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# Cognitive Enhancing, Anti-Acetylcholinesterase and Antioxidant Properties of *Tagetes erecta* against Diazepam Induced Amnesia in Rodents

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## ABSTRACT

Oxidative stress can be involved in cognitive dysfunction associated with neurodegenerative disorders. Diazepam (DZP) administration has been chosen to simulate the memory impairment. The aim of this study was to evaluate Anti-amnesic activity of methanolic extract of *Tagetes erecta* flower heads using in-*vitro* and *in-vivo* models. The extract was also evaluated for its anti-oxidant potential. Anti-amnesic activity of the extract was screened by using diazepam induced (acute) amnesic model using actophotometer and cook's pole climbing apparatus. *In-vitro* anticholinesterase (AChE) using Ellman's assay was estimated. Anti-oxidant potential of the extract was evaluated by using reducing power and lipid peroxidation assays. The acute toxicity studies revealed that the extract was safe up to 2000 mg/kg bd. wt. The METE at two doses levels 200 and 400 mg/kg bd. wt reversed the memory deficit induced by diazepam in mice models. The extract significantly scavenged the free radicals in dose dependant manner. The presence of active constituents like flavonoids, terpenoids, steroids, alkaloids and phenols in methanolic extract of flower heads of *Tagetes erecta* might be responsible for its anti-amnesic, anti-cholinesterase and anti-oxidant activity.

**Keywords:** *Tagetes erecta*, anti amnesic, AChE inhibition, diazepam, antioxidant.

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## INTRODUCTION

Dementia is an umbrella term that covers different types of clinical syndromes such as Alzheimer's disease (AD), vascular dementia, dementia with Lewy bodies, frontotemporal dementia (FTD), Parkinson's disease etc. [1] Memory, one of the most complex brain functions, involving multiple neuronal pathways and neurotransmitters is considered the ability of an individual to record, retain, and recall the information when needed. [2-3] 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). [4-6]

AD is a neurodegenerative disorder characterized by cognitive and memory deterioration, progressive impairment of activities of daily living and a multiplicity of behavioural and psychological disturbances. [7]

The primary causes of AD appear to be (i) decreased cholinergic activity; (ii) deposition of amyloid-beta peptides in the brain (iii) oxidative stress. Acetylcholinesterase (AChE) plays a key role in the regulation of the cholinergic system and hence, inhibition of AChE has emerged as one of the most promising strategies for the treatment of AD. One of the major therapeutic strategies is to inhibit the AChE and hence to increase the acetylcholine level in the brain. [8] The imbalance between the generation of free radicals and antioxidants has also been claimed to be a cause of AD. [9]

Tagetes is a genus of annual or perennial, mostly herbaceous plants in the sunflower family (Asteraceae). Flowers of *Tagetes erecta* Linn used traditionally from ancient times and are used in folk medicine to cure various types of diseases. The flower is used to cure fever, epileptic fits and according to Ayurveda, it is said to purify blood and flower juice is given as a remedy in cold, rheumatism and bronchitis. [10-11] Its different species have been found to show different activities like antibacterial, anti-depressant, anti-inflammatory, antimycotic, larvacidal, insecticidal, mosquitocidal, and nematicidal activity. Its flower is choice of medication in many cases. [12] The plant has been found to contain various secondary metabolites which show numerous pharmacological activities. It includes flavonoids, carotenoids, polyphenol, lutein, xanthophylls, essential oils, etc. [13] Being rich source of many bioactive components and wide availability, *T. erecta* is now one of the prime targets of researchers working on chemistry of natural products. The major pigments present in *T. erecta* are basically flavonoids and carotenoids. [14] Many Indian herbs are being used in traditional practices to cure various human ailments. Tagetes species belonging to family Asteraceae, are most common in plant kingdom, which is used in different areas like cosmetic preparation, medicines as well as it is most widely used as ornamentals. Thus, the present work focused on evaluating the Anti-amnesic effect of *Tagetes erecta* against diazepam induced cognitive impairment and oxidative damage.

## MATERIALS AND METHODS

### Preparation of extract

The powdered crude material (200 g) was extracted with methanol by Soxhlation At the end of the extraction process; the extract obtained was filtered and evaporated to solid mass. The extract was preserved in desiccators to remove remaining moisture, if present, and finally stored in air tight containers for further use.

