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Effect of botanical extracts containing carnosic acid or rosmarinic acid on learning and memory in SAMP8 mice

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ABSTRACT

Oxidative damage is one of the hallmarks of the aging process. The current study evaluated effects of two proprietary antioxidant-based ingredients, rosemary extract and spearmint extract containing carnosic acid and rosmarinic acid, respectively, on learning and memory in the SAMP8 mouse model of accelerated aging. The two rosemary extracts contained carnosic acid (60% or 10% carnosic acid) and one spearmint extract contained 5% rosmarinic acid. Three doses of actives in each extract were tested: 32, 16, 1.6 or 0 mg/kg. After 90 days of treatment mice were tested in T-maze foot shock avoidance, object recognition and lever press. Rosemary extract containing 60% carnosic acid improved acquisition and retention in T-maze foot shock, object recognition and lever press. Rosemary extract with 10% carnosic acid improved retention in T-maze foot shock avoidance and lever press. Spearmint with 5% rosmarinic acid improved acquisition and retention in T-maze foot shock avoidance and object recognition. 4-hydroxynonenal (HNE) was reduced in the brain cortex after treatment with all three extracts ($P < 0.001$) compared to the vehicle treated SAMP8. Protein carbonyls were reduced in the hippocampus after administration of rosemary with 10% carnosic acid ($P < 0.05$) and spearmint containing 5% rosmarinic acid ($P < 0.001$). The current results indicate that the extracts from spearmint and rosemary have beneficial effects on learning and memory and brain tissue markers of oxidation that occur with age in SAMP8 mice.

1. Introduction

As the number of elderly adults (>65 years of age) worldwide is expected to more than double by the year 2030 and individuals are staying in the workforce longer, the need to stay cognitively fit is increasing. Thus, the development of natural interventions aimed at slowing or preventing cognitive decline naturally associated with aging is critical. Botanical ingredients and nutrients have been investigated as a solution to this growing concern.

Oxidative damage is considered one of the hallmarks of the aging process [1]. The neuronal dysfunction present in diseases associated with aging such as Alzheimer's disease is thought in large part to be from oxidative stress [2–4]. Free radicals which lead to oxidative stress are thought to be responsible for aging [5]. Aging and its related diseases are consequences of free radical damage to cellular molecules and the inability of endogenous antioxidants to counter balance these changes. Oxidative stress leads to changes in the mitochrondria, membrane fatty acid composition, protein oxidation and inflammation [6].

The senescence-accelerated mouse (SAM) is a model of accelerated senescence that was established through phenotypic selection from a common genetic pool of AKR/J strain [7] SAMP8 mice have age-related impairment in learning and memory that coincides with increased levels of lipid and protein oxidation [8]. Antioxidants have been found to reverse learning and memory deficits in SAMP8 mice. Alpha-lipoic acid and *n*-acetyl cysteine enhance memory and reverse indices of oxidative stress in these mice [9]. Alpha-lipoic acid significantly decreased carbonyl levels of lactate dehydrogenase B, dihydro pyrimindase-like protein and alpha enolase [10].Plant extracts that contain antioxidants, such as extra virgin olive oil, have also been found to improve learning and memory in SAMP8 mice and reverse indices of oxidative stress [11].

Carnosic acid (CA) and rosmarinic acid (RA) are polyphenol plant extracts that have been found to be neuroprotective and preventative against oxidative stress [1,12,13]. CA has been shown to prevent inflammation and cell death [13,14]. CA prevents inflammation produced by lippopolysacchride administration [15] and cell death produced by 6-hydroxydopamine [16]. *Rosemarinus officinalis* L. leaf extract, which

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contains CA, has been found to inhibit acetylcholinesterase (AChE) activity [17]. What makes these antioxidants unique is that they are converted to their active forms by oxidative stress [18].

CA and RA have been reported to exert behavioral effects in rodents including memory enhancement, anti-stress and antidepressant like effects [19,20]. Protection against oxidative stress and inflammation has been associated with improved memory in diseases of aging [11]. RA improved memory in the Morris water maze spatial memory task and passive avoidance [17,21]. CA and RA reduced immobility in the tail suspension test and altering monoaminergic and cholinergic function and gene expression in the brain associated with depression [20].

