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ETHANOLIC EXTRACT OF ACORUS CALAMUS LINN AND EUGENOL COMBINATION IMPROVED THE COGNITIVE FUNCTIONS OF STREPTOZOTOCIN ADMINISTRATED RATS THROUGH ATTENUATION OF LIPID PEROXIDATION AND ACETYLCHOLINE ESTERASE LEVEL

Irene James, Chennu Manisha, Tenzin Choephel, Gaddam Narasimha Rao, Chintha Narendar, Jeyabalan Jeyaram Bharathi, Elango Kannan, Asha Jose, Santilna Karolina Sahadevan, Antony Justin*

Department of Pharmacology, JSS College of Pharmacy, Ooty 643001,Tamilnadu,India
(A Constituent College of JSS Academy of Higher Education and Research, India)
Corresponding author E-mail:* **justin@jssuni.edu.in**

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ABSTRACT

Objective: The scope of the present study is to assess the cognitive enhancement activity of Ethanolic extract of *Acorus calamus Linn* (EtAC) and Eugenol (EU) combination in intracerebroventricular (ICV) administration of Streptozotocin (STZ) in rat model of Alzheimer's disease (AD).

Methods: Totally five groups were taken in this study and each group entailed 6 rats. The first group was served as sham-operated (SO) group that received 10µl of artificial cerebrospinal fluid (aCSF) through ICV route. The second group was administered only with STZ (3mg/kg/10µl aCSF). The third group was treated with 200mg/kg of EtAC. The fourth group was treated with Eugenol 100mg/kg and the fifth group was administered with a combination of both EtAC (200mg/kg) and EG (100mg/kg) in STZ injected rats. The treatment was initiated in third, fourth and fifth groups respectively after the 14^{th} day of STZ administration and was continued till the 28^{th} day. The cognitive function of animals assessed on days 1, 7, 14, 21 and 28 by radial arm test. After the behavioral study, the brains were isolated for neurobiochemicals analysis in hippocampus region to estimate the lipid peroxide (LPO) and Acetylcholine esterase (AChE) levels with respect to different treatments.

Results: The administration of STZ remarkably decreased the cognitive functions in the rats which were characterized through increased memory errors in radial arm maze test. Interestingly, the treatment with EtAC, EG, and combination of EtAC with EG significantly reduced the memory errors in STZ administered rats indicate the increased cognitive functions. The treatment with combination therapy has significantly decreased the lipid peroxide (LPO) and Acetylcholine esterase (AChE) levels in hippocampus region of the brain in comparison to *pre se* treatment in ICV-STZ rat model shows the synergistic activity exhibited by EtAC-EG combination.

Conclusion: It can be concluded that the combination of EtAC-EG exerted remarkable improvement in the cognitive functions of STZ administered rats through attenuating lipid peroxide (LPO) and Acetylcholine esterase (AChE) levels in the hippocampus region of the brain.

INTRODUCTION:

Alzheimer's disease (AD) is a progressive neurodegenerative disease due to excessive accumulation of neurofibrillary tangles and amyloid senile plaques in a specific region of the brain leading to neuronal loss. It mainly affects the elderly patient which is characterized by severe dementia **(Serrano-Pozo et al., 2011).** General features of AD are severe memory loss, awkward Behavior, personality instability and declination of cognitive function **(Markesbery et al., 1997).** Through the epidemiological aspects