### Identification of phytochemical constituents

Phytochemical screening of methanolic extract of *Tagetes erecta* (METE) was carried out by using standard tests.

### Acute toxicity testing

Acute toxicity study was carried out in order to check the toxic effects of methanolic extract of *Tagetes erecta* flowers. Acute toxicity studies were carried out as per the OECD 425 guidelines.

### Experimental animals

Adult Swiss albino mice (20-25 g) were used for the present study. They were kept in polypropylene cages at 25 ± 2°C, with relative humidity 45-55% under 12 h light and dark cycles. All the animals were acclimatized to the laboratory conditions for a week before use. They were fed with standard animal feed and water *ad libitum.* All the pharmacological experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) with (Reg. No. 1175/PO/ERe/S/08/CPCSEA).

### Materials

Aluminium chloride (Himedia laboratories, Mumbai), Donepezil (Dr. Reddy's laboratories, Hyderabad), Thiobarbituric acid (S. D. Fine Chemicals Ltd., Mumbai), were used. All other chemicals and reagents unless specified were of analytical grade.

### *In-vivo* methods for evaluation of Anti- amnesic activity

The *in vivo* evaluation of anti-amnesic activity of the methanolic extract of *Tagetes erecta* flower heads was evaluated in diazepam induced (acute) amnesic model using Actophotometer and Cook's pole climbing apparatus.

### Diazepam induced (acute) amnesic model

30 Healthy Swiss albino mice of either sex weighing 20-25 g were selected and these animals were divided into 5 groups (6 in each group) as follows.

**Table 1: Study design for Diazepam induced acute amnesic model**

| S. No | Group | Treatment |
|---|---|---|
| 1 | Group – I | Normal saline |
| 2 | Group – II | Diseased control - Diazepam (1 mg/kg bd. wt, *i.p.*) on 8th day |
| 3 | Group – III | METE (200 mg/kg bd. wt, *p.o.*) for 1- 8 days + Diazepam (1 mg/kg bd. wt, *i.p.*) on 8th day |
| 4 | Group – IV | METE (400 mg/kg bd. wt, *p.o.*) for 1- 8 days + Diazepam (1 mg/kg bd. wt, *i.p.*) on 8th day |
| 5 | Group –V | Donepezil (1 mg/kg bd. wt, *p.o.*) for 1- 8 days + Diazepam (1 mg/kg bd. wt, *i.p.*) on 8th day |

At the predetermined time intervals, i.e. 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.

### Locomotor activity by Actophotometer

In order to detect the association of decreased activity in Actophotometer with changes in motor activity, the locomotor activity was recorded for a period of 5 min using actophotometer. The actophotometer consist of a square arena (30 × 30 × 25 cm) with wire mesh bottom, in which the animal moves. Six lights and six photocells

were placed in the outer periphery of the button in such a way that mice can block only one beam. The movement of animal interrupts a beam of light falling on a photocell during which a count was recorded and displayed. [15]

### Cook's pole climbing method

The basic operational mode of this method is that following an auditory warning stimulus, the animal learns to avoid the foot shock delivered through the cage floor by jumping to pole. This method has long been accepted as a reliable technique to evaluate learning and memory in experimental animals. The training and testing of mice was conducted in 25 × 25 × 40 cm chamber that is enclosed in a dimly light, sound attenuating box. The mice had learned to jump on a pole to avoid foot shock. A tone 50 Hz was used as a conditional stimulus and foot shock of 1 mA - 2 mA was the unconditioned stimuli. In the training procedure, the animal was initially allowed to adopt in the chamber for 1 min. This was followed, in succession by conditioned and unconditioned stimuli for a period of 10 sec each. The trial ended either after the animal responded by jumping on the pole after 10 seconds or after 30 seconds each whichever was earlier. The animal was given such trial every day for 10 days, mice were initially trained to escape the foot shock by climbing on the pole, i.e. the shock free zone and only those mice, which could climb the pole and escape the foot shock, were included in the study. Retention of the memory of the painful stimuli established in learning procedure was tested before and after drug treatment. It was quantified as the percentage of animals avoiding shock by jumping on the pole. [15]