The current study was designed to test if these two antioxidants found naturally in rosemary extract and spearmint extract, CA and RA, respectively, could improve learning and memory in the SAMP8 mouse model of accelerated aging. We have previously shown the ability of antioxidants to improve learning and memory deficits in the SAMP8 mice and reverse signs of oxidative damage [9,11]; therefore, the SAMP8 mice are an excellent model to test these antioxidant compounds [22].

2. Methods

2.1. Mice

At the start of treatment, the subjects for the experiments were 9 month old SAMP8 and 9-month old 50% SAM male mice from the breeding colony at the VA Medical Center, St. Louis. The SAMP8 were generously provided to our laboratory from Dr. T Takeda of Kyoto University in Japan in the late 1980′s and maintained as an inbred strain. The SAM 50% is a cross between a male SAMP8 and a female CD-1 mouse which do not show age-related impairment in learning and memory. Sentinels from the facility were tested regularly to ensure our facility was virus- and pathogen- free. Food (Richland 5001) and water were available on an ad libitum basis, and the rooms had a 12 h light-dark cycle with lights on at 0600 h. Experiments were conducted between 0730 and 1400 h. These studies were conducted at VA Medical Center, St. Louis and were approved by the Animal Care and Use Committee at the VA Medical Center, St. Louis, MO.

2.2. Treatment

Nine month old SAMP8 male mice received rosemary extract containing either 60% or 10% CA or spearmint extract containing 5% RA (all extracts obtained from Kemin Foods, L.C.) for 16 weeks once/day via oral gavage at approximately 0900 h ($n = 12$ per group at the start of the study). All extracts were tested for solvent, heavy metal, microbial and pesticide analysis. In addition, the rosemary extracts standardized for carnosic acid contained less than 0.1% rosmarinic acid while the spearmint extract with rosmarinic acid standardized for rosmarinic acid contained nondetectable levels of carnosic acid. In the first study, we examined the effect of 60% CA (CA60). The SAMP8 mice were randomly assigned to one of 4 treatment groups: 1.) CA60 - 32 mg/kg of carnosic acid, 2.) CA60 - 16 mg/kg carnosic acid, 3.) CA60 - 1.6 mg/kg and 4.) vehicle (sunflower oil). The control strain received 5.) vehicle (sunflower oil) treated 50%SAM. In the second study, we examined rosemary extract containing 10% CA (CA10). The SAMP8 mice were randomly assigned to one of 4 treatment groups: 1.) CA10–32 mg/kg of carnosic acid, 2.) CA10–16 mg/kg carnosic acid, 3.) CA60–1.6 mg/kg and 4.) vehicle (sunflower oil). The control strain received 5.) vehicle (sunflower oil) treated 50%SAM. The third study, we examined the effects of spearmint extract containing 5% rosmarinic acid (RA). The SAMP8 mice were randomly assigned to one of 4 treatment groups: 1.) RA - 32 mg/kg of rosmarinic acid, 2.) RA - 16 mg/kg rosmarinic acid, 3.) RA - 1.6 mg/kg rosmarinic acid and 4.) vehicle (water). The control strain received 5.) vehicle (water) treated 50%SAM.

Sunflower oil was used as the vehicle control for the rosemary extracts containing CA because sunflower oil was used in the standardization of the extract and water was used as the vehicle control for the spearmint extract with RA. All treatments were administered via oral gavage. Body weights were recorded weekly throughout the study.

2.3. Behavioral testing

Behavioral testing was started in the twelfth week of treatment. Mice were first trained in T-maze foot shock avoidance during week 12 (T-maze session I) and T-maze (session 2) was performed in week 13, followed by object recognition in week 13. Lever press was performed during weeks 14 and 15. All testing was performed and recorded by someone that was blinded to the treatment groups.

