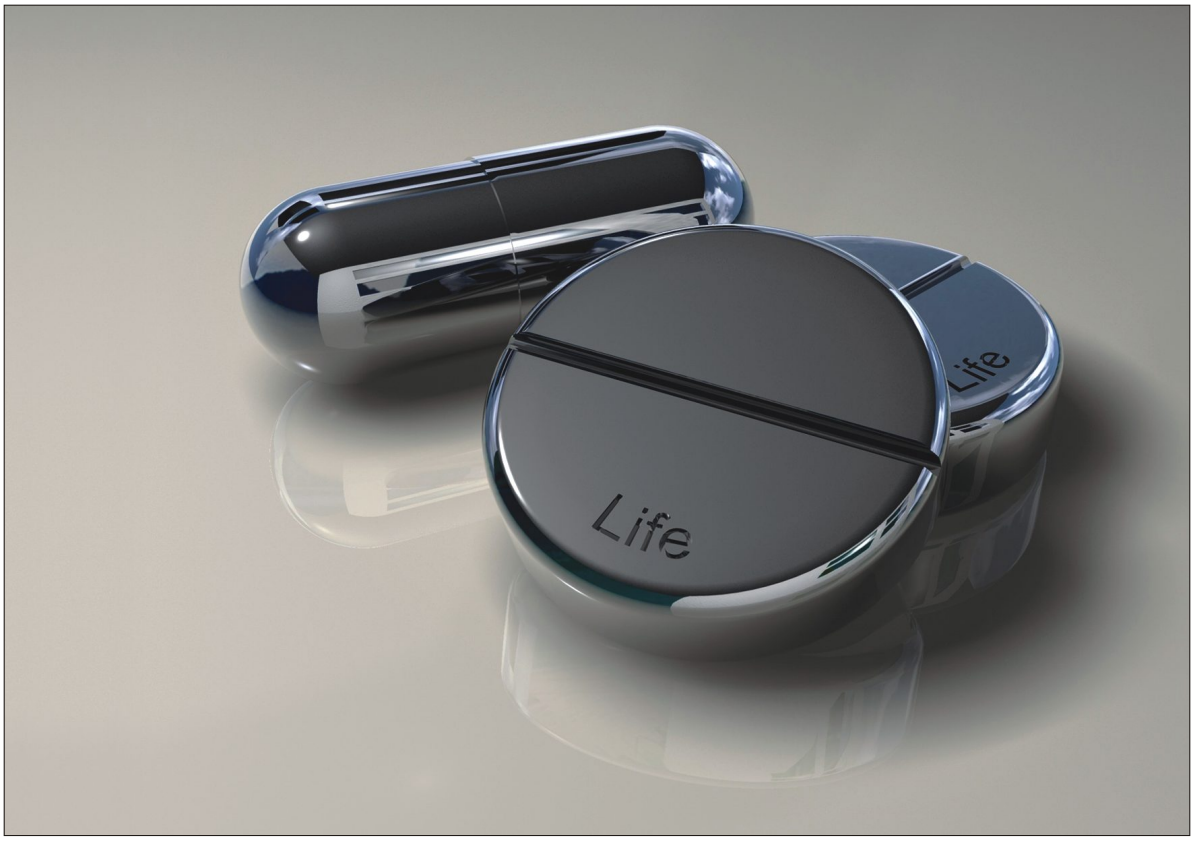

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Research Article
Modulation of Pro-inflammatory Mediators by Eugenol in $AlCl_3$ Induced Dementia in Rats