### *In-vitro* AChE inhibition assay

AChE activity was measured by using spectrophotometer based on Ellman's method. [16] The enzyme hydrolyses the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2- nitrobenzoate which can be detected at 412 nm. In test tube 10 µL of 50 mM Tris–HCl buffer pH 8.0 and 250 µL of plant extract at the concentrations of 10 – 50 µg/ mL, 10 µL 6.67 UmL-1 AChE and 20 µL of 10 mM of DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]) in buffer were added. Positive control namely Donepezil was prepared in serial concentration as same as test extract by dissolving in 50 mM Tris–HCl buffer pH 8.0. The mixture was incubated for 15 min at 37°C. Then, 10 µL of acetylthiocholine iodide (200 mM) in buffer were added to the mixture and the absorbance was measured at 412 nm every 10 sec for 3 mins, for a blank with buffer instead of enzyme solution was used.

The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V= Abs/Δt) the calculation as follows.

$$\text{Inhibition (\%)} = \frac{\text{100 - Change of sample absorbance}}{\text{Change of blank absorbance}} \times 100$$

The experiment was done in triplicate and concentrations of the test extract that inhibit the hydrolysis of the substrate (acetylcholine) by 50% ($IC_{50}$) were determined by linear regression analysis between the inhibition percentage versus the extract concentration by using the excel program.

### Statistical analysis

Graph Pad prism 7 software and MS excel was used for statistical analysis of data. All the results were expressed as mean ± standard error of mean (SEM), analyzed for ANOVA and Dunnett's test (Multiple). Differences between test groups and standard group were considered significant at $p<0.05$ and $p<0.01$ levels.

**Table 2: Results of phytochemical analysis**

| Phytochemical constituents | Results |
|---|---|
| Terpenoids | ++ |
| Flavonoids | ++ |
| Phenols | ++ |
| Steroids | ++ |
| Alkaloids | + |
| Tannins | -- |
| Cardiac glycosides | - |
| Saponins | - |

**Table 3: Effect of METE on basal activity score using Actophotometer**

| Groups | Treatment | Basal activity scores | |
|---|---|---|---|
| | | 8th day | 9th day |
| I | Normal control | 282 ± 0.76 | 287.66 ± 0.33 |
| II | Negative control (Diazepam) | 189.66 ± 0.55** A | 180 ± 0.25** A |
| III | METE (200 mg/kg) | 199.66 ± 1.35** A, a | 276.6 ± 1.14** A, a |
| IV | METE (400 mg/kg) | 226 ± 0.57** A, a | 279 ± 0.44** B, a, |
| V | Donepezil (1 mg/kg) | 249.5 ± 0.34** a | 281.8 ± 0.65** a |

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnet's t-test by comparing with control, negative control & standard. Significant values are expressed as control group (**$p<0.01$), negative control (a=$p<0.01$) & standard (A=$p<0.01$, B= $p<0.05$).

## RESULTS

### Preliminary phytochemical analysis

The preliminary phytochemical investigation of methanolic extract of *Tagetes erecta* flower heads showed the presence of flavonoids, terpenoids, phenols, steroids and alkaloids.

### Acute Toxicity Studies

The methanolic extract of *Tagetes erecta* did not exhibit any signs of toxicity and mortality even up to 2000 mg/kg, bd. wt. All animals were safe even after 14 days of observation. The two doses selected for the present study are 200 and 400 mg/kg b. wt

### Diazepam induced amnesia

### Effect of METE on basal activity using Actophotometer

The results were shown in Table 3.

### Effect of METE on passive avoidance time using cook's pole climbing apparatus

The results were shown in Table 4.

### *In-vitro* AChE Inhibition

Since AD pathogenesis is potentially attributed to acetylcholine decline and Aβ formation, the anti-amnesic effects of METE were evaluated by inhibition of AChE. [17] METE exhibited AChE-inhibitory activity with $IC_{50}$ values of 34.5µg/ml and donepezil exhibited the AChE inhibition with an $IC_{50}$ value 31.5µg/ml, respectively. These results indicate that METE may reverse diazepam induced cognitive impairment through inhibition of cholinesterase enzyme as implicated in Alzheimer's disease.