2.3.1. T-Maze training and testing procedures

The T-maze has previously been used by us to access learning and memory in the SAMP8 mouse [11,23–29]. The task requires an intact hippocampus to perform well. This is a hippocampal-dependent task, as damage to 30% of the hippocampus impairs both learning and memory performance in the T-maze using the same design as in the current study [30]. The T-maze is a complex task. The idea of the task is that the mice learn to avoid the shock by moving from the start box to the clear escape box located at the end of one arm before the start of the shock which occurs 5 s after the simultaneous opening of the door and the initiation of the buzzer. The T-maze consisted of a black plastic alley with a start box at one end and two arms at the other end. The start box was separated from the alley by a black plastic guillotine door that prevented movement down the alley until the door was raised by the investigator at the onset of training. A clear plastic liner was used as a "goal box". It was placed at the end of one of the two arms based upon conditions described below. An electrifiable floor of stainless steel rods ran throughout the maze to deliver a mild scrambled foot-shock.

Mice were not permitted to explore the maze prior to session 1. A block of trials was initiated when a mouse was placed into the start box. The guillotine door was raised and a cue buzzer sounded simultaneously; 5 s later foot-shock was applied. The arm of the maze entered on the first trial was designated "incorrect" and the mild foot-shock was continued until the mouse entered the other arm where the goal box had been placed at the end, this becomes the designated "correct" side for the goal box location for that particular mouse for the remainder of the experiment. At the end of each trial, the mouse is removed from the goal box and returned to its home cage until the next trial.

Mice were trained in session one until they succeeded in avoiding the foot shock one time. Avoidance of the shock occurs when once the buzzer has sounded and the door opened (simultaneously), and the mouse traversed down the alley entering the correct arm and into the goal box in less than 5 s. In session one, the latency to escape the shock on trial, as well as number of left/right errors for the entire session and the number of trials attempted until the mouse makes one avoidance of the shock were recorded. Training used an inter-trial interval of 35 s, the buzzer was a door-bell type which sounded at 55 dB, and shock was set at 0.35 mA (Coulbourn Instruments scrambled grid floor shocker model E13-08). A second T-maze session was performed one week later by continuing training until mice made 5 avoidances of the foot shock in 6 consecutive trials. The lower the number of trials to 5 avoidances during session 2 the greater retention of the information learned during session one. The results for the T-maze session two were reported as the number of trials to make 5 avoidances in 6 consecutive trials.

2.3.2. Object-place recognition

Object-place recognition is a memory task that involves the hippocampus when, as performed here, the retention interval being 24 h after initial exposure to the objects [11]. Mice were habituated to an empty apparatus for 5 min a day for 3 days prior to entry of the objects.

During the training session, the mouse was exposed to two similar objects (plastic frogs) which it was allowed to examine for 5 min. The apparatus and the objects were cleaned between each mouse. The time spent exploring the two objects was recorded. Twenty-four hours later, the mouse was exposed to one of the original objects and a novel object in a new location. The amount of time spent examining the objects was recorded. The percentage of time spent exploring the novel object based on total time spent exploring either object was reported. The novel object was made out of the same material as the original object and of the same size, but a different shape. This eliminated the possibility of the smell associated with a particular object being a confounding factor. The underlying concept of the task is based on the tendency of mice to spend more time exploring new, novel objects rather than familiar objects. Thus, the more time spent with the novel object the greater the retention/memory if the previously exposed object at 24 h.

2.3.3. Lever press for milk reinforcement

Mice were first habituated to milk the week prior to the lever press by removing food and water overnight for 3 nights and verifying that the mice drank at least 15 ml by the morning on the third night. Any mouse not drinking milk was not used in the lever press. Lever press is a procedural (operant) associative learning and memory task. Mice were placed into a fully automated lever press chamber. Pressing a lever on one wall of the compartment caused a light and an arm with a liquid dipper with 100 μl of milk to appear in an opening in the wall where is can place its head located on the opposite wall. Prior to the start of testing on day one only, the mouse is placed in the apparatus and the lever is activated by the investigator for the mouse to learn the food cup when visible contains milk. Once the mouse has drunk the milk one time the session begins. The pretrial training on the availability of milk does not train the mouse how to press the lever to activate the arm which rises up into the box displaying the food cup. On day 1 the mice had 11 s to obtain the reward; on all subsequent days, mice had 6 s obtain the reward once it had press the lever. The reward availability was on a fixed ratio schedule, one press of the lever leads to one 100 μl reward (FR1) for the entire study. Mice were given 40 min training sessions on M, W, F for two weeks. Mice were food deprived 16 h prior to the start of the test to provide motivation to perform the task. Food was returned immediately upon completion of a session. The number of rewarded level presses was recorded by Graphic State 2, Coulbourn Instruments (Whithall, PA). Acquisition was measured as the number of rewarded lever presses [22].

