



Effects of Fermented Coffee Extracts on Spatial Cognition and Memory of Alzheimer's Disease Induced Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The current study hypothesizes that ingestion of polyphenols-enriched preparation such as yeast fermented extracts of green and roasted coffee beans will demonstrate neuroprotective and stimulatory function.

Study Design: Optimizing yeast (*Saccharomyces cerevisiae*) mediated fermentation conditions of *C. Arabica* beans → Extraction by the sonication-agitation method before and after roasting to obtain Fermented Green Coffee Extracts (FGCE) and Fermented Roasted Coffee Extracts (FRCE) → Phytochemical profiling of the fermented extracts was performed → Animal Study (*in vivo* evaluation).

Place and Duration: The research work was conducted during December, 2019 to May, 2020 at the Department of Biotechnology, Sri Venkateswara College of Engineering, Post Bag No.1, Pennalur, Sriperumbudur Tk, Kancheepuram Dt TN-602117, India.

Methodology: The FGCE and FRCE extracts were prepared and subjected to comparative phytochemical profiling for *in vitro* analysis. Further, the *in vivo* analysis was performed on 24 Albino Wistar rats, which were divided into four groups (Group I (Control group) received normal diet; Group II (AD induced group) received $AlCl_3$; Group III received FGCE and $AlCl_3$, Group IV received FRCE and $AlCl_3$). In order to represent the most exact model that mimics AD the rats were

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injected with $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ at dose 70 mg / kg I.P for 6 weeks. During induction the rats were subjected to spatial memory tests (T-maze) and motor co-ordination tests (burrowing assay).

Results: The FGCE had a higher amount of polyphenols (1.20 ± 0.02 GAE mg / ml) compared to FRCE (0.99 ± 0.047 GAE mg / ml). Also, the ferric reducing anti-oxidant potential was higher in FGCE (5.14 ± 0.17 mmol Fe^{2+} / g) than in FRCE (3.7 ± 0.2 mmol Fe^{2+} / g). As a result of behavioural analysis, the fermented green coffee extract consumption in Alzheimer induced rats had a prominent positive effect on memory retention and motor co-ordination.

Conclusion: This study elucidates the potential nature of FGCE in decelerating the progression of AD at a higher rate than FRCE.

Keywords: Alzheimer's disease; fermented coffee; chlorogenic acid; polyphenols; nutraceutical.

1. INTRODUCTION

Considering the impact of the various socio-demographic transitions in the world, especially the fortuitous increase in lifespan over the last decades has lead to an increase in the risk of age-related cognitive impairments such as dementia, including neurodegenerative diseases, e.g. Alzheimer's disease (AD) and Parkinson's disease (PD). Alzheimer's disease is the most complex yet common form of dementia, and it is unlikely that any one drug or other intervention will successfully treat it [1]. The epidemiological data states that the ratio of AD occurrence is approximately one to nine in individuals of age < 65 years and one in three over 85 years of age.

The pathophysiology of AD encompasses the accumulation of Amyloid-beta plaques ($\text{A}\beta$) which are extracellular deposits of $\text{A}\beta$ in the brain parenchyma and in the cerebral blood vessels where it is known as cerebral amyloid angiopathy (CAA). Another possibility is the presence of neurofibrillary tangles (NFT) composed largely of paired helical tangles that disturb the cytoskeleton by disrupting cell structure destroying axonal transport and synaptic viability leading to neuronal dysfunction [2].

Currently, there is no effective treatment capable of slowing down disease progression. In recent times, the main focus of AD treatment by novel pharmacotherapies is to deplete $\text{A}\beta$ production through the inhibition of β and γ secretase enzymes; and to induce dissolution of existing cerebral $\text{A}\beta$ plaques. However, these approaches attempting to treat these lesions have not achieved permanent successful results. Thus, investigating strategies that may prevent or delay the progression of Alzheimer's disease is a matter of the utmost importance [3].

Since no disease-modifying intervention is available, nutritional strategies which might slow

down the development of dementia can be implemented in a routine diet [4]. Nutritional interventions for treating Alzheimer's majorly focus on insulin resistance, dyslipidemia, and oxidative stress. Over the last decade, there is a substantial surge in evidence indicating oxidative stress as a major AD potentiating factor, which can be counteracted by consuming anti-oxidant rich diet [5,6]. Meanwhile, numerous polyphenolic compounds have been inculcated in diets, due to its richness in antioxidants, which has been described to be neuroprotective. Polyphenols are typically involved in both anti-oxidant activity and anti-inflammatory activity, which are vital in the alleviation of Alzheimer's disease. Yet, the complexity of dietary polyphenol composition is deteriorated by their high volatility and the capability to convert into various reaction products when the plant cells are damaged, for instance, by processing the food or during the extraction process [7]. So, these transformations must be of primary regard in any study [8].

Coffee is one of the most popular beverages with a rich source of bio-active compounds which is loaded with bioactive anti-oxidant compounds especially polyphenols, such as phenolic acids, mostly chlorogenic acids (in green beans), and caffeic acids (occurring after roasting). Every single cup of coffee holds upto 70–350 mg of CGAs and accounts for about 13g / 100g coffee [9]. The plasma concentration of unmetabolized CGA followed by the ingestion of coffee peaked at 300 mg in 30 mins which then exerts neuroprotective effects once it crosses the blood-brain barrier (BBB). The vital impact of CGA on the brain range from neuronal differentiation enhancement; disaggregation of Amyloid beta-protein, reduction of Reactive oxygen species (ROS), and also betterment in cognition [10,11]. But, Green coffee beans which are abundant in phenolics, especially the Chlorogenic Acid (CGA) once roasted forms Melanoidins bound CGA are formed during coffee roasting [12,13].