**Table 4: Effect of METE on passive avoidance time using cook's pole climbing apparatus**

| Groups | Treatment | Time taken to climb the pole (sec) | |
|---|---|---|---|
| | | 8th day | 9th day |
| I | Normal control | 20± 0.36 | 18.83±0.30 |
| II | Negative control (Diazepam) | 31.66±0.42** A | 38.33±0.42** A |
| III | METE (200 mg/kg) | 26.33±0.66** A, a | 25.16±0.30** A, a |
| IV | METE (400 mg/kg) | 25.83±0.74** A, a | 23.5±0.42** A, a |
| V | Donepezil (1 mg/kg) | 22.83±0.79* a | 20.66±0.33* A |

Values were expressed as mean ± SEM (n=6). Statistical analysis was performed by using ANOVA followed by Dunnetts t-test by comparing with control, negative control & standard. Significant values are expressed as control group (**$p$<0.01, *$p$<0.05), negative control (a=$p$<0.01) & standard (A=$p$<0.01)

**Table 5: *In-vitro* AChE inhibition of methanolic extract of *Tagetes erecta* flower heads**

| S. No | Compounds | Concentrations (µg/mL) | % Inhibition | $IC_{50}$ Values |
|---|---|---|---|---|
| 1. | METE | 10 | 9.78 ± 1.61 | 34.5 |
| | | 20 | 21.84 ± 2.26 | |
| | | 30 | 46.04 ± 1.69 | |
| | | 40 | 57.49 ± 1.17 | |
| | | 50 | 77.7 ± 1.42 | |
| 2. | Donepezil | 10 | 13.05 ± 0.86 | 31.5 |
| | | 20 | 23.84 ± 0.86 | |
| | | 30 | 43.78 ± 0.84 | |
| | | 40 | 64.17 ± 0.56 | |
| | | 50 | 84.31 ± 1.13 | |

Values were expressed as mean ± SEM (n=3).

## *In-vitro* Antioxidant Assays

The methanolic extract of *Tagetes erecta* flower heads was subjected to *in-vitro* antioxidant activity. *In vitro* anti-oxidant activity was performed using reducing power assay & Lipid peroxidation assays.

### Reducing power assay

The *in-vitro* antioxidant activity was performed using reducing power assay. The increase in absorbance with increase in concentration indicates the reducing capacity. The results were expressed in Table 6.

In reducing power assay the METE & Ascorbic acid were tested at different concentrations 10, 20, 30, 40 and 50µg/ml. The lowest concentration of METE & ascorbic acid showed an absorbance of 0.57 & 0.61 respectively, whereas the highest concentration of METE & ascorbic acid showed a maximum absorbance of 0.92 & 0.95 respectively. This clearly shows with increase in concentration the absorbance also increases. Early reports also suggest that increase in absorbance indicates increase in reducing capacity. From this the METE possesses significant anti-oxidant activity.

### Lipid peroxidation assay

The *in-vitro* antioxidant activity was performed using Lipid peroxidation assay. The results were expressed in Table 7.

**Table 6: Reducing power assay of methanolic flower extract of *Tagetes erecta.***

| S. No | Compounds | Concentration | Absorbance |
|---|---|---|---|
| 1. | METE | 10 | 0.573 ± 0.003 |
| | | 20 | 0.610 ± 0.006 |
| | | 30 | 0.770 ± 0.006 |
| | | 40 | 0.833 ± 0.007 |
| | | 50 | 0.920 ± 0.006 |
| 2. | Ascorbic acid | 10 | 0.613 ± 0.007 |
| | | 20 | 0.770 ± 0.006 |
| | | 30 | 0.850 ± 0.006 |
| | | 40 | 0.883 ± 0.009 |
| | | 50 | 0.957 ± 0.003 |

Values were expressed as mean ± SEM (n=3).

**Table 7: Lipid peroxidation assay of methanolic flower extract of *Tagetes erecta***

| S. No | Compounds | Concentration | Absorbance | $IC_{50}$ Values |
|---|---|---|---|---|
| 1. | METE | 50 | 15.85 ± 0.54 | 172.5 |
| | | 100 | 19.28 ± 0.80 | |
| | | 150 | 35.10 ± 0.82 | |
| | | 200 | 53.50 ± 0.83 | |
| | | 250 | 69.10 ± 1.09 | |
| 2. | Ascorbic acid | 50 | 12.24 ± 1.17 | 156.5 |
| | | 100 | 23.80 ± 1.80 | |
| | | 150 | 54.42 ± 1.79 | |
| | | 200 | 69.38 ± 2.35 | |
| | | 250 | 78.20 ± 1.79 | |

In lipid peroxidation assay, the METE was tested at different concentrations like 50, 100, 150, 200, and 250µg/mL. The lowest concentration of 50 µg/mL showed a percentage inhibition of 15.85 whereas the highest concentration of 250µg/mL showed a percentage inhibition of 69.10 The $IC_{50}$ value for the METE was found to be 172.5µg/mL which is compared with standard ascorbic acid having $IC_{50}$ value of 156.5µg/mL respectively.