2.4. Oxidative stress

2.4.1. Sample preparation

Brain samples were briefly homogenized with a Wheaton tissue homogenizer in an ice-cold lysis buffer (pH 7.4) containing 320 mM sucrose, 1% mM Tris-HCl (pH 8.8), 0.098 mM MgCl2, 0.076 mM EDTA, and proteinase inhibitors leupeptin (0.5 mg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 mg/ml) and PMSF (40 μg/ml) and a phosphatase inhibitor cocktail. The homogenized samples were then diluted 2× with lysis buffer. After homogenization and dilution, a small aliquot of homogenized samples were sonicated for 10 s at 20% power with a Fisher 550 Sonic Dismembrator (Pittsburgh, PA, USA) and frozen. The remaining homogenate was centrifuged at 3000*g* for 5 min and the supernatant cytosolic and membranous fractions were transferred out into another set of tubes. Following the addition of 400 μl of lysis buffer, the remaining pellet nuclear fraction was centrifuged at 3000 x g for 5 min and supernatant removed. The pellet was suspended in 20 μl of lysis buffer and inhibitor. The supernatant cytosolic and membranous fractions were centrifuged at 10,000 ×*g* for 10 min, and the resulting supernatant cytosolic fraction was transferred out into another set of tubes leaving the pellet membranous fraction. All sonicated samples and fractions were stored at −70 °C until used for further experiments. Protein concentrations were measured through Pierce Bicinchoninic Acid (BCA) method.

2.4.2. Slot blot analysis

2.4.2.1. Protein carbonyls. For protein carbonyl detection, slot blot analysis of the 2,4-dinitrophenyl hydrazone (DNP) Schiff-base adduct of the carbonyls was employed. Sample aliquots of 5 μl were incubated at room temperature with 5 μl of 12% sodium dodecyl sulfate and 10 μl of 2,4-dinitrophenylhydrazine (from OxyBlot™ Protein oxidation kit, Chemicon-millipore, Billerica, MA, USA) for 20 min, followed by the addition of 7.5 μl of neutralization solution containing Tris (2 M) in 30% glycerol to each sample. Following derivatization samples were diluted to 1 μg/ml using 1× phosphate buffer solution (PBS) containing sodium chloride, mono, and dibasic sodium phosphate. The corresponding sample solutions (250 μl) were rapidly loaded as duplicates onto a nitrocellulose membrane through water vacuum pressure. The resulting protein-bound nitrocellulose membrane was then blocked with fresh blocking solution containing 750 mg of bovine serum albumin (BSA) in 25 ml of wash blot containing 35.2 g sodium chloride, 1.77 g monobasic sodium phosphate, 9.61 g dibasic sodium phosphate and 1.6 ml TWEEN, diluted to 4 L with deionized water for 90 min. The membrane was then incubated with polyclonal Rb × DNP (from OxyBlottm Protein oxidation kit, Chemicon-millipore, Billerica, MA, USA, dilution 1:100) in wash blot for 2 h. After three 5 min washes with fresh wash blot, the membrane was then incubated with polyclonal anti-rabbit IgG alkaline phosphatase (Chemicon, Temecula, CA, USA, dilution 1:8000) for 1 h and washed with fresh wash blot in three increments of 5, 10 and 10 min. After washing, the membrane was developed colorimetrically using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium reagent solution for alkaline phosphatase secondary antibody. After development, blots were dried and scanned on a CanoScan8800F (Canon) scanner using Adobe Photoshop and analyzed using Scion Image software (Scion Corporation).