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Abstract

Background and Objective: Alzheimer disease is one of the common types of dementia, which involves progressive neuronal cell's degeneration. The main objective of this study was to evaluate the *in vitro* anti-choline esterase activity of four phenyl propanoids derivatives (Eugenol, Gallic acid (GA), Sinapic acid and Scopolin) and evaluation of Eugenol in $AlCl_3$ induced dementia in rats. **Materials and Methods:** The four phenyl propanoids derivatives (Eugenol, Gallic acid (GA), Sinapic acid and Scopolin) were evaluated for *in vitro* anti-choline esterase activity. For *in vivo* screening the rats were treated with aluminum chloride at a dose of 175 mg kg^{-1}, for a period of 25 days to produce dementia. Then rats divided in 4 different groups and further evaluated for 10 days of treatment , i.e., Negative control, Standard group and one group received sub maximal dose of rivastigmine along with Eugenol (1.25 mg kg^{-1} p.o+ 50 mg kg^{-1} Eugenol, p.o) and other group received Eugenol (50 mg kg^{-1}, p.o) alone. All rats were observed until the 35th day of experimental protocol. The different behavioral and biochemical parameters like GSH, TBARS and Nitrite level were also determined. Neuro-protective activity of eugenol against neuro inflammation produced by $AlCl_3$ was accessed by estimations of two pro inflammatory cytokines (TNF-α, IL-1β) in brain tissues. **Results:** Rats treated with aluminum chloride (175 mg kg^{-1}, p.o.) produced a significant decline in behavioral and biochemical parameters in rats. The rats treated with Eugenol showed significant reversal of memory deficit Dementia of AD type. **Conclusion:** The study concluded that Eugenol had a synergistic effect with rivastigmine when used in combination and showed neuro protection against $AlCl_3$ induced dementia of AD type in rats and modulates the neuro inflammation produced by $AlCl_3$.

Key words: Cell degeneration, neuro inflammation, alzheimer's disease, neuronal cell, rivastigmine, eugenol, neuro protective

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alzheimer's disease (AD) is a type of neuro-degenerative disease, affecting memory and cognitive functions. The most people affected by this disease are at the age 60 plus. Patients become to enable of functions like judgment, decision making, emotional changes and other behavioral changes. The treatment and regimen of the disease include use of anti-choline esterase drugs like donepezil, rivastigmine and galantamine. As per findings suggested some of the herbals also provide symptomatic relief in the AD Type in rats[1-4]. The neuro-transmitter, acetylcholine deficiency in AD created an urge among scientists to find the suitable compounds as choline esterase inhibitors to increase the availability of it at the synapse[5,6]. Now, it becomes a major approach for the treatment and added ground-breaking results in AD treatment in patients of AD[7]. In a search of suitable bio markers, Reactive Oxygen Species (ROS) also plays an important role to cause cellular degeneration so the use of compounds, which prevent cell damage also showed a better improvement in pathogenesis of AD[8,9]. The natural anti-oxidants like polyphenols, flavonoids, lignins and stilbene which are part of human diets. Traditionally, their supplements were consumed with large population to cure various diseases and some of them are present in edible food to give a prophylactic response to oxidative stress. These compounds are proven for their interaction with several enzymes[10]. Several other studies suggested that the interaction of polyphenols with enzymes is due to their complex structure and better bioavailability[11-13]. So the objective of present study was to screen some of the competent polyphenol molecules (Eugenol, Gallic acid (GA), Sinapic Acid and Scopolin) as anti-choline estarse in Alzheimer's disease and based on results, the effective one is further evaluated for *in vivo* activity in rats. The study also evaluated the interaction of Eugenol with standard drug (rivastigmine) available for AD Therapy. Choline esterase is most culprit enzymes in neuro-degenerative disease like dementia of AD type in rats so current study will stabilize the role of Eugenol in AD. The study also provides the protective role of Eugenol in neuro inflammation produced by aluminum chloride in dementia.

MATERIALS AND METHODS

All the work related to this study was conducted in affiliated institutes. The analytical part and animal study was conducted in a month of May-August, 2015 and other work was completed on August, 2018.

Drugs and chemicals: The standard compounds like scopoline (99.7%), gallic acid (99.9%), eugenol (99.7%) and sinapic acid (98.9%) were procured from Sigma Aldrich (Germany) (S and G Lab. supplies). Drug, rivastigmine was provided as a gift sample (Ref. Sun/G/254-5 gm) from Sun pharmaceutical Pvt., Ltd., India. The other chemicals like DTNB, acetylthiocholine iodide, trichloroacetic acid, thiobarbituric acid and sodium carboxy methyl cellulose was procured from Merk and S.D. Fine Chemicals Ltd., (Mumbai) India (Analytical Grade).