of the disease, it is the most prevalent form of dementia worldwide and according to WHO report, it is the most expensive chronic disease in 2018. In 2005, the estimated AD patients were around 24.3 million and in 2012, more than 35.6 million people suffered from AD throughout the world approximately. It was estimated that there were 46.8 million people worldwide living with dementia in 2015 and by 2050, dementia cases are expected to triple and the number of AD population rate will be around 115.4 million due to natural process of aging and an increase other risk factors associated with AD **(Prince et al., 2013).** In early mild and moderate stages of AD, people may experience irritability, anxiety or depression. In later severe stages, other symptoms may occur including sleep disturbances, physical or verbal outbursts, emotional distress, yelling, delusions, hallucination and disorientation to time, space and location. In advanced Alzheimer's, people need assistance with daily activities. Those in the final stages of the disease lose their ability to communicate, fail to recognize loved ones and become bed-bound and reliant on care **(Helpern et al., 2004)**. AD is clinically classified into two types i.e., familial AD and sporadic AD. The development of early onset of the familial AD is primarily caused by missense mutations in the chromosome, results in an imbalance in kinase/phosphatase activity at the level of tau protein, hence results in hyperphosphorylation of tau protein leading to the formation of neurofibrillary tangles which too contribute to memory loss **(Iversen et al., 1987).** The late onset of sporadic AD is mainly due to aging of brain which leads to deterioration or gradual declination of cerebral glucose or energy metabolism and its regulation in molecular and genetic level, thus the conduction of neurotransmission starts slowing down and impulse conduction in the brain cease to function. Therefore, in cerebral cortex region, mass number of neurons will lose its conduction tendency and various neurotransmitters especially Acetylcholine which has seen to be a hallmark in the AD patients **(Tanzi et al., 2005; McGinnis et al., 2013).** It comes to the core issue of pathological features and mechanism of AD, there are four predominant hypotheses which brought more depth of insight about the understanding of pathological aspects like β-amyloid hypothesis, cholinergic hypothesis, oxidative stress hypothesis, and the chronic inflammation hypothesis. According to the β-amyloid hypothesis, the victim's brain has shown β-amyloid plagues deposition in the parenchyma cell of the brain and cerebral blood vessel through autopsy. In the cholinergic hypothesis, the cholinergic neuron is drastically reduced; in some cases, the loss of neurons exceeds more than 75% leads to severe acetylcholine depletion. Acetylcholine is one of the most significant neurotransmitter which is responsible for the cognitive functions of the brain. The depletion of Acetylcholine is the most critical change in the AD. Hence the depletion of a cholinergic neuron is one of the most validated pathology hypotheses of AD leading to attenuation of Acetylcholine level resulting in memory loss **(Imbimbo Bet al., 2005)**.

In another way, through the aspect of oxidative theory, due to the induction of lipid peroxidation, the reactive nitrogen and oxygen species leads to the development of free radicals. These free radicals tend to cause cellular and molecular damage. The brain is exceptionally delicate to damage from oxidative stress because of its high rate of oxygen consumption and mass lipid content and relative inadequate amount of anti-oxidant enzymes as compared to other organs. In neurons, oxidation can result in numerous problems including up-regulation of pro-inflammatory cytokines and DNA damage. In chronic inflammation-features of pathology, the microglia are activated to release potentially cytotoxic molecules, such as pro-inflammatory cytokines, reactive oxygen species, proteinases, and complement proteins. This is a normal response to cellular damage, in AD, this appears to proceed uninhibited, causing more harm than protection. Cytokines

stimulate inflammatory processes that may promote apoptosis (programmed cell death) of neurons and oligodendrocytes and induce myelin damage. It is interesting to note that the inflammation in the AD is chronic and localized to discrete areas of the brain **(Heneka et al., 2015)**. In Ayurveda, various herbs are being used to enhance the memory, but many of them lack scientific evidence to prove them for treating AD. In fact, *Acorus calamus Linn* (Sweet flag) an ayurvedic drug is traditionally used for its purgative, diuretic, sedative and carminative properties. It has been reported to have potential antioxidant, antimicrobial, insecticidal, and anti-aging properties. Also, it is used in memory disorders on learning performance and reducing lipid peroxidation content and acetylcholinesterase inhibitory activity. In Ayurvedic medicine (AM), the rhizome of *Acorus calamus* has been utilized for the enhancement of cognitive functions. Hydro-Ethanolic and Ethanolic extracts from *Acorus calamus* rhizomes exhibited neuroprotection and sedative effects **(Venkatramaniah et al., 2016) (Balakumbahan et al., 2010).** Eugenol, the chief chemical constituent of clove bud; is highly used for its anti-oxidant and anti-inflammatory properties. It was reported for the inhibition of amyloid beta-induce excessive flood of calcium ion into the neurons resulted in neuronal death, which is the major pathogenesis of AD **(Irie et al., 2006; Said et al., 2017).** It clearly indicates that *Acorus calamus* and Eugenol could be promising neuroprotective agents in neurodegenerative conditions like AD through multiple targeting efficacies. In this background, the current study is designed to investigate the beneficial effects of Ethanolic extract of *Acorus Calamus Linn* (EtAC) and Eugenol (EG) combination in intracerebroventricular (ICV) administration of Streptozotocin (STZ) in rat model of Alzheimer's disease (AD). The neuroprotective assessments were made through evaluating cognitive function and measuring the glutamate, lipid peroxide (LPO), Nitric Oxide (NO) and interleukin-1β (IL-1β) levels in the hippocampus region of the brain.