2.4.3. 4-Hydroxy-2-trans-noneal (HNE)

Levels of protein-bound HNE are used as a marker of lipid peroxidation and were determined as previously described [29]. For slot blot analysis of protein-bound HNE detection, sample aliquots of 5 μl were incubated at room temperature with 5 μl of 12% sodium dodecyl sulfate and 10 μl of Laemmli buffer for 20 min, followed by dilution to 1 μg/ml using 1× phosphate buffer solution (PBS) containing sodium chloride, mono, and dibasic sodium phosphate. The corresponding sample solutions (250 μl) were rapidly loaded as duplicates onto a nitrocellulose membrane through water vacuum pressure. The resulting protein-bound nitrocellulose membrane was then blocked with fresh blocking solution for 90 min. The membrane was then incubated with polyclonal anti-HNE (Alpha diagnostic, San Antonio, TX, USA, dilution 1:5000) in wash blot for 2 h. After three 5 min washes with fresh wash blot, the membrane was then incubated with polyclonal anti-rabbit IgG alkaline phosphatase (Chemicon, Temecula, CA, USA, dilution 1:8000) for 1 h and washed with fresh wash blot in three increments of 5, 10 and 10 min. After washing, the membrane was developed colorimetrically using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium reagent solution for alkaline phosphatase secondary antibody. After development, blots were dried and scanned on a CanoScan8800F (Canon) scanner using Adobe Photoshop and analyzed using Scion Image software (Scion Corporation).

2.4.4. 3-Nitrotyrosine (3-NT)

3-NT levels were used as an additional marker of protein oxidative damage [5,28]. Samples (5 μl) were incubated at room temperature for 20 min in 5 μl of 12% SDS and 10 μl of Laemmli buffer (0.125 M Trizma base, 4% SDS, 20% glycerol), for 20 min. Samples (250 ng of protein) per slot were blotted onto a nitrocellulose membrane. A primary rabbit antibody (Sigma-Aldrich) specific for 3-NT (1:1000) was used.

The same secondary goat anti-rabbit (Sigma-Aldrich) antibody was then used for detection of each primary antibody. Blots were developed and quantified as described above for protein carbonyls. The developing and detection were performed as described above for protein carbonyls.

2.4.5. Triglycerides

We have previously shown that elevated triglycerides are associated with impaired learning and memory [22]. Serum triglyceride was quantitated using an enzymatic assay system from Pointe Scientific, Inc. (Canton, MI) which incorporated a linear, endpoint color reaction. Triglycerides in the sample are hydrolyzed by lipase to glycerol. The glycerol is then phosphorylated by glycerol kinase and ATP to glycerol-3-phosphate (G_3P) and ADP. The G_3P is converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine (4-AAP) and 3-hydroxy-2,4,6-tribomobenzoic acid (TBHB) in a reaction catalyzed by peroxidase to yield a red colored quinoneimine dye. The intensity of color produced was measured at 540 nm using a Bio-Rad microplate reader (Hercules, CA).

2.5. Statistics

Results were analyzed using analysis of variance (ANOVA) to examine the effect among groups. The measure of acquisition and retention in the T-maze were the number of trials to reach criterion. The results for object recognition are presented in percentage of time spent exploring the novel object out of total exploration time. Lever press was analyzed by a two-way repeated measures ANOVA. Results are expressed as means ± standard errors. Dunnett's or Tukey's post hoc analyses were used as indicated in the results section. Tukey's post hoc analyses were used to compare means in the two-way ANOVAs. Dunnett's was used after one-way ANOVAs for comparison to the SAMP8 vehicle control group. The brain tissue oxidative stress parameters were analyzed using a Mann-Whitney *U* test.

3. Results

3.1. Rosemary extract containing 60% carnosic acid (CA60)

3.1.1. Behavioral testing

3.1.1.1. T-maze. The one-way ANOVA for trials to first avoidance in session one for CA60 produced a significant treatment effect $F(4, 48)$ 8.98, $P < 0.001$ (Fig. 1a). Dunnett's post hoc test indicated that the SAMP8 mice that received 32 mg/kg CA60 took significantly fewer trials to make one avoidance than the mice that received vehicle. One-way ANOVA for latency to escape shock on trial 1 was not significant $F(4,47) = 1.12$, *p NS*. The number of side choice errors by treatment during session 1 was not significant $F(4,47) = 2.39$, *p NS*. The one-way ANOVA for trials to criterion in the T-maze session 2 showed a significant treatment effect $F(4,47) = 7.25$, $P < 0.001$ (Fig. 1d). Dunnett's post hoc analysis indicated that the SAMP8 mice that received 32, 16