Recently several kinds of research have been focusing on examining the bidirectional gut-brain interaction, the neuroprotective value of the fermented food and its positive impact on cognition is becoming evident. Commercial value and applications of fermented foods are expanding as a natural remedy to cognitive problems [14]. In fact, according to a recent study, green coffee beans gained higher functionality with additional processing steps such as soaking in fruit extracts and fermentation. Yeast fermentation of green coffee beans for 24 h was effective in fortifying the functionality of coffee by inducing a significant increase in antioxidant activity, and phenol content [15]. The extraction method also plays a vital role in improving the phenol content. Cold-brewed coffee is traditionally, extracted with water for 24 h, but this procedure is both time-consuming and uneconomic. Non-conventional extraction methods involving a combination of ultrasonication and agitation had considerably positive effects on organic acid content and antioxidant activity in cold-brewed coffee [16].

Therefore the objective of this study is to analyze the neuroprotective efficacy of yeast fermented coffee extracts in Alzheimer induced rats by co-administration of Aluminium chloride and fermented coffee extracts. The protective effects of both fermented green and fermented roasted coffee extracts are examined by behavioural tests, analyzing working, reference memory.

2. MATERIALS AND METHODS

2.1 Chemicals

Aluminium chloride hexahydrate ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$), Sodium carbonate (Na_2CO_3), Monosodium phosphate (NaH_2PO_4), Disodium phosphate (Na_2HPO_4), Folin-Ciocalteu reagent, Gallic acid Quercetin, 2, 5-Dinitrosalicylic acid (DNS), YPD agar, and YPD broth were purchased from the Sigma-Aldrich Chemical Company.

2.2 Coffee Fermentation, Drying, Roasting, Brewing

Green Coffee Beans (900 g, *Arabica green coffee beans*) was purchased from Alpha Global Enterprises. The green coffee beans were then immersed in distilled water (1,350 ml), and inoculated with yeast (*S. Cerevisiae*, (11.25 ml; 1×10^7 CFU / ml)) in a 2 litre Büchner flask. This mixture was fermented for 24 hours at 30°C. At the end of which, the fermented coffee beans

were washed thrice using sterile distilled water. Then, they were dried in the hot air oven at 600°C until the moisture content is reduced to 10%. The fermented green coffee beans were divided into two batches, where one batch was subjected to roasting in a roaster. At maximum heating power (1,600W for 16 minutes). The roasted coffee beans were kept at room temperature for three days. The two batches, fermented green, and fermented roasted coffee beans were ground in a coffee grinder at medium speed. Cold brewing was opted in order to facilitate extraction of coffee at a higher concentration, 15 g of ground coffee powder of each batch were soaked in 100 ml of water at 4°C for 12 hours. Finally, the cold brew of both FGCE and FRCE were extracted by ultrasonication for 35 minutes 80 KHz and agitation using a magnetic stirrer at 50 rpm for 50 minutes. The extracts were finally filtered using a cotton filter initially, and then filtered using *Whatman* filter paper no.1 [15-17].

2.3 Studies on Fermentation Characteristics

The impact of fermentation on the characteristics of fermentation solution was checked by measuring the pH, to analyze the change in organic acid quantity. To assess the change in reducing sugar content, in the green coffee beans DNSA test was performed [18]. 2 ml coffee ferment sample (0th hour and 24th hour) was mixed with 6 ml DNS solution and heated at 100°C for 5min. Water (3 ml) was added to the reaction solution and its absorbance was measured at 550 nm. D-Glucose solutions (0.1 – 2.5mg / ml) were used for a standard curve (R2 = 0.997). Finally, to check the proliferation of yeast, the fermentation media post inoculation were subjected to serial dilution plating in YPD agar (0th and 24th hour), resultantly the colony forming units (CFU) were counted using colony counter.

2.4 Phytochemical Screening of GCE, RCE, FGCE and FRCE

2.4.1 Total polyphenol content (TPC)

The total polyphenol content (TPC) of green coffee extracts (GCE), roasted coffee extracts (RCE), fermented green coffee extracts (FGCE), and fermented roasted coffee extracts (FRCE) were measured using modified Singleton's method (1977) [19]. 0.02 ml of the coffee extracts were diluted with 1.58 ml of distilled water. Diluted coffee extract (0.16 ml) was mixed with

Folin-Ciocalteu's phenol reagent (0.01 ml) and allowed to stay for 8 min. 20% Na₂CO₃ solution (0.03 ml) was added and the mixture was incubated in a dark room for 2 h. Distilled water was used as blank instead of the coffee extract. The absorbance was measured at 765nm. Gallic acid solutions (200-1200 µg / ml) were used to generate a standard curve (R² = 0.997). The concentrations of polyphenols in all four coffee extracts were presented as mg gallic acid equivalent/ml of coffee extract (mg GAE/ml).

2.4.2 Total flavonoid content (TFC)

This assay was carried out with reference to Dewanto (2002). All the four coffee extracts (GCE, RCE, FGCE, and FRCE) were individually subjected to dilution to a ratio of 1:100 in distilled water. 1ml of each of this extract were then taken in test tubes, and 4 ml of distilled water was added into each test tube along with 0.3ml of NaNO₃ (50g/l). After the 5 mins reaction time, 0.3 ml AlCl₃(100g/l) was added. Further, they were incubated for 6 minutes and 2ml of NaOH (1 mol/l) was added along with 2.4 ml of distilled water. OD was measured at 510 nm with quercetin extract as standard. Stock quercetin solution (5 mg / ml) was prepared, then the standard solutions of quercetin were prepared by serial dilutions using water (200-1200 µg / mL). The concentration of flavonoids in all four coffee extracts, were presented as mg quercetin equivalent / mL of coffee extract (mg QE/ml) [20].