***In vitro* ache assay:** This assay was performed as per the method described by Nascimento and Martins[11] and modified by Albano *et al.*[12] and Owokotomo *et al.*[13]. The samples measured at 412 nm in an X-Rite 640B spectrophotometer (Grand Rapids MI, 49512, USA). Rivastigmine was used as positive control. The percentage inhibition of enzyme activity was calculated by comparison with the negative control. The IC_{50} values are indicative of 50% inhibition of enzyme hydrolysis, which is determined by spectrophotometry method (Table 1). The samples were tested in triplicates. To calculate the IC_{50} values, each sample was assayed at 5 concentrations (30, 20, 10, 5 and 2.5 mg mL^{-1}). The IC_{50} values were obtained from dose effect curves by linear regression. The inhibition factor (strength of inhibition) for test and positive control were calculated using the equation[13]:

$$\text{Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where, A_0 was the absorbance of the negative control (enzyme+methanol) and A_1 was the absorbance of the sample (enzyme+solution of test). Tests were carried out in triplicate and data were analyzed using descriptive statistics:

$$\text{Inhibition (\%)} = \frac{IC_x \text{ of reference inhibitor}}{IC_x \text{ of test compound}}$$

where, IC_x is the concentration of test substance that inhibited x% of AChE activity.

Animals: The Wistar rats (180-200 g, 7-8 weeks) of either sex were selected and provided 12 h light-dark cycle with free access of food (Standard pellet diet) and water. All animals were trained in noise-free environment and trials were conducted between 9:00 AM-2:00 PM. The experiment was ethically approved and carried out as per official guidelines of experimentation in animals (IAEC/2015/17-18).

Acute toxicity study (LD_{50}): The acute toxicity (LD_{50}) of Eugenol was calculated as per standard guidelines issued by organization for economic cooperation and development (OECD) following the up and down method (OECD guideline No. 423)[10-12].

Experimental design

Aluminium chloride-induced dementia: This model was followed by the methods described by Bais *et al.*[3]. Day 1- 4, all animals were gone for training under Morris Water Maze and on 5th day (Prove day), rats were randomly divided in order to separate groups (n = 6) (Table 2). Details of groups given:

- **Group I:** Control group received normal saline (5 mL kg^{-1}, p.o)
- **Group II:** Negative control (II) ($AlCl_3$ induced-untreated rats)
- **Group III:** Standard group (III) [($AlCl_3$ Induced+ Rivastigmine treated rats-(2.5 mg kg^{-1}, p.o.)]
- **Group IV:** Eugenol alone treated group ($AlCl_3$+Eugenol-50 mg kg^{-1}, p.o. treated rats)
- **Group V:** Eugenol along with sub-maximal dose of Rivastigmine (IV) ($AlCl_3$+Eugenol-50 mg kg^{-1}+ Rivastigmine-1.25 mg kg^{-1}, p.o. treated rats)

All rats were accessed for learning, memory and ambulatory movements. Morris water maze test was used to access the memory deficit caused by neuronal damage. The test was performed via well-known specifications of a water maze consisted of a circular (150 cm diameter and 40 cm height)[13]. This test were performed on 5, 16, 26 and 36th day of experimental trial and later; various parameters like Time elapsed in escaping to the NW quadrant, i.e., escape latency time (ELT) and total time (TT) time spent in NW quadrant were measured during the retention trials[3]. The animals were sacrificed to study various biochemical parameters like extent of oxidative stress and acetylcholine esterase (Ache) activity[14].

Evaluation

Sampling and preparation of tissue homogenate: At the end of experiment, rats were dissected and their brain were removed immediately and maintained under temperature of 2-8°C to prevent further enzyme degradation. Then it was washed thoroughly with ice cold isotonic saline. A 10% tissue homogenate was prepared using 0.1 M phosphate buffer (pH 8, stored 2-8°C) and used for various estimations.

Biochemical assessment

Acetyl choline esterase (AchE) level: The AchE level in brain tissue homogenate samples were processed and determined according to the method described by Morris *et al.*[14] and Kangtao *et al.*[15].

Glutathione (GSH) level: The GSH level in brain tissue homogenate samples were processed and determined according to the method described Beutler *et al.*[16] and Bais and Prashar[17] with slight modifications. Results were expressed as nmol of GSH/mg of protein.

Catalase level: The catalase level in brain tissue homogenate samples were determined using method described by Owokotomo *et al.*[13] and Luck [18].

Lipid peroxidation (TBARS) level: The TBARS levels in brain tissue homogenate samples were determined via spectrophotometry method[19]. The content level was expressed in terms of nmol malondialdehyde.