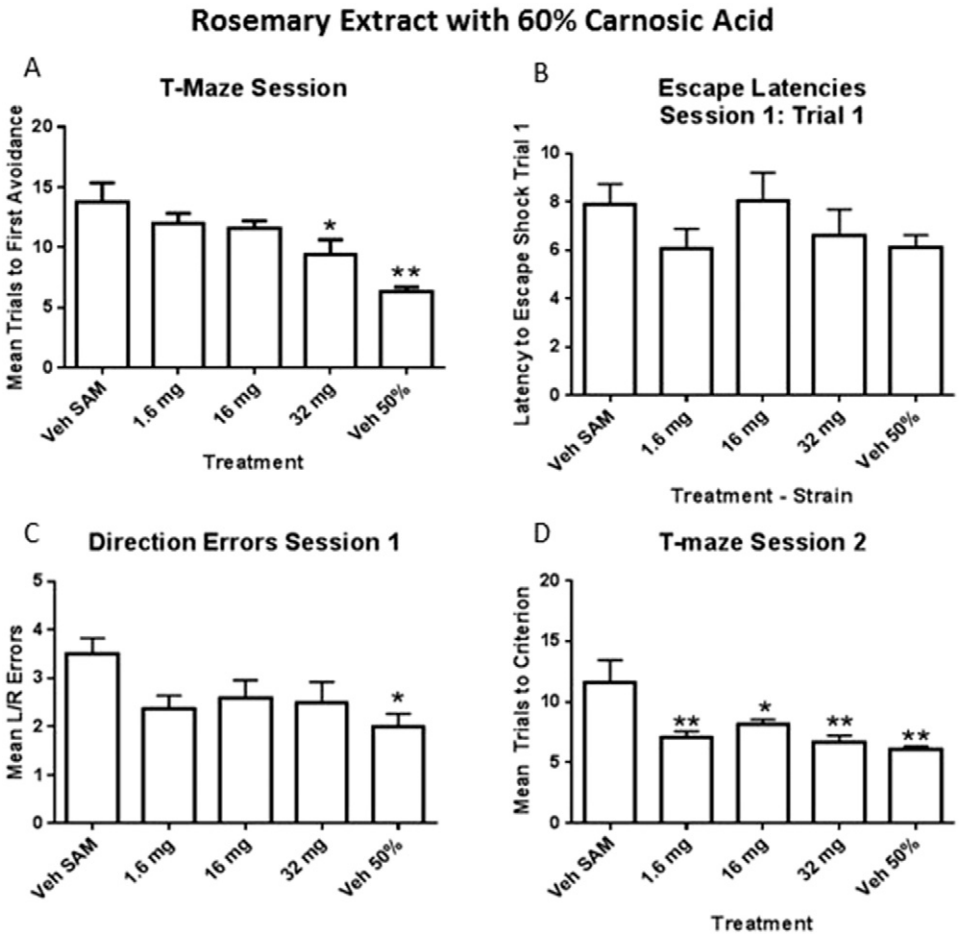

Fig. 1. Effects of rosemary extract containing 60% carnosic acid on T-maze foot shock avoidance. Rosemary extract containing 60% carnosic acid had a greater impact on memory in session 2 than learning in session 1 with only 32 mg/kg CA60 improving performance in session 1 (a) whereas 1.6, 16 and 32 mg/kg CA60 improved performance in session 2 compared to the SAMP8 vehicle controls (d). There was no effect on escape time in trial one of session one suggesting that the extract had no effect on either the ability to detect shock nor the motivation to escape it (b). There was also no effect on the number of trials it took each group to learning the correct side of the maze with the location of the escape box suggesting that the temporal component was the most difficult to learn and remember (c). The * indicates $P < 0.05$ and ** indicates $P < 0.01$. The values represent means + SEM.

and 1.6 mg/kg CA60 took significantly fewer trials to reach criterion than the SAMP8 mice that received vehicle.