METHODS

Chemicals

Ethanol, acetylcholine iodide, and acetylthiocholine iodide were procured from Sisco Research Laboratories, Chennai, India. DTNB (5,5'-dithiobis-(2-nitrobenzoic acid), Streptozotocin, Thiobarbituric acid, and Trichloroacetic acid were purchased from Sigma-Aldrich, St. Louis (USA). Eugenol of pure grade was purchased from Spectrochem Pvt. Ltd Mumbai, India.

Plants Materials and extract preparation

Acorus calamus Linn. (AC) is a species of flowering plant, a tall wetland monocot of the *Acoraceae* family, Species A calamus, in the Genus Acorus. The plants were collected from the Palakkad region of Kerala. The plants were properly authenticated by botanist and brought for the investigation. The plants were properly authenticated by botanist and brought for the investigation. The plants were dried and rhizomes were separated for extract preparation. About 300g of rhizomes of *Acorus calamus* was weighed and placed into a flask. Ethanol solvent (96%) was supplemented to the flask until the plant materials were submerged by the solvent. The extraction process continued for 2 hours and the solvent was boiled. The procedure was repeated for three times and the ethanol extract was combined together and evaporated using a rotary evaporator.

Experimental Animals

Adult male Wistar rats of weight ranging from 180-230 g were obtained from the central animal house facility of JSS College of Pharmacy, Ooty, Tamilnadu, India. The animals were housed in polyacrylic cages [38x23x10cm] with not more than 4 animals per cage under standard laboratory conditions at a favorable temperature of 25±2ºc and 45- 55% of relative humidity. All the animals were maintained on a 12:12hr light: dark cycle and had free access to food and water. Animals were adapted to laboratory conditions prior to the tests were carried out. The food was withdrawn 12-18hr prior to the surgical procedure. The experimental protocols were authorized by

the Institutional Animal Ethics Committee (IAEC) and conducted as per following the guidelines of Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. IAEC approval number for this is JSSCP/IAEC/M.Pharm/Pharmacology/02/2017-2018.

Grouping of Animals

Totally five groups were taken in this study and each group entailed 6 rats. The first group was served as sham-operated (SO) group that received 10µl of artificial cerebrospinal fluid (aCSF) through the ICV route. The second group was administered only with STZ (3mg/kg/10µl aCSF). The third group was treated with 200mg/kg of EtAC. The fourth group was treated with Eugenol 100mg/kg and the fifth group was administered with a combination of both EtAC (200mg/kg) and EG (100mg/kg) in STZ injected rats. The EtAC and Eugenol were suspended in 0.3% Carboxymethyl cellulose (CMC) and administered through the oral route. STZ was dissolved in aCSF and infused through intracerebroventricular (ICV) route.

Intracerebroventricular induction of STZ in rats – AD model

The animals were anaesthetized with ketamine hydrochloride (80 mg/kg, i.p) and xylazine (10 mg/kg, i.p). After anesthesia, the animals were placed on a stereotactic frame. A middle sagittal incision was made on the scalp and burr holes were drilled on both sides of the skull with coordinates at -0.8 mm posterior to bregma and ±1.5 mm lateral to the sagittal suture. Streptozotocin (STZ) were dissolved in artificial cerebrospinal fluid (127 mM NaCl, 1.0 mM KCl, 1.2 mM KH2PO4, 26 mM NaHCO3, 10 mM D-glucose, 2.4 mM CaCl2, 1.3 mM MgCl2). The rats were injected with 5µl of STZ bilaterally (both side 5µl – total 10µl) at a depth of 2.4 mm by using 10-µl microsyringe (Hamilton, USA) fitted with a 26-gauge needle. The sham-operated animals were administered with the same volume of aCSF without STZ. Then the animals were left for 14 days for recovery period and induction of AD. After 14 days of recovery period, the treatment was started and was continued till the 28th day **(Correia et al., 2013)**.