2.4.3 Antioxidant Activity (AA)

The FRAP assay was carried out in all four extracts (GCE, RCE, FGCE, FRCE) as described by Durmaz and Alpaslan (1950). Initially, 1 ml of the extract was mixed with 2.5 ml of phosphate

buffer (0.2 M/L, pH 6.6) and 2.5 ml of 1 % (w/v) K₃Fe(CN)₆ and incubated at 50 °C for 20 min. Then, 2.5 ml of 10 % (w/v) trichloroacetic acid was added and centrifuged (15,000×g, 10 min). After centrifugation, 2.5 ml of the upper layer was taken and mixed with 2.5 ml of water and 0.5 ml of 0.1 % (w/v) FeCl₃. The absorbance was measured at a wavelength of 700 nm. Obtained results were expressed as equivalents of Fe²⁺ (µM).

2.5 In-vivo Experiment

The study was conducted on twenty-four matured male *Wistar* strain male albino rats (Obtained from TANUVAS, Tamil Nadu, India), 3 months of age, weighing about 180 ± 5 g. Prior to the experiment, the animals were acclimated for a period of 15 days in laboratory conditions. Rats were housed in colony cages (6 rats per cage), at an ambient temperature of 25 ± 2 °C with 12 h light: 12 h dark cycle. Rats had free access to standard food and water *ad libitum*. CPCSEA is followed throughout the duration of the experiment.

2.5.1 Drug and treatment schedule

Aluminium chloride hexahydrate and the fermented coffee extracts (FGCE, FRCE) were prepared freshly, right before the induction. Rats administered with AlCl₃.6H₂O dissolved in Milli-Q water at dose 70 mg / kg I.P for 45 days [21]. The fermented coffee extracts are dissolved in Milli-Q water and administered at dose 600 mg / kg I.P for 45 days [22,23]. The twenty-four rats were divided into four groups. Each group had six rats for the experimental process, as follows:

Table 1. Grouping of animals

GROUP	DESCRIPTION
GROUP I (Control group)	Rats of this group were left unexposed to any drugs in order to preserve natural brain morphology
GROUP II (Alzheimer induced group)	Intra-peritoneal injection of AlCl ₃ (70 mg / ml water / kg body weight / rat) for 45 days.
GROUP III (FGCE Test group-1)	Co-Administration of AlCl ₃ and fermented green coffee bean extract (FGCE) (600 mg / ml water / kg body weight / rat) for 45 days. (AlCl ₃ was administered 30 minutes after the FGCE).
GROUP IV (FRCE Test group 2)	Co-Administration of AlCl ₃ and fermented roasted coffee bean extract (FRCE) (600mg / ml water / kg body weight / rat) for 45 days. (AlCl ₃ was administered 30 minutes after the FRCE).

2.6 Behavioural Assessment

Once a week, the condition of each animal was assessed by measuring body weight, food consumption, physical state, and clasping reflex. During and after treatment, animals were subjected to hippocampus-dependent spatial memory tests and motivation behaviour assay using the T maze and burrowing capacity test respectively. Three rats from each group were subjected to behavioural assessment by T-maze and the remaining three in each group underwent burrowing assay [24,25,26,27].

2.6.1 T-maze

The T-maze apparatus was shaped in form of the letter 'T', and it is structurally divided into 1 stem (50cm ×15cm) which consists of a sliding guillotine door 17 cm from the tip, the region between the tip and the gate is the starting point. A cloth screen was placed 5 cm from the end of the stem as a source of diversion. Finally towards the end, there are two arms, wherein each arm's end there is a food cup (50cm×10cm). Further, during the performance of the forced arm choice task and left-right discrimination assay, a detachable barrier was placed in the arm of choice for a transient period [24] (Fig. 1).

Prior to the analysis the rats were given only half its usual diet for a day and the maze was cleaned thoroughly in order to prevent external olfactory cues. The analysis by T-maze was performed in three distinct phases. To embark on, the rats were subjected to **habituation** (1 day) and **pre-training** phase for five times a day

(4 days). While in the habituation phase, the food-pellets were placed in every region of the maze (6 pellets) and were allowed to explore for 30 mins (no barrier). A day later, the rats were subjected to the pre-training phase where, the exploration was allowed in presence of barriers. And, once the pre-training phase was complete they were introduced to **training phase** for both working memory testing and reference memory testing by developing the ability to alternate between arms and make choices.

2.6.1.1 Forced alternation task (Working memory assay)

This task consisted of 9 trials (1.5 - 3 mins / trial / rat), each had a forced choice run (with barrier) and a free choice run (no barrier) (30 sec interval). During the forced trial, one baited arm was blocked. The guillotine door was lifted slowly, for the rat to enter, after which it is closed cautiously. Once it started moving along the stem, it passed through the curtain and while nearing towards the arm it realizes one arm was blocked and chooses the other arm. After the rat eats the pellet on one arm, it was brought back to the starting point. Followed by the forced choice run (after 5secs interval), the free choice run was done, where both the arm barriers are removed and the rat was allowed to make a choice. If it chose the arm opposite to the previously chosen arm chosen arm, then the choice was marked as a correct choice. The results are recorded as Working Memory Index (WMI) [24,25].

Working Memory Index (WMI) = Number of correct choices / Number of total trials

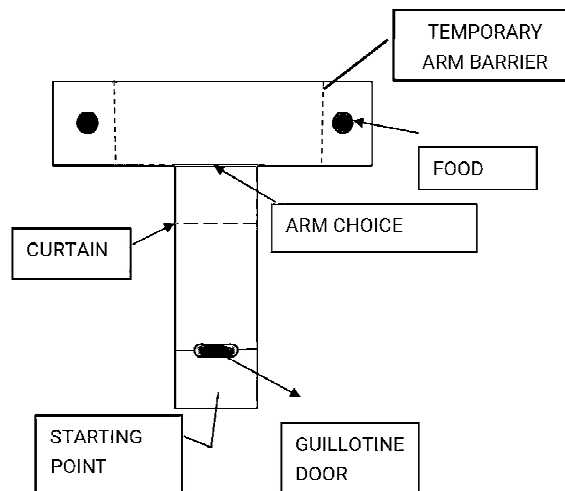


Fig. 1. Labelled T-maze layout [25]

2.6.1.2 Left-Right discrimination task (Reference memory assay)

The rats were subjected to 7 trials in this task, all the trials were free choice trials (1.5 - 2.5 mins / trial / rat). The goal arm was not varied throughout the trial. Also, once the rat entered the starting area, all the barriers were lifted and once the rat passed the curtain, it was allowed to make a free choice between the two arms. Once, the baited arm was chosen, and the rat eats the pellet, it is brought back to the starting point. During the next free choice trial, all the barriers were lifted and if the rat retained the correct goal arm in memory, then it is the right choice. Else, if the rat chooses the unbaited arm or if it gets distracted by the external cues, there is an error in reference memory. The results were recorded as Reference Memory Index (RMI).