3.1.1.2. Object recognition. The one-way ANOVA for total time spent exploring the two similar objects on day 1 was not significant $F(4,46) = 1.33$, *p NS* (Fig. 2a). The one-way ANOVA for time spent exploring the novel object on the 24 h retention test produced a significant treatment effect $F(4,46) = 4.88$, $P < 0.003$ for mice administered CA60 (Fig. 2b). Dunnett's post hoc test indicated that the SAMP8 mice which received 32 and 16 mg/kg CA60 spent significantly greater amount of time exploring the novel object than the SAMP8 mice which received vehicle $P < 0.05$. In addition, the SAMP8 mice which received 32 and 16 mg/kg CA60 were not significantly different from each other or the SAM 50% controls.

3.1.1.3. Lever press. The two-way repeated measures ANOVA, treatment x day, for number of rewarded lever presses produced a significant effect for treatment $F(4257) = 17.27$, $P < 0.001$ and day $F(5257) = 15.31$, $P < 0.001$ for mice administered CA60 (Fig. 2c). The interaction treatment x day was not significant $F(20,257) = 3.78$, $P > 0.05$. Tukey's post hoc analysis indicated that on days 3, 4, 5 and 6, SAMP8 mice which received 32 mg/kg CA from CA60 received significantly more rewards than the SAMP8 mice which received sunflower oil (vehicle). The SAMP8 mice which received 16 mg/kg CA60 received significantly more rewards on days 4, 5, and 6 compared to the SAMP8 mice which received vehicle.

3.1.2. Triglyceride levels

The one-way ANOVA for triglyceride levels was not significant $F(4,46) = 2.35$, $P > 0.05$ for mice administered CA60 (Fig. 2d).

3.1.3. Oxidative stress

Mann-Whitney *U* test indicated that CA60 significantly decreased 4-hydroxynonenal (HNE) in the cortex at 1.6 and 32 mg/kg CA from CA60 in comparison to SAMP8 administered sunflower oil as a vehicle control (Table 1). There was no significant effect of CA60 on 3-nitrotyrosine (3-NT) or protein carbonyls within the cortex. CA60 significantly increased protein carbonyls in the striatum at 16 mg/kg CA60, but had no effect on HNE or 3-NT. CA60 significantly increased protein carbonyls in the hippocampus at 32 and 16 mg/kg CA60. CA60 had no effect on 3-NT in the hippocampus in SAMP8 mice compared to the vehicle treated SAMP8.

3.2. Rosemary extract containing 10% carnosic acid (CA10)

3.2.1. Behavioral testing

3.2.1.1. T-maze. The one-way ANOVA for trials to criterion during session 1 in the T-maze produced a significant effect for group $F(4, 44)$ $F = 5.914$, $P < 0.001$ (Fig. 3a). Dunnett's post hoc test indicated that the 50% SAM mice that received the vehicle took significantly fewer trials to reach criterion than the SAMP8 mice which received 32, 1.6 mg/kg CA10 or the vehicle. The ANOVA for latencies to escape shock on trial 1 of session 1 was not significant $F(4,44) = 0.35$, P *NS*. The ANOVA for side choice errors during session 1 produced a significant effect $F(4,44) = 2.58$, $P < 0.05$. Dunnett's post hoc test indicated that the 50% SAM mice made significantly fewer side choice errors than the SAM Veh mice. There were no differences between the SAM Veh and any of the SAM mice receiving the dietary supplements. The ANOVA for trials to criterion during session 2 of the T-maze retention test indicated a significant effect of treatment $F(4, 44) = 4.04$, $P < 0.007$ (Fig. 3d) following administration of CA10. Dunnett's post hoc test indicated that the mice which received 16 mg/kg CA10 and the 50% SAM that received vehicle took significantly fewer trials to reach criterion than the SAMP8