Table 1: Summary of *in vitro* AchEI by phenyl propanoids derivative

Compounds	IC_{10} (mg L^{-1})	IC_{50} (mg L^{-1})	IC_{90} (mg L^{-1})	Inhibition factor (IF) (IC_{50})	Inhibition factor (IF) (IC_{90})
Rivastigmine	ND	7.52	11.21	1	1
Gallic Acid	ND	8.41	12.01	1.01	0.99
Sinapic Acid	1.52	7.61	10.98	1.10	1.07
Scopolin	ND	9.51	13.52	0.90.	0.89
Eugenol	2.12	5.52	10.45	1.46	1.12

ND: Not shown in test

Table 2: Effect of eugenol on aluminium chloride induced dementia of Alzheimer's type in rat using morris water maze (Escape latency time)

Groups/Treatments	ELT (sec) 5th day	16th day	26th day	36th day
I (Control group)	3.98±0.1	4.04±0.1	5.03±0.1	4.15±0.1^{b***}
II (Untreated $AlCl_3$-affected rats)	3.879±0.1	13.0±0.05	25.28±0.27	22.12±0.1^{a***}
III (Rivastigmine-treated $AlCl_3$-affected rats)	3.98±0.13	10.1±0.08	18.32±0.28	5.1±0.1$^{a***\ b***}$
IV (Test-A) $AlCl_3$ (175 mg kg^{-1} p.o.)+EUGENOL (50 mg kg^{-1} p.o.)	3.87±0.07	10.0±0.12	17.83±0.22	8.12±0.1$^{a***\ b***}$
V (Test-B) $AlCl_3$ (175 mg kg^{-1} p.o.)+EUGENOL (50 mg kg^{-1})+Rivastigmine (1.25 mg kg^{-1} p.o.)	3.97±0.04	10.94±0.04	20.87±0.07	7.13±0.03$^{a***\ b***}$

Data are Mean±SEM values, n = 6, data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 and ^{ns}p>0.05. ^{a}Compared with control, ^{b}Compared with inducer, ^{NS}Not significant, ELT: Escape latency time

Superoxide dismutase (SOD) level: The SOD level in brain tissue homogenate samples were determined using enzyme diagnostic kits (RANDOX, Ransod enzyme kit, U.K.). This method was based on reaction of generated superoxide radicals with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium-chloride to form the red formazan dye[20].

Nitrite level: It was measured by the method described by Lyle *et al*.[21].

Estimation of pro-inflammatory cytokines: The TNF-α level and IL-1β were estimated by ELISA kit [13].

Statistics and data analysis: The IC_{10}, IC_{50} and IC_{90} were calculated from the dose-response equations obtained in reference to standard compound. All data analysis was done using M.S. Excel software. The experiment data was expressed as means±standard error of the mean (SEM). Data were analyzed via non-parametric analysis of variance by two-way (ANOVA) followed by Turkey multiple comparison tests and other data were evaluated using Graph Pad PRISM Version 7, software. A p-value<0.05 was considered statistically significant.

RESULTS

Effects of phenyl propanoids derivatives on *in vitro* AchEI capacity: The *in vitro* AchEI was calculated and presented as IC_{10}, IC_{50} and IC_{90}. From the experimental findings, gallic acid and sinapic acid showed a potent AchEI activity which was matched with standard rivastigmine. Eugenol showed a potent inhibition at lower concentration with less IC_{50} values. Other compound like scopoline was not found much potent at lower concentration when compared with rivastigmine (Table 1).

Effects of eugenol on behavioral parameters

Morris water maze test: All the rats were tested for memory deficit in Morris water maze apparatus with randomized training trials for initial four days (probe day). The negative control group (II) showed memory deficit accessed by raised escape latency time (ELT) on 16th and 25th day and reduced time spent in target quadrant when compared with a control group. The animals treated with Eugenol (50 mg kg^{-1}, p.o.) (Alone) in group IV showed reversal of ELT and TT changes and this effect were found more significant ($p \leq 0.01$) in Group V, when treated in combination with rivastigmine (Table 2, 3).

Effect of eugenol on AchE level: The AchE level was found higher in aluminum treated (Group II) rats as compared with control rats (Group I). Rivastigmine treated rats (Group III) found less concentration of AchE in brain tissues. The rats treated with Eugenol (Group IV) also showed less concentration but the AchE level was much improved when given in combination (Group V) with rivastigmine and found almost twice to Eugenol alone (Group IV). The response observed in combination Eugenol and rivastigmine (Group IV), (AchE level) gave significant amelioration when compared to negative control (Group II) (Table 4).