## DISCUSSION

In the present study methanolic extract of *Tagetes erecta* flower heads was evaluated for anti-amnesic activity by using diazepam induced amnesic animal model and various behavioural parameters like basal activity score and passive avoidance time were evaluated. One of the characteristic changes that occur in AD is increase in acetyl cholinesterase (AChE) activity, the enzyme responsible for acetylcholine hydrolysis, from both cholinergic and non-cholinergic neurons of the brain. [18] However AChE activity has been shown to be increased within and around amyloid plaques to promote the assembly of amyloid beta-peptides into fibrils and to increase the cytotoxicity of these peptides. During the study, the cognitive functions were evaluated by measuring basal activity score and

passive avoidance time of all the animals. At the end of the study basal activity score is increased whereas the passive avoidance time was found to be decreased in groups treated with the extract and the standard drug which indicates acquisition of memory which can be due to decreased GABA levels which is the possible mechanism to induce anterograde amnesia by diazepam.

The various phytochemicals identified in the methanolic extract of *Tagetes erecta* flower heads are phenolics (syringic acid and gallic acid), terpenoids (β-amyrin and erythrodiol), flavonoids (quercetin, kaemferol), steroids (β-sitisterol, stigma sterol), caretonoids (lutein and zexanthin) and tocopherol (α and β tocopherol). It can be stated that the above said active constituents like phenolics, flavonoids, terpenoids and steroids have shown improvement in cognitive function by inhibiting AChE as reported. [19-20] The inhibition of the lipid peroxides like nitric oxide and superoxide anions might be another possible mechanism responsible for its anti- amnesic activity as reported. [17]

The reducing ability of a compound generally depends on the presence of reductants which have been exhibiting antioxidative potential by breaking the free radical chain and donating a hydrogen atom. The reducing capacity of a compound can be known by measuring $Fe^{3+}$- $Fe^{2+}$ complex. $Fe^{3+}$ reduction is due to electron donating activity of METE, which is an important mechanism of antioxidant action. Increase in absorbance indicates an increase in reductive ability of METE. The reducing power activity of METE might be due to presence of phenols and flavonoids in the extract with adequate number of hydroxyl groups. [21]

Currently, lipid peroxidation is considered as the main molecular mechanisms involved in oxidative damage to cell structures and in the toxicity process that lead to cell death. It involves formation and propagation of lipid radicals, the uptake of oxygen, rearrangement of double bonds in unsaturated lipids and eventual destruction of membrane lipids, with the production of a variety of break down products including alcohols, ketones, alkanes, aldehydes and ethers. [22] In pathological situations the reactive oxygen and nitrogen species are generated at higher than the normal rates and as a consequence lipid peroxidation occurs with alpha-tocopherol deficiency. Oxidative stress is understood as an imbalance situation with increased oxidants or decreased anti-oxidants. [23] The reducing property of METE indicates they can be used as electron donors which reduced the oxidised intermediates of lipid peroxidation processes, therefore it can be used as an anti-oxidant. The phenolic compounds identified in the METE might have suppressed lipid peroxidation through different mechanisms like free radical quenching, electron transfer, radical addition or radical recombination. [24]

Oxidative stress has been shown to affect amyloid-beta generation in the AD pathogenesis. Upregulation of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE 1) gene transcription by oxidative stress may contribute to the pathogenesis of AD. [25] METE was found to possess antioxidant activity through scavenging of free radicals. Thus, METE produced significant memory enhancing effect in mice probably due to its antioxidant property by virtue of which susceptible brain cells get exposed to less oxidative stress resulting in reduced brain damage and improvement of neuronal function. In conclusion, METE showed memory enhancing activity in mice probably by inhibiting brain acetylcholinesterase activity, through involvement of GABA-benzodiazepine pathway and due to its antioxidant activity. From the above results METE possess antiamnesic, antiacetylcholinesterase and antioxidant properties.

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