Cognitive behavioral assessment

Radial Arm Maze

Radial arm maze task is most extensively used to investigate specific aspects of spatial working and reference memory. This task is based upon the premise that animals have evolved an optimal strategy to explore their environment and obtain food with the minimum amount of effort. For testing the animals, bait any four arms of the maze with food should be done. In this study, the baited arms were 2, 4, 6 & 8. The animals were placed on the central platform of the maze and all guillotine doors were closed. All doors were simultaneously raised to allow the rats to freely explore the cage, collect the food rewards and finish the task. Then the parameters (within 10 min) like number of correct entries by rats into baited arms, the number of animal entries into un-baited arms and the number of re-entries into baited arms were noted. Time elapsed between the beginning of the test session and the rat obtained all available food rewards were calculated. *Scores:* Entries into un-baited arms were considered as reference memory errors (RME), re-entries into baited arms were considered as incorrect working memory errors (IWME) and less time-no fear, active, correct entries were considered as correct working memory errors (CWME) i.e., good memory **(Venkatramaniah et al., 2016)**

Brain isolation

After behavioral studies, the animals were sacrificed with excess anesthesia. Then brain samples were isolated and the hippocampus region was micro-dissected, rinsed with isotonic saline and weighed. The hippocampus region was subjected to lipid peroxide (LPO) and pro-inflammatory cytokine interleukin-1β (IL-1β) estimation.

Neurobiochemicals estimation

Tissue preparations

The brain was homogenized with 10% ice-cold KCl (a quantity of 100µl KCl for a quantity of 10mg tissue) for other neurobiochemicals analysis,

Lipid peroxide assay (LPO) assay

Lipid peroxidation was evaluated by measuring the TBAR content according to the thiobarbituric acid (TBA) test described by **Ohkawa et al. (1979)** with slight modifications. The incubation mixture consists of 0.5 ml of aliquot, 0.2ml of 8% sodium dodecyl sulphate, 1.5 ml of 0.9% aqueous solution of thiobarbituric acid and double distilled water bath for 30 min. After cooling, the red chromogen was extracted into 5 ml of mixture of n-butanol and pyridine (15.1 v/v) centrifuged at 4000 rpm for 10 min. The absorbance of the organic layer was taken at 532 nm (Shimadzu, Japan). 1,1,3,3-Tetra ethoxy propane was used as an external standard in the concentration range of 80–240 nmol. TBAR content was expressed in nmol/mg tissue.

Anticholine esterase (AChE) estimation

The AchE level was measured in the hippocampus region of the brain using standard protocol as described in **Ellman et al., 1961.**

Statistical Analysis

The data were represented as mean ± standard error mean (SEM). The parameters were subjected to statistical analysis by one-way ANOVA and post-test analysis by Bonferroni's multiple comparison tests. Values were considered to be significant when $P < 0.05$. The statistical analysis was carried out using the software Graphpad Prism, version 6.

Results

Radial arm maze

a. Reference Memory Error - RME

The STZ (3mg/kg, ICV) treated rats showed a significant increase in the RME on the 28th day when compared to 1st day. The treatment with EtAC (200mg/kg) has significantly decreased the RME on 14th, 21st and 28th day ($p<0.001$) respectively which is represented in Table 1. Similarly, the group of animals treated with EU (100mg/kg) showed a significant ($p<0.01$) decrease in the RME on 14th and 21st day. The same treatment group showed a more significant reduction on RME at the end of the 28th day which showed the efficacy of EU in continuous treatment. The combination of EtAC (200mg/kg) and EU (100mg/kg) showed a significant ($p<0.05$) decrease in the RME on the 7th day itself but were more significant ($P<0/001$) effect on 21st and 28th day. The treatment groups when compared with the normal animals exhibited a significant reduction in the RMEs and specifically on the 28th day showing a significant ($p<0.01$) effect. The study indicated the memory-enhancing activity of EtAC and EU combination.