Reference Memory Index (RMI) = Number of correct choices / Number of total trials [24,25].

2.6.2 Burrowing assay

The apparatus used was a plastic downpipe which was slightly elevated at one end and sealed at the other end to prevent accidental outpour of food pellets. Burrows made from plastic downpipe were of dimensions: 6 cm diameter cut into 20 cm long lengths. 5 cm long screws were used to elevate the other end of the tube off the floor. Two holes were drilled 1 cm from the open end of the burrowing tube and the screws were inserted perpendicularly and tightened. The burrowing assay was performed in three phases; initially the rats were acclimatized with the burrowing tube, where each group was provided with one tube. Once acclimatized, the rats were subjected to training and testing phase, where they were exposed to the burrowing activity individually in separate cages. The burrow is filled with 400g of pellets, and is left overnight (5pm to 9 am) then the results were recorded. To avoid overwhelming, the test was conducted once in five days, with habituation one day before. The results were recorded as Weight of pellet displaced. The results were analysed by Dunnet's multiple comparisons test [26,27].

Weight of pellet displaced = Weight of Pellets after experiment-Initial weight (200g)

2.7 Statistical Analysis

Data was expressed as the Mean \pm SD and statistical analysis was carried out by one way

ANOVA followed by Tukey multiple comparisons test for phytochemical assays of extracts and Dunnet's post-hoc multiple comparison test to calculate significance of the difference between treatments by behavioural analysis. P Values < 0.05, **P<0.01, and ***P<0.001 were considered to be statistically less significant, moderately significant, and highly significant respectively. And, the significant differences of each group were recorded in comparison to the control group. All statistical analyses were performed and graphs were sketched using Graph Pad Prism (ISI®, USA) software (version 8) computer program.

3. RESULTS AND DISCUSSION

3.1 Fermentation Characteristics of GCE, RCE, FGCE and FRCE

The evaluated fermentation characteristics showed the fermentation has taken place appropriately (Fig. 2). The pH of immersed coffee beans decreased from 7.02 at 0th hour, to 5.89 at 24th hour. This decrease in pH is due to increase in soluble organic acids produced by the fermentation such as Chlorogenic acid (CGA) [28]. The reducing sugar content in the medium did not change much over the period of fermentation. There was a definite increase in the number of colony forming units / ml of the fermentation media at 0th hour and 24th hour of inoculation, indicating proliferation of yeast cell and involvement in fermentation process.

3.2 Phytochemical Screening

3.2.1 Total polyphenol content (TPC)

There were significant differences between the TPC of all four extracts (GCE, RCE, FGCE, FRCE represented by a, b, c, d respectively) due to factors like roasting and fermentation. In decreasing order, the total phenolic content in the four extracts were: FGCE (1.20 \pm 0.02 GAE mg / ml of coffee); FRCE (0.99 \pm 0.047 GAE mg / ml of coffee); GCE (0.96 \pm 0.05 GAE mg / ml of coffee) and RCE (0.82 \pm 0.03 GAE mg / ml of coffee) (Fig. 3). The net phenolic content varies due to the following reasons (i) Fermentation produces soluble phenolic compounds, hence, strongly bound phenolic compounds in the cell wall tend to be weakened making them easier to extract. Hence the phenolic content tends to increase in the fermented coffee extracts [29,30]. (ii) With an increase in roasting temperature,

degradation of phenolic content occurs and gets associated with melanoidins; hence, the contribution of CGA to the antioxidant activity of the whole green coffee extract was higher than that of melanoidin-bound phenolic compounds in roasted extracts [31].

3.2.2 Total flavonoid content (TFC)

There were significant differences in flavonoid content of all four extracts (GCE, RCE, FGCE, FRCE represented by a, b, c, d respectively),

due to roasting and fermentation. In decreasing order, the total flavonoid content of coffee beans was FRCE (1.19 ± 0.025 QE mg / ml of coffee); FGCE (0.98 ± 0.065 QE mg/ml of coffee); RCE (0.92 ± 0.037 QE mg / ml of coffee) and GCE (0.79 ± 0.02 QE mg / ml of coffee) (Fig. 4). Yeast fermentation is effective in increasing the quantity of flavonoids in the coffee extracts as insoluble phenolic compounds are converted into soluble flavonoids during the process [32].

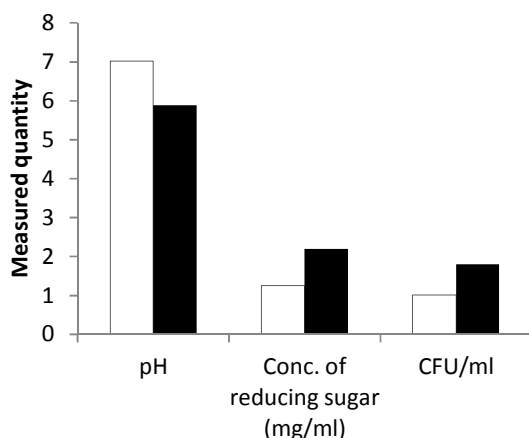


Fig. 2. Fermentation characteristics of green coffee beans (0th hour & 24th hour)
 □ indicates values taken at the start (0th hour) of fermentation and ■ indicates values taken at end (24th hour) of fermentation