Effect of eugenol on biochemical parameters: The effects of Eugenol on the different enzyme's level were determined (Table 4). Aluminum treated (Group II) rats showed increased level of TBARS and Nitrite. The rats treated with rivastigmine (Group III) and Eugenol (Group IV and V) showed significant reduction in raised level of TBARS and nitrite, when compared control rats (Group I) on 36th day of trial.

The level of catalase, SOD and GSH were found reduced in aluminum treated (Group II) rats as compared to control rats, which were further improved by the treatment of Eugenol and rivastigmine.

Effect of eugenol on pro-inflammatory cytokines: The rats affected with aluminium chloride showed increased levels of TNF-α in brain tissues (Fig. 1). This level was further reduced when animals treated with Eugenol when compared with negative control rats. Interleukin-1 (IL-1) level was found high

Table 3: Effect of eugenol on aluminium chloride induced dementia of Alzheimer's type in rat using morris water maze

	T.T (sec)			
Groups/Treatments	5th day	16th day	26th day	36th day
I (Control group)	2.53±1.01	3.18±0.04	2.55±0.05	$3.42\pm0.18^{b***}$
II (Untreated $AlCl_3$-affected rats)	2.65±0.1	3.15±0.13	1.99±0.04	$1.20\pm0.06^{a***}$
III (Rivastigmine-treated $AlCl_3$-affected rats)	2.62±0.09	2.88±0.04	1.78±0.02	$2.8\pm0.15^{a, ns, b***}$
IV (Test-A) $AlCl_3$ (175 mg kg^{-1} p.o.)+Eugenol (50 mg kg^{-1} p.o.)	2.57±0.12	2.60±0.05	1.50±0.01	$1.98\pm0.2^{a* b***}$
V (Test-B) $AlCl_3$ (175 mg kg^{-1} p.o.)+Eugenol (50 mg kg^{-1})+Rivastigmine (1.25 mg kg^{-1} p.o.)	2.64±0.11	3.22±0.05	1.90±0.02	$3.2\pm0.23^{a* b***}$

Data are Mean±SEM values, n = 6, data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 and $^{ns}p>0.05$. ^{a}Compared with control, ^{b}Compared with inducer, ^{NS}Not significant, T.T: Total time

Table 4: Effect of Eugenol on aluminium chloride-induced dementia of Alzheimer's type in rat using various biochemical parameters

Groups/Treatments	AchE (nM/L/min/g of tissue)	GSH (Nm/mg of protein)	TBARS (Nm/mg of protein)	Nitrite (Nm/mg of protein)	SOD (Units/mg of protein)	Catalase (μM of H_2O_2 decomposed/min/mg of protein)
I (Control) 0.9% Nacl (5 mL kg^{-1} p.o.)	$4.8 \pm 0.18^{b***}$	$9.15 \pm 0.42^{b***}$	$1.9 \pm 0.151^{b***}$	$1.71 \pm 0.24^{b***}$	$48.7 \pm 3.5^{b***}$	$1.0 \pm 0.023^{b***}$
II (Inducer) $AlCl_3$ (175 mg kg^{-1} p.o.)	$8.80 \pm 0.18^{a***}$	$3.2 \pm 0.27^{a***}$	$4.3 \pm 0.039^{a***}$	$4.53 \pm 0.29^{a***}$	$11.76 \pm 2.3^{a***}$	$0.21 \pm 0.04^{a***}$
III (Standard) $AlCl_3$ (175 mg kg^{-1} p.o.)+ Rivastigmine (2.5 mg kg^{-1} p.o.)	$5.77 \pm 0.27^{a, ns, b**}$	$7.9 \pm 0.22^{a* b***}$	$2.65 \pm 0.047^{a*** b***}$	$2.3 \pm 0.14^{a, ns, b***}$	$43.34 \pm 1.5a^{ns\ b***}$	$0.85 \pm 0.01^{a** b***}$
IV (Test-A) $AlCl_3$ (175 mg kg^{-1} p.o.)+ Eugenol (50 mg kg^{-1} p.o.)	$6.69 \pm 0.38^{a* b***}$	$5.15 \pm 0.19^{a*** b***}$	$3.28 \pm 0.037^{a*** b***}$	$3.1 \pm 0.27^{a** b**}$	$32.7 \pm 2.9^{a** b***}$	$0.68 \pm 0.04^{a*** b***}$
V (Test-B) $AlCl_3$ (175 mg kg^{-1} p.o.)+Eugenol (50 mg kg^{-1})+ Rivastigmine (1.25 mg kg^{-1} p.o.)	$4.9 \pm 0.37^{a** b***}$	$6.93 \pm 0.12^{a*** b***}$	$2.9 \pm 0.052^{a** b***}$	$2.9 \pm 0.12^{a* b***}$	$39.0 \pm 3.8^{a, ns, b***}$	$0.79 \pm 0.028^{a*** b***}$