b. ***Incorrect Working Memory Errors - IWME***

The STZ treated group showed a significant increase in the IWME when compared to the 14th, 21st and 28th days of the SO group. The AD induced animals treated with EtAC, showed a significant ($P<0.05$) decrease in IWME on the 7th, 14th day ($p<0.01$), 21st day and 28th day ($p<0.001$) respectively in comparison to STZ group. Similarly, a group of animals treated with EU showed a significant ($p<0.05$) reduction in IWME on 14th, 21st day ($p<0.01$) and 28th day ($p<0.001$). The combination of EtAC and EU in the AD animals showed significantly (*$p<0.05$) decreased the IWME score in the 7th day and 14th day ($P<0.01$). Further, It shows the most significance ($P<0.001$) decrease on IWME score in the 21st and 28th day which is summarized in table 2, hence this showed the efficacy of combined effect of EtAC and EU in cognitive functions of Alzheimer's disease induced animals.

c. Correct Working Memory Errors- CWME

The results indicated that the STZ treated group had showed a significant reduction in CWME on day 28 when compared to the 1st day of the SO group. The treatment with EtAC (200mg/kg) showed a significant ($p<0.05$) increase in the CWME on the 7th day, 14th day ($p<0.01$), 21st day ($p<0.001$) and 28th day when compared to STZ group. The EU (100mg/kg) treatment showed

significant ($p<0.05$) increase in CWME on 14th day and 28th day ($p<0.001$) in comparison to STZ treated group. The combination group showed a significant ($p<0.01$) increase in the CWME on the 14th day and progressively consistent to 28th day ($p<0.001$). Overall the result indicates that there is a remarkable increase in the CWME score in combination groups when compared to STZ treated which indicates that EtAC and EU are the potent cognitive-enhancing combination in memory declined conditions.

Lipid peroxide (LPO) level

Intracerebroventricular administration of STZ has remarkably increased the LPO ($P<0.001$) in the hippocampal region of the brain indicate the level of oxidative stress. Treatment with EtAC, EU and combination of EtAC+EU have significantly decreased the hippocampal LPO level ($P<0.001$) in comparison to STZ treated rats. Interestingly, the treatment with EtAC+EU has shown a significant reduction in LPO level ($P<0.01$) when compared to EtAC treated group indicate the synergistic effect exhibited by combination group is depicted in figure 1.

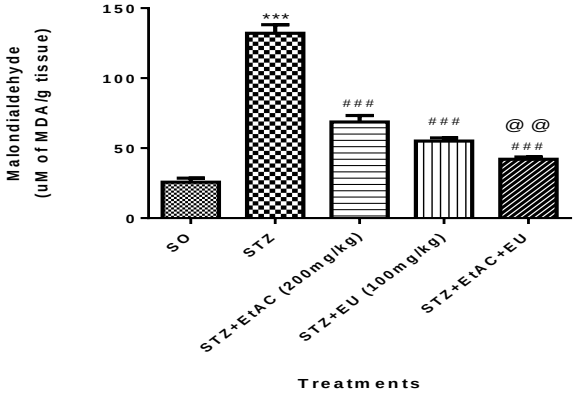

Figure 1: Effect of EtAC, EU and its combination of lipid peroxide (LPS) level in the hippocampus region of the brain.The Data are expressed as mean ± SEM. The statistical analysis was done with one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\#}P<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}P<0.00$ vs SO; $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$vs STZ and $^{@}P<0.05$, $^{@@}P<0.01$,$^{@@@}P<0.001$vs STZ+EtAC groups respectively.

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
SO	1.83±0.47	2.00±0.36	2.00±.0.51	2.16±0.30	2.33±0.21
STZ (3mg/kg)	2.84±0.45	3.00±0.35	3.50±0.34	3.61±0.37	3.35±0.25
STZ+EtAC (200mg/kg)	2.5±.22	2.00±.36	$1.66±.30^{***}$	$1.66±.33^{***\#}$	$0.83±.30^{\#\#***}$
STZ+EU(100mg/kg)	2.40±0.30	2.10±0.28	$1.9±0.31^{**}$	$1.5±0.25^{**\#}$	$1.2±.20^{\#\#***}$
STZ+ EtAC +EU	2.2±0.22	1.6±.205*	$1.61±.35^{***}$	$1.3±0.28_{***\#}$	$0.7+0.35^{\#\#\#***}$

Table 1: Effect of EtAC, EU and its combination of Radial Arm Maze (RAM) test-Reference memory error (RME). The Data are expressed as mean ± SEM. The statistical analysis was done with one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\#}P<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}P<0.00$ vs SO and $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$vs STZ group respectively.