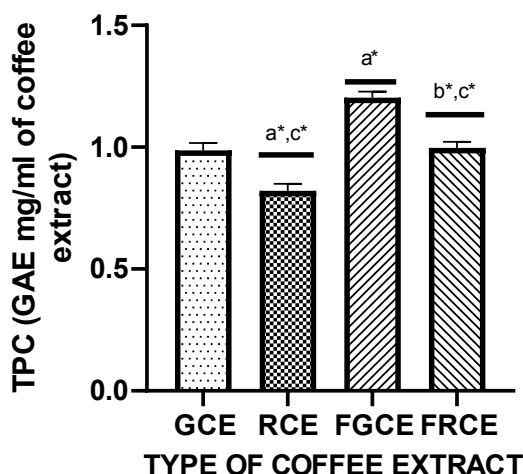


Fig. 3. Total polyphenol content (TPC) (GAE mg / ml of coffee extract)
 Values were represented as Mean values \pm SD from 3 repetitions. a, b, c, d: Significantly different from GCE, FGCE, RCE, and FRCE respectively at * $P < 0.05$ using one way ANOVA followed by Tukey multiple comparison test. □ Green Coffee Extract (GCE); ■ Roasted Coffee Extract (RCE); ▨ Fermented Green Coffee Extract (FGCE); ▩ Fermented Roasted Coffee Extract (FRCE)

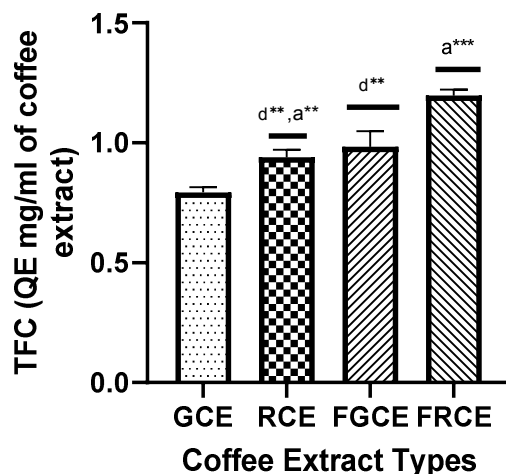


Fig. 4. Total Flavonoid content (TFC) (QE mg / ml of coffee extract)

Values are represented as Mean values \pm SD from 3 repetitions. a, b, c, d: Significantly different from GCE, FGCE, RCE, and FRCE respectively at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ using one way ANOVA followed by Tukey multiple comparison test. Green Coffee Extract (GCE); Roasted Coffee Extract (RCE); Fermented Green Coffee Extract (FGCE); Fermented Roasted Coffee Extract (FRCE) Also, the green coffee extracts ($P = 0.0016$) have lower flavonoid content compared to roasted ($P = 0.0002$), since there is an increase in flavonoid only with increase in thermal degradation

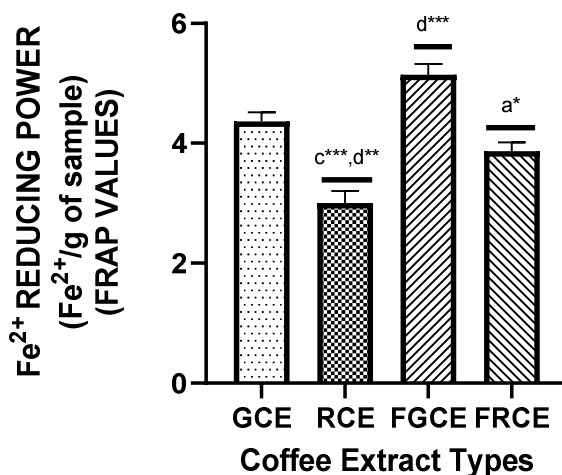


Fig. 5. Total Antioxidant Activity (AA) (Fe²⁺ g / ml of coffee extract)

Values are represented as Mean values \pm SD from 3 repetitions. a, b, c, d: Significantly different from GCE, FGCE, RCE, and FRCE respectively at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ using one way ANOVA followed by Tukey multiple comparison test. Green Coffee Extract (GCE); Roasted Coffee Extract (RCE); Fermented Green Coffee Extract (FGCE); Fermented Roasted Coffee Extract (FRCE)

3.2.3 Antioxidant Activity (AA)

All the coffee extracts showed remarkable differences in ferric reducing antioxidant potential (FRAP) (i.e., Radical scavenging activity) as a

result of fermentation. The FRAP values of all four extracts (GCE, RCE, FGCE, FRCE represented by a, b, c, d respectively), in decreasing order were: fermented green coffee extract (5.14 ± 0.17 mmol Fe²⁺ / g of sample);

green coffee extract (4.3 ± 0.15 mmol Fe^{2+} / g of sample); fermented roasted coffee extract (3.7 ± 0.2 mmol Fe^{2+} / g of sample) and roasted coffee extract (2.8 ± 0.04 mmol Fe^{2+} / g of sample) (Fig. 5). It was found that the FRAP values of roasted coffee extracts were lesser compared to that of the green coffee extract. This was due to the decrease of CGA content with the initiation of roasting at high temperature. Also, the fermented coffee extracts tend to show an increase in scavenging activity, due to the increase in antioxidant capacity compared to the non – fermented coffee extracts. Though the increase in antioxidant activity post-fermentation is almost equally significant in green coffee extracts

($P = 0.0038$) and roasted coffee extracts ($P = 0.007$), the overall contribution of Green coffee extracts were of higher value than melanoidin-bound phenols of roasted coffee [31].

The phytochemical profiling of all four extracts (GCE, RCE, FGCE, FRCE respectively) determines the increased polyphenol, flavonoid content and antioxidant activity in fermented coffee extracts (Table 2). Wherein the fermented green coffee extracts hold a higher content of polyphenol and exhibits higher antioxidant activity.