Data are Mean ± SEM values, n = 6, data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 and ^{ns}p>0.05, ^{a}Compared with control, ^{b}Compared with inducer, ^{NS}Not significant

Fig. 1: Effect of Eugenol on TNF-α level in brain cortex

All values were represented as Mean±SEM, data are Mean±SEM values, n = 6, data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 and ^{ns}p>0.05, ^{a}Compared with control, ^{b}Compared with inducer, ^{NS}Not significant

Fig. 2: Effect of Eugenol on IL-1β level in brain cortex

All values were represented as Mean±SEM, data are Mean±SEM values, n = 6, data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test, *p<0.05, **p<0.01, ***p<0.001 and ^{ns}p>0.05, ^{a}Compared with control, ^{b}Compared with inducer, ^{NS}Not significant

in negative control rats when compared with control rats. The rats treated with Eugenol, either alone or in combination with rivastigmine showed decreased levels of IL-1 level in the rat's brain (Fig. 2).

DISCUSSION

The present study proved the additive effect of Eugenol when given along rivastigmine with fewer side effects. The findings will promote the use of nutraceuticals with synthetic drugs in a regimen of AD disease. The choline esterase inhibitors are the only synthetic drugs approved by Food

Drug Administration (FDA). The use of rivastigmine is well-established and proven drug for therapy in AD disease[22-27]. The pathogenesis of AD disease showed impaired cholinergic transmission, which results in memory deficit. This impairment occurs by two ways, (i) Reduction in Ach release[28-32] and (ii) Change in acetyl choline transferase activity, which increases scarcity of Ach[33-37]. The elevated level of AchE, also increase the progression of AD disease so there always a need of a drug regimen that controls its activity. The study also supports the neuro-protection against pro-inflammatory cytokines (TNF-α, IL-1β) to prevent the neuro-inflammation which is an important factor in pathogenesis of AD[35].

Flavonoids and polyphenols are the common anti-oxidants available in a human diet but the concentration may vary according to the sources. The plants with rich polyphenols content must be the priority for researchers to explore. These polyphenols are divided into different classes as phenolic acids, flavonoids, lignins and stilbene. These compounds found abundantly in the plant kingdom with structural similarities, compounds like caffeic acid, gallic acid, p-coumaric acid, vanillic acid, ferulic acid and Eugenol have common carboxylic group which may have interaction with different enzymes[38-40]. Eugenol belongs to bioactive carboxylic acids and found as a potent inhibitor of AchE. Eugenol acts by reducing oxidative stress so why it found effective against cerebral hypoxia[39]. The effect activates the cholinergic function to improve memory deficit in aluminum chloride induced dementia[41-43].

Current study also showed similar findings with impaired behavioral changes by aluminium chloride administration. The results showed significant interaction of Eugenol with AchE and showed decreased escape latency and increased time spent in target quadrant [NW]. The hypothesis suggested the rivastigmine and Eugenol antagonizes the memory deficit caused by aluminium. The study concludes that the synergistic effect of Eugenol with rivastigmine is due to its chelating effects of the enzyme which is supported by a study published by Birks[4] stated, the electrostatic interaction of Eugenol with AchE and confirms its AchEI property. Thus, Eugenol may be considered as a potential candidate in treatment of various memory disorders.

CONCLUSION

The study concluded that Eugenol has significant neuro-protective effects in Dementia of AD type and its synergistic effect is due to its interaction AchE. There are further studies need to be done to explore the molecular mechanism of Eugenol with possible mode of action.

SIGNIFICANCE STATEMENT

This study discovered the mechanism of Eugenol in AD disease and stabilizes the therapeutic interaction with AchE that can be beneficial for other scientist to explore the molecular mechanism of Eugenol interaction. This study will help the researchers to uncover the possible cause of synergistic effect of Eugenol with rivastigmine and help many researchers to design the better therapeutic regimen in AD disease.

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