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
SO	2.1±0.36	2.15±0.35	2.05±0.26	2.1±0.31	1.9±0.27
STZ (3mg/kg)	3.6±0.21	3.8±0.35	$3.92±0.28^{\#}$	$3.95±0.36^{\#}$	$3.98±0.24^{\#}$
STZ+EtAC (200mg/kg)	2.5±0.34	$2.3±0.2^{*}$	$1.74±0.30^{**}$	$1.59±0.38^{***}$	$1.48±0.70^{***}$
STZ+EU(100mg/kg)	2.8±0.29	2.5±0.31	$2.0±0.34^{*}$	$1.9±0.26^{**}$	$1.8±0.2^{***}$
STZ+ EtAC +EU	2.3±0.28	$2.1±0.26^{*}$	$1.8±0.25^{**}$	$1.5±0.3^{***}$	$1.2±0.31^{***}$

Table 2: Effect of EtAC, EU and its combination of Radial Arm Maze (RAM) test-Incorrect working memory error (RME). The Data are expressed as mean ± SEM. The statistical analysis was done with one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\#}P<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}P<0.00$ vs SO and $^{*}P<0.05$, $^{**}P<0.01$,$^{***}P<0.001$vs STZ group respectively.

Groups	Day 1	Day 7	Day 14	Day 21	Day 28
SO	2.0±.23	2.23±0.35	2.5±0.21	2.6±0.2	2.4±0.3
STZ (3mg/kg)	1.8±0.23	1.6±0.3	1.5±0.29	1.3±0.35	1.0±0.26
STZ+EtAC (200mg/kg)	2.6±0.36*	2.5±0.25*	2.0±0.21**	3.0±0.3$^{***\#\#}$	3.2±0.28$^{***\#\#}$
STZ+EU(100mg/kg)	2.4±0.35	2.8±0.31*	3.1±0.2$^{**\#}$	3.3±0.24 $^{***\#}$	3.5±0.28$^{***\#\#}$
STZ+ EtAC +EU	3.5±0.2#	3.4±0.31$^{*\#}$	3.0±0.24$^{**\#}$	3.8±0.27 $^{***\#\#}$	3.9±0.34$^{***\#\#}$

Table 3: Effect of EtAC, EU and its combination of Radial Arm Maze (RAM) test- Correct working memory error (CWME). The Data are expressed as mean ± SEM. The statistical analysis was done with one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\#}$P <0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$ P<0.00 vs SO and *P<0.05, **P<0.01,***P<0.001vs STZ group respectively.

Acetylcholine esterase (AChE) level

The significant elevation of AChE level (P<0.001) in the hippocampus region of STZ administered rats was observed which indicates the STZ induced cholinergic dysfunction in the AD brain. Administration of EtAC, EU and combination of EtAC+EU have significantly decreased the hippocampal AChE level (P<0.001) level in comparison to STZ treated rats. Fascinatingly, the treatment with EtAC+EU has shown a significant reduction in AChE level (P<0.01) when compared to EtAC and EU alone treated group indicate the synergistic effect exerted by combination treatment.

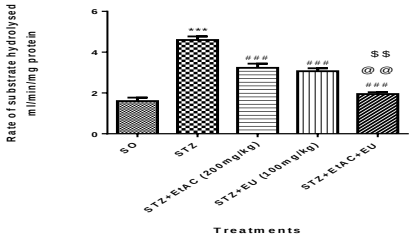

Figure 2: Effect of EtAC, EU and its combination on Acetylcholine esterase (AChE) level in hippocampus region of the brain.The Data are expressed as mean ± SEM. The statistical analysis was done with one-way ANOVA followed by Bonferroni's multiple comparison tests. $^{\#}$P <0.05, $^{\#\#}$ p<0.01, $^{\#\#\#}$ P<0.00 vs SO; *P<0.05, **P<0.01,***P<0.001vs STZ, $^{@}$P<0.05, $^{@@}$P<0.01,$^{@@@}$P<0.001vs STZ+EtAC and $^{\$}$P<0.01, $^{\$\$}$P<0.05, $^{\$\$\$}$P<0.001vs STZ+EU groups respectivel