3.3 Behavioural Analysis

3.3.1 T-Maze Test

There was a significant day \times treatment (Control; AD Induced; AD+FGCE; AD+FRCE) interaction ($F(6, 16) = 6.208$, $P = 0.0016$) and a significant treatment effect of ($F(3, 8) = 42.71$, $P < 0.0001$) for the working memory. The reference memory resulted in a significant day \times treatment effect ($F(6, 16) = 10.17$, $P = 0.0001$) and a significant treatment effect of ($F(3, 8) = 24.33$, $P = 0.0002$). Significant differences were observed in both forced alternation task and left-right

discrimination task. The results of statistical analysis are presented in form of Mean \pm SD.

3.3.1.1 Forced alternation task (Working memory task)

The forced alternation task was performed in every group, wherein each group responded in distinctive manner. All the four groups were subjected to maze adaptation and trained where the control group could reach 80% working memory meanwhile the AlCl_3 induced AD mimic model showed drastic decline in WMI despite the training. During the testing period, alternation was observed to be frequent in control group, and its learning ability improved with time. The Alzheimer induced groups showed slight changes in learning tendency until the second week of induction, after which there was prominent decrease in the ability to retain memory and increase in fatigue were observed. On the fifteenth day of assessment, the AlCl_3 induced group showed lesser WMI (0.29 ± 0.06) when compared to the control group (0.66 ± 0.11), hence reaching a significant difference. Meanwhile, the FGCE treated group showed a WMI of 0.47 ± 0.06 indicating a significant difference in comparison to the AD induced group. But, the group treated with FRCE showed no significant difference when compared to AD induced group. The significant differences between FGCE treated vs. AD induced seemed to increase with time indicating the counteracting efficacy of FGCE. Meanwhile the FRCE treated group did retain memory slightly better than AD induced group, but the difference was not significant and the overall tendency to learn decreased with time. Since, the group treated with FGCE showed an increase in learning capacity with time, it is proving the neuroprotective function of the fermented green coffee extract. The statistical analysis is diagrammatically represented in (Table 3) and (Fig. 6).

Table 2. Phytochemical screening of the fermented and non-fermented coffee extracts

Extract	Total polyphenol content (GAE mg/ml of coffee)	Total flavanoid content (QE mg/ml of coffee)	Ferric reducing antioxidant potential (MMOL fe^{2+} /g of sample)
Green coffee extract	0.96 ± 0.05	0.79 ± 0.02	4.3 ± 0.15
Roasted coffee extract	0.82 ± 0.03	0.92 ± 0.037	2.8 ± 0.04
Fermented green coffee extract	1.20 ± 0.02	0.98 ± 0.065	5.14 ± 0.17
Fermented roasted coffee extract	0.99 ± 0.047	1.19 ± 0.025	3.7 ± 0.2

Table 3. Working Memory Index of all four groups of rats

	Control Group			Alzheimer Induced Group			FGCE+AD Treated Group			FRCE+AD Treated Group		
DAY 15	Correct choices	Total trials	WMI	Correct choices	Total trials	WMI	Correct choices	Total trials	WMI	Correct choices	Total trials	WMI
Rat 1	5	9	0.55	3	9	0.33	4	9	0.44	4	9	0.44
Rat 2	7	9	0.77	3	9	0.33	5	9	0.55	3	9	0.33
Rat 3	6	9	0.66	2	9	0.22	4	9	0.44	4	9	0.44
MEAN			0.66±0.11			0.29±0.06***			0.47±0.06**			0.40±0.06
DAY 30												
Rat 1	7	9	0.77	3	9	0.33	4	9	0.44	4	9	0.44
Rat 2	7	9	0.77	2	9	0.22	5	9	0.55	3	9	0.33
Rat 3	6	9	0.66	2	9	0.22	5	9	0.55	2	9	0.22
MEAN			0.73±0.06			0.25±0.06***			0.51±0.06***			0.36±0.08
DAY 45												
Rat 1	7	9	0.77	2	9	0.22	4	9	0.44	3	9	0.33
Rat 2	8	9	0.88	1	9	0.11	6	9	0.66	3	9	0.33
Rat 3	8	9	0.88	1	9	0.11	5	9	0.55	2	9	0.22
MEAN			0.84±0.05			0.14±0.06***			0.55±0.11***			0.29±0.11

The WMI values are expressed as Mean \pm SD ($n = 3$), $P < 0.05$, $**P < 0.01$, $***P < 0.001$ using two way ANOVA and Dunnet's multiple comparison test. Significant difference recorded by comparing AD induced vs. Control; Treated groups vs. AD induced

Table 4. Reference Memory Index of all four groups of rats

Control Group			Alzheimer Induced Group			FGCE+AD Treated Group			FRCE+AD Treated Group			
DAY 15												
	Correct choices	Total trials	RMI	Correct choices	Total trials	RMI	Correct choices	Total trials	RMI	Correct choices	Total trials	RMI
Rat 1	4	7	0.57	3	7	0.42	4	7	0.57	3	7	0.42
Rat 2	5	7	0.71	2	7	0.28	4	7	0.57	4	7	0.57
Rat 3	4	7	0.57	2	7	0.28	3	7	0.42	2	7	0.28
MEAN			0.61±0.08			0.32±0.08**			0.52±0.09*			0.42±0.14
DAY 30												
Rat 1	5	7	0.71	2	7	0.28	4	7	0.57	3	7	0.42
Rat 2	6	7	0.85	1	7	0.14	5	7	0.71	3	7	0.42
Rat 3	5	7	0.71	1	7	0.14	5	7	0.57	2	7	0.28
MEAN			0.8±0.08			0.18±0.08***			0.61±0.06**			0.37±0.07*
DAY 45												
Rat 1	6	7	0.85	1	7	0.14	4	7	0.57	2	7	0.28
Rat 2	6	7	0.85	1	7	0.14	5	7	0.71	3	7	0.42
Rat 3	5	7	0.71	0	7	0	4	7	0.71	1	7	0.28
MEAN			0.9±0.08			0.09±0.08***			0.66±0.08***			0.32±0.08*

