study the effect of drug on CNS observed by the number of movements that the test animal makes during a period of 5 min of the study. The activity is a measure of the level of excitability of the CNS; increased activity results from excitation of the central nervous system and also from ability to learn.

The percent reduction in locomotion was calculated from the basal score and is comparatively represented in Figure 2.

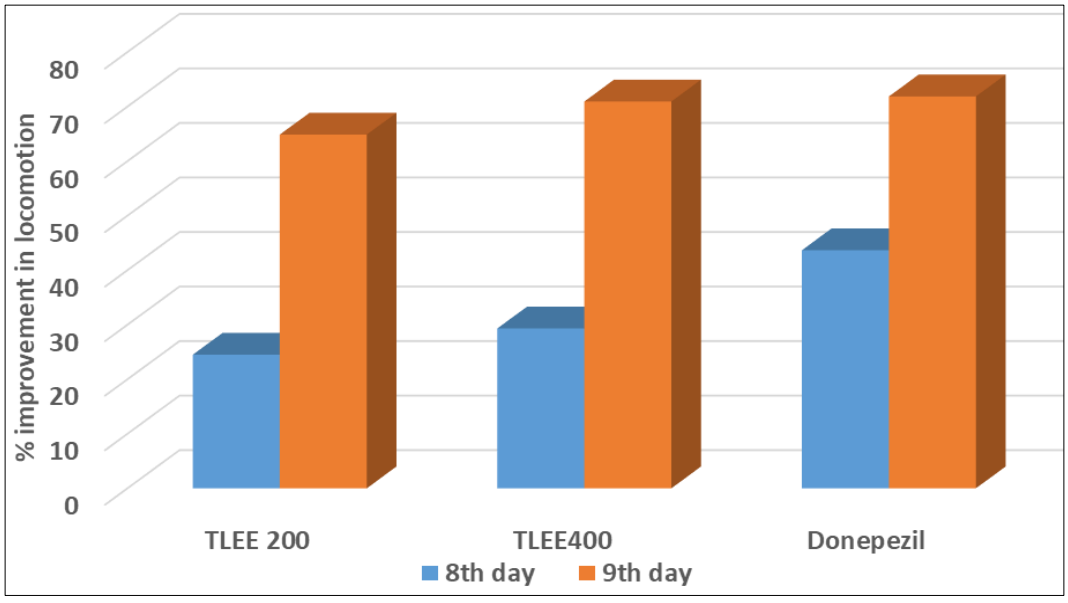

Fig 2: Percent improvement in locomotion by TLEE and standard drug

Effect of passive avoidance

The amnesia inducing drug Diazepam resulted increased the passive avoidance time to climbing. The passive avoidance time was found to be 1.52 times on the 8th day and 2.08 times on the 9th day in comparison to the normal control. The increase in passive avoidance time represents the amnesia in the mice.

Mice treated with TLEE were able to reduce the passive avoidance time by 13.29% at 200 mg/kg dose and 23.42% at 400 mg/kg dose on the 8th day whereas on the 9th day the time was further reduced by 27.75% at 200 mg/kg dose and 41.89% at 400 mg/kg dose on the 9th day as compared to the disease control suggesting a significant improvement in memory. The standard drug was able to produce 29.11% and 47.64% reduction in the passive avoidance time (Figure 3).

The reduced passive avoidance time represents the ability of TLEE to counter the amnesic effect of diazepam thereby letting the mice retain the memory of shock. The percent reduction in passive avoidance time was calculated and is comparatively represented in Figure 4.