DISCUSSION:

The present study mainly focused to assess the cognitive enhancement activity of combination of *Acorus calamus* (AC) and Eugenol in STZ induced Alzheimer's disease model in rats. Alzheimer's disease, a neurodegenerative disorder marked by significant memory loss, occurs due to various pathological changes such as the β-amyloid accumulation, oxidative stress, deficiency of acetylcholine, and neuroinflammation. STZ, when given directly into the hippocampus through ICV, induces cognitive decline by intracellular glucose oxidation and ROS production which in turn leads to glucose toxicity and oxidative stress resulted in neurodegeneration **(Yamasaki et al., 2012)**.

Present experimental findings show that STZ administration exhibited remarkable memory loss within the 14th day which is evidenced from significant increase in the number of incorrect working memory errors (IWME) and reference memory errors (RME) and reduction in correct working memory error (CWME) when compared to the SO group. The animals treated with EtAC showed remarkable changes in the behavioral studies, it was observed that the cognitive behavior of STZ administered rats are increased. EtAC has shown a significant reduction in the number of IWME and RME and increased the CWME on 7, 14, 21 and 28 days in STZ treated rats. In similar to the above results, animals treated with Eugenol also showed a significant reduction in the number of IWME and RME and increased the CWME on 7, 14, 21 and 28 days in comparison to STZ treated rats. The AD animals, when treated with the combination AC and Eugenol, showed significant changes in cognitive behavior when compared with the STZ group shows the efficacy of EtAC+EU combination in AD like conditions.

In RAM test, it was observed that there was significant decrease in the memory errors in combination-treated groups from the 7th day onwards which continued to decrease progressively till 28th day, while the number of CWME significantly increased on the 14th day which continuously increased till 28th day when compared to STZ treated animals. The treatment with EtAC+EU has improved the cognitive behavior from 7th day onwards when compared to alone treated group indicate the synergistic effect of EtAC+EU combination in AD like conditions.

In the present study, the lipid peroxidation levels were remarkably increased in the STZ administered rats while it was reduced in the treatment groups. Interestingly, the EtAC+EU combination group attenuated the hippocampal LPO level in STZ administered rats in comparison to per se treatment group indicate the synergistic effect exhibited by combination in AD like conditions. Hence, it was evident that EtAC and EU have a strong antioxidant activity which may help in the suppression of neurodegeneration by preventing oxidative stress. The enzyme AChE a key factor that contributes to the pathogenesis of AD, it degrades the acetylcholine which essential for memory and learning process **(Irie et al., 2006; Vijayapandia et al., 2013).** The present findings have shown the significant elevation of AChE level in the hippocampal region of the STZ administered animals. The elevated level of AChE would have depleted the cholinergic transmission acetylcholine which may decline the cognitive functions in the behavioral test. Treatment with EtAC, EU and its combination have significantly reduced the AChE level in the STZ infused animals, but the effect was more in combination group in comparison to individual treatment groups depict the synergistic beneficial effect of EtAC+EU combination in cholinergic transmission of AD animals which evidences from earlier study **(Swathi et al., 2015)**.

Previous studies implicated that the ability of EU to inhibit apoptosis and pro-inflammatory cytokine secretion and subsequent protection of neuronal cells was related to its effect to counterbalance the oxidative stress, also the histological examination revealed a neuroprotective potential of EU against neuronal damage **(Said et al., 2017)**. In the present study, the efficacy of EU has increased when combined with EtAC, therefore it can be concluded that the combination of EtAC+EU produced a remarkable synergistic effect on improvement of cognitive functions of STZ administered AD animals. The mechanism for cognitive enhancement is might be attributed through attenuation of lipid peroxide-induced oxidative stress and inhibition of AChE mediated cholinergic dysfunction resulted in prevention of further degeneration of neurons. The protective effect of EtAC+EU combination in the memory regions of the brain like cortex and hippocampus would have improved the cognitive behaviors and memory and learning functions in the radial arm maze test.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest

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