The RMI values are expressed as Mean ± SD (n = 3), P < 0.05, **P < 0.01, ***P < 0.001 using two way ANOVA and Dunnet's multiple comparison test. Significant difference recorded by comparing AD induced vs. Control; Treated groups vs. AD induced

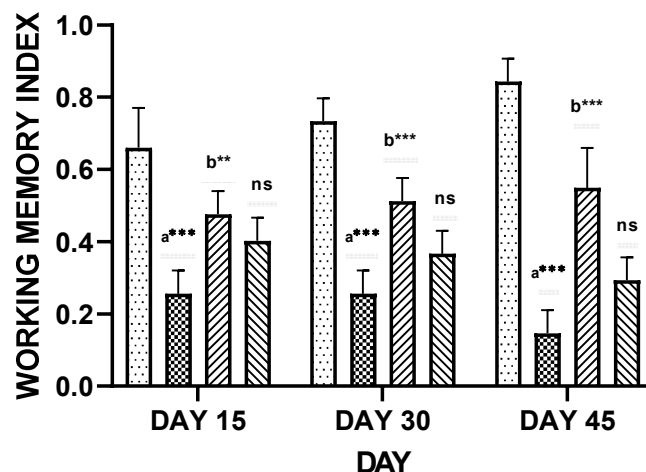


FIG. 6. Working memory index of control, ad induced, fgce treated and frce treated. fgce treated group, depicting improvement in wmi with time

Data expressed as Mean \pm SD ($n = 3$). a, b, c, d: Significantly different from Control, AD induced, FGCE Treated and FRCE Treated, FGCE treated group respectively at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ using two way ANOVA and Dunnett's multiple comparison test. Significant difference recorded by comparing AD induced vs. Control; Treated groups vs. AD induced. Control Group; AD Induced; FGCE + AD Treated Group; FRCE + AD Treated Group

3.3.1.2 Left-Right Discrimination task (Reference memory task)

The reference memory indices, changed gradually between treatments and with time for a 45 days' time period. The reference memory index of control group was significantly higher, as the control group rats could retain the choice of arm in memory for a prolonged duration with an RMI of upto 80%. And its ability to navigate through the maze was very swift and natural. Meanwhile, the AD induced group showed deterioration in reference memory and ability to navigate through cues. The deterioration pattern for AD induced group was slow during the first two weeks similar to the observation in working memory task. At the end of the second week of induction, the rats belonging to AD induced group reached a mean reference memory index (0.32 ± 0.08), which was almost 50% lesser than the mean RMI of the control group (0.61 ± 0.088), this contrast accounted for a significant difference ($P = 0.012$). On the other hand, the group treated with FGCE showed significant difference when compared to the AD induced group, there was a prominent increase in the significant difference and the reference memory index of FGCE treated group through the testing period. Meanwhile, the FRCE treated group did not show much significant difference when

compared to AD induced group, and despite the slight increase in RMI over time, there was no increase in learning ability. Since, during the last four weeks the rats underwent gradual decline in sensory processing, and most importantly they experienced fatigue. Similar to the working memory test, the group treated with FGCE showed neuroprotective effect, and reached a RMI value of 0.66 ± 0.08 at the end of six weeks. The FRCE treated group on the other hand reached an RMI value of 0.37 ± 0.07 . The results recorded as reference memory index are represented in form of a table (Table. 4) and chart (Fig. 7).

From the obtained working and reference memory indices, it can be inferred that the rats in all four groups were responsive to olfactory and sensory cues, but at different levels. These differences were observed despite the equal training duration, and controlled external cues including olfactory cues and spatial cues. The AD induced group, experienced gradual loss of navigation ability, where the rats instead of reaching for the food pellet, it tried returning back to the start area or spent prolonged time in the arm region. $AlCl_3$, which was injected to induce Alzheimer's disease result in free radical production, giving rise to cognitive decline and difficulty with task response in AD induced rats

[33,34]. Moreover, impairment of learning ability and spatial memory indicates the accumulation of $AlCl_3$ in different regions of the brain especially the hippocampus which is the domain for memory and learning [35,36]. Since, the ability to retain sensory cues in short term and long term memory was disrupted, the AD induced rats re-entered the previously chosen arm without alternation (forced alternation task) and showed behavioural fluctuation even in repeated trials (Left-right discrimination task). With time, the AD induced rats could not choose or alternate since it was tired and indecisive. Another main feature was fatigue and depression, especially during the last two weeks of analysis, since neuropsychiatric co-morbidities like depression during the course or development of AD is common, mainly due to disrupted dopaminergic pathway from VTA to hippocampus [37]. The groups treated with fermented coffee extracts, did not perform as efficient as the control group, but it showed protective effect, since they performed better than the AD induced group. The neuroprotective effect of coffee polyphenols (CPP) have been tested and proven evidently by several *in vivo* and *in vitro* studies, the main

polyphenol is chlorogenic acid (CGA) which is the major portion of CPPs. Also coffee polyphenols play a significant role in amyloid beta disintegration reducing cytotoxicity [38,39]. Fermented products eventually have more concentration of polyphenols [14,15,17] hence consumption of these antioxidant rich polyphenols, tends to improve cognition and memory [40]. The group administered with FGCE showed improvement in both working memory index (WMI) and reference memory index (RMI) with time, but in a gradual fashion, exhibiting therapeutic effects. Whereas, the group treated with FRCE did not show development in WMI and RMI but it showed a mild protective effect since the decline was not rapid as AD induced group. The free polyphenols like CGA in whole green coffee is proven to contribute more to the antioxidant activity of the extract than the melanoidin bound polyphenols that occur as a result of roasting. Therefore green coffee polyphenols possess better antioxidant activity and bioavailability than roasted [31]. The high concentration of CGA found in green coffee extract, not only improves memory function but also executive function [41].