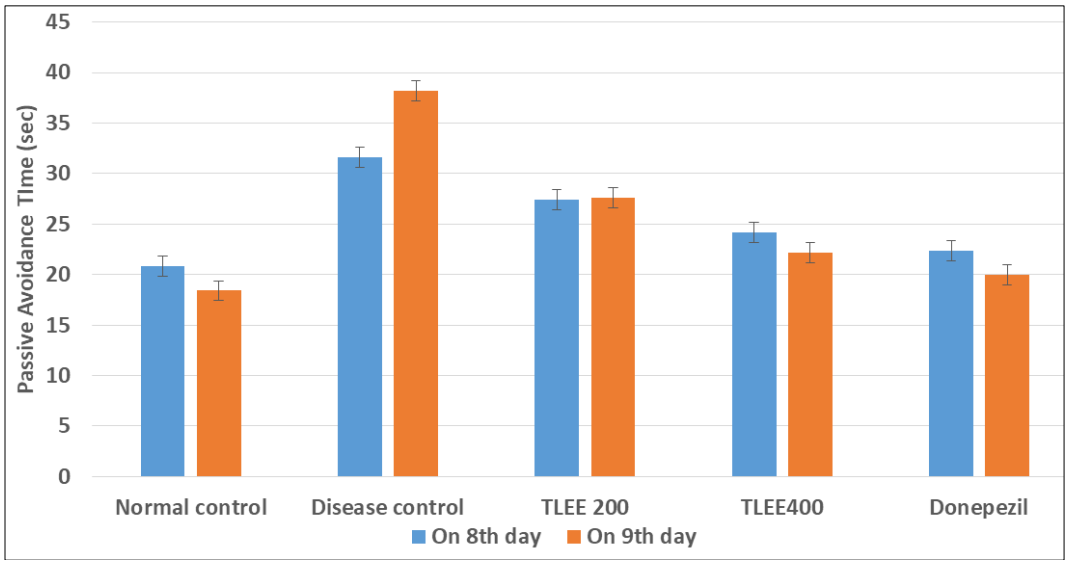

Fig 3: Effect of TLEE on passive avoidance time to climb

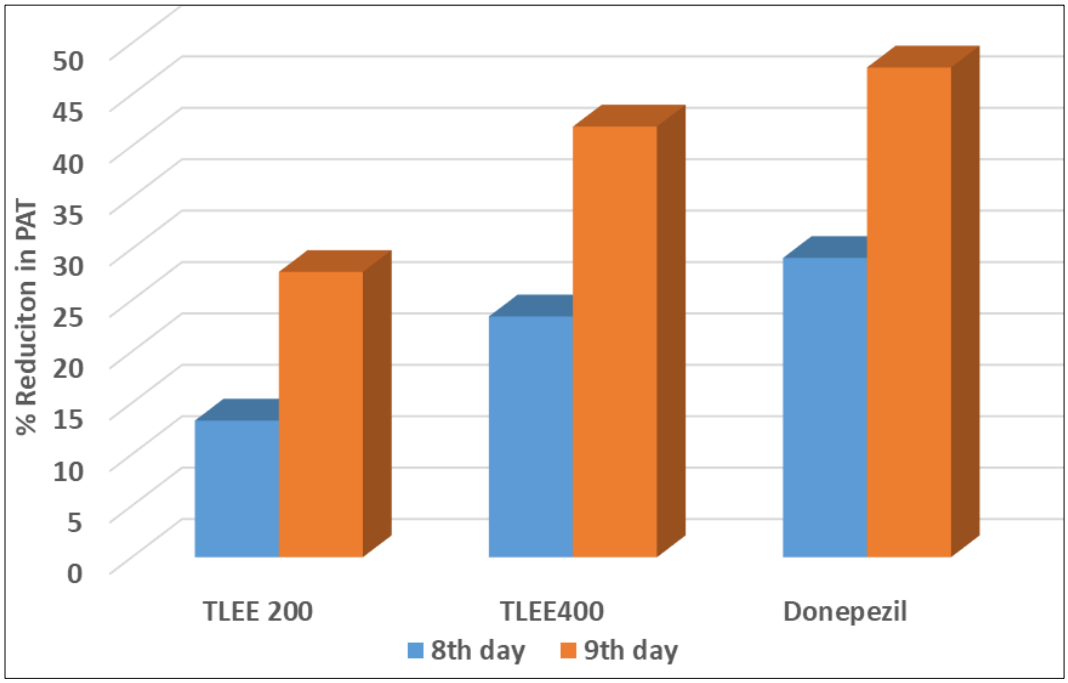

Fig 4: Percent reduction in PAT by TLEE and standard drug

Anti-oxidant action

The capability of the extracts to reduce potassium ferrocyanide (Standard) at different time periods has been depicted (Figure 5). Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. The pale yellow color obtained from the standard was deepened (darkened) on interaction with the contents of the capsule.

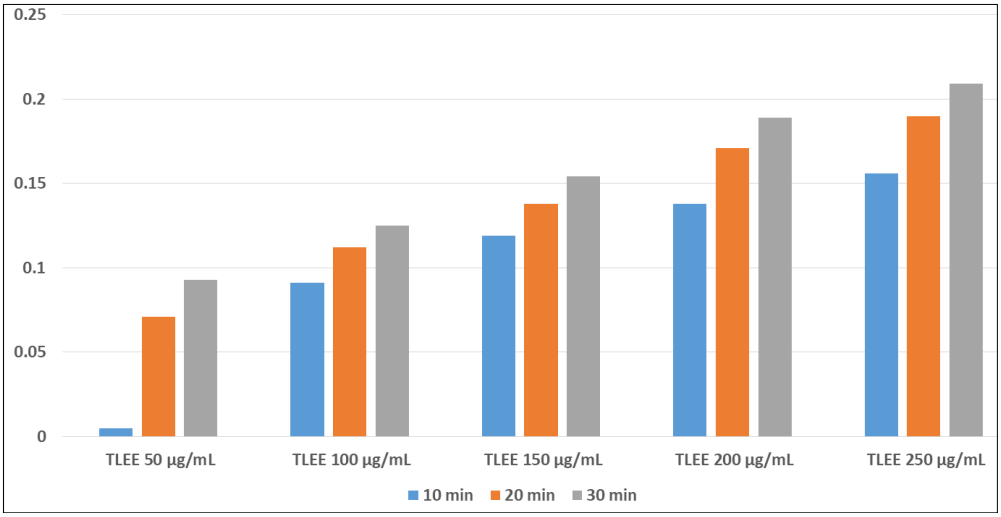

Fig 5: Reducing Power of TLEE and standard

The ethanol extract from leaves of *Tabernaemontana divaricata* was able to produce a dose and time dependent reduction of potassium ferrocyanide to potassium ferricyanide. The maximum reduction occurred at 30 min post interaction.

Conclusion

The ethanolic leaf extract of *Tabernaemontana divaricata* exhibited good antioxidant activity primarily due to the presence of phenolics, flavonoids and tannins. From findings of the study it has been concluded that the ethanol extract of leaf extract *Tabernaemontana divaricata* has good cognition enhancement capabilities and could be of high value in formulating herbal products that could be used for treatment of amnesia produced in neurodegenerative conditions like Alzheimer's disease.

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