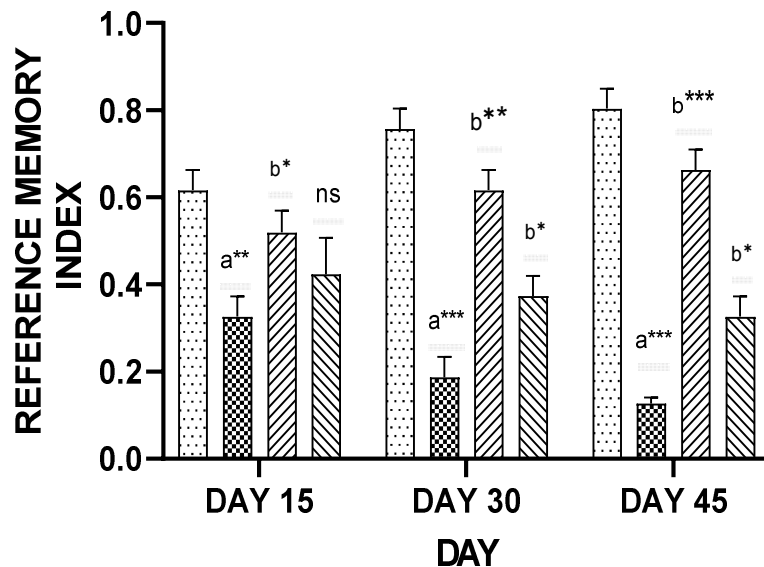


Fig. 7. Reference memory index of control, ad induced fgce treated and frce treated. fgce treated group, depicting improvement in rmi with time

Data expressed as Mean \pm SD (n = 3). a, b, c, d: Significantly different from Control, AD induced, FGCE Treated and FRCE Treated, FGCE treated group respectively at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ using two way ANOVA and Dunnet's multiple comparison test. Significant difference recorded by comparing AD induced vs. Control; Treated groups vs. AD induced. Control Group; AD Induced; FGCE + AD Treated Group; FRCE + AD Treated Group

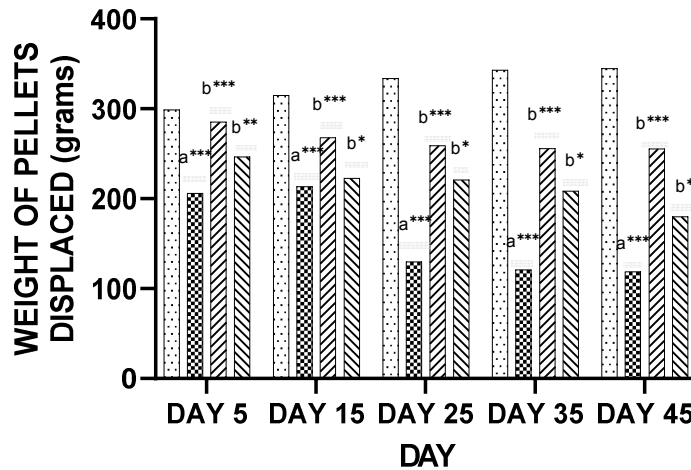


Fig. 8. Burrowing rate of control, ad induced, fgce treated and frce treated groups

Data is expressed in grams ($n = 3$). A, b, c, d: significantly different from control, ad induced, fgce treated and frce treated, fgce treated group respectively at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ using two way anova and dunnet's multiple comparison test. Significant difference recorded by comparing ad induced vs. Control; treated groups vs. Ad induced. control group; ad induced; fgce + ad treated group; frce + ad treated group

3.3.2 Burrowing Assay

The four groups responded quite similarly to the task, yet the rate of displacement increased with time in case of controls while it reduced drastically in the AD induced group as presented in Fig. 8. The fermented coffee treated groups showed better burrowing tendency than the AD induced group, the group treated with FGCE showed better results than FRCE treated group, there was not spontaneous decline in burrowing rates of treated groups, the decline was gradual in FRCE treated group, while the FGCE treated group showed constant rate during the last three weeks. Two-way repeated measures ANOVA showed a main effect of ($F(3, 12) = 30.74$, $P < 0.001$). The AD induced rats underwent attenuated burrowing rate with the progression of time, indicating the neurodegeneration process caused due to $AlCl_3$ induction. The $AlCl_3$ stimulated loss of motivation and depletion in dopamine, necessary for motor co-ordination [42]. The impairment in burrowing activity and reduced motivation was observed prominently at the end of two weeks, and was persistent in case of AD induced rats. The FGCE treated group with increased bio-availability of CGA must have counteracted the plaque and fibril formation caused by $AlCl_3$ [43]. Despite the mild protective effect of the FRCE induced group, the overall burrowing capacity was lesser than the FGCE induced group. Hence, the fermented green

coffee extracts seemed to exhibit better therapeutic efficacy by increasing organizational and executive function when it came to burrowing.

CONCLUSION

On the whole, considering the steady increase in the number of elderly citizens in society, there is an increase in search for dietary sources which can be ingested on a regular basis to extend mental agility rather than consuming medication with side effects. From several epidemiological studies conducted, it has been reported that there is a protective association between coffee intake with either decreased risk of cognitive decline or developing AD. Fermented coffee being a rich source of antioxidant, it can be beneficial to several health conditions when taken on a daily basis. In this study, fermented green coffee was proven to have better effect on AD induced rat than fermented roasted coffee. Evidenced by behavioural analyses, the neuroprotective effect of fermented green coffee was due to a high anti-oxidant activity contribution by coffee polyphenols, which also served as a better decelerating agent in Alzheimer's disease progression than the fermented roasted coffee polyphenols. Thus, the data of this research supports the potential nature of FGCE as a nutraceutical supplement or dietary beverage which could be conformed on further clinical investigations.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental study was conducted at Animal House Facility of Sri Venkateswara College of Engineering with the protocol approved by IAEC SVCE: Protocol 2 (Ref.: 9/IAEC/Dated 24th December 2019). Instruction given by Institutional Animal Ethical Committee IAEC was followed regarding injection and other treatment of the experiment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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