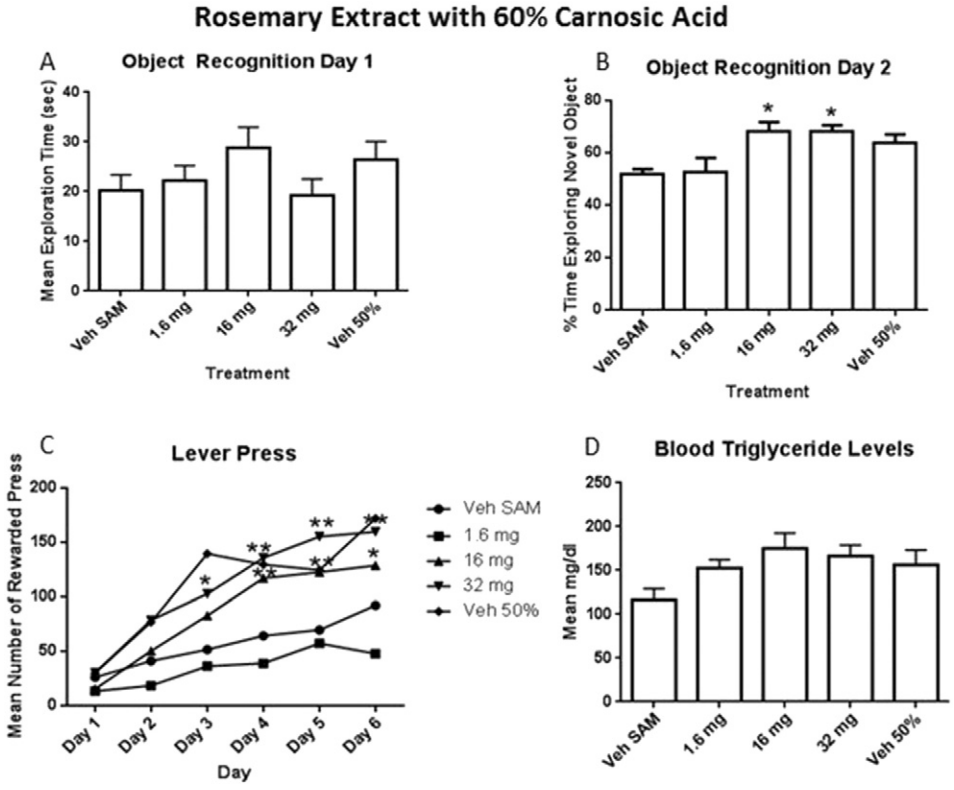

Fig. 2. The effects of rosemary extract containing 60% caronic acid on retention object recognition and learning in the operant lever press. Rosemary extract containing 60% carnosic acid had no effect on exploration time on day 1 with 2 like objects in novel object recognition (a). The extract improved object recognition retention on day 2, the mice that received 16 and 32 mg/kg spent significantly more time with the novel object compared to the SAMP8 mice that received vehicle (b) and operant learning in the lever press (FR1) on day 3, 4 and 5 at 32 mg/kg CA60 and days 4 and 5 at 16 mg/kg compared to the SAMP8 vehicle control (c). The * indicates $P < 0.05$ and the ** indicates $P < 0.01$ compared to the Veh SAM. Values represent means + SEM in object recognition. Only the means are represented in the lever press for graph readability.

Table 1
The levels of 4-hydroxynoneal (HNE), 3-nitrotyrosine (3-NT) and protein carbonyls (PC) in the cortex, striatum and hippocampus after chronic treatment with extracts containing carnosic and rosmarinc acid in 12 month old SAMP8 mice receiving treatment compared to the vehicle treated 12 month old SAMP8 mice. The * indicates $P < 0.05$, the ** indicates $P < 0.01$ and the *** indicates $P < 0.001$. Values represent means.

	1.6 mg/kg	16 mg/kg	32 mg/kg
Rosemary containing 60% carnosic acid			
Cortex			
HNE % controls	80.6***	91.8	74.3*
3-NT % controls	92	106	103
PC % controls	96	99	99
Striatum			
HNE % controls	104	104	107
3-NT % controls	99	99	90
PC % controls	100	115*	108
Hippocampus			
3-NT % controls	93	87	94
PC % controls	104.7	106.1*	113.4***
Rosemary extract containing 10% carnosic acid			
Cortex			
HNE % controls	98.4	98.2	83***
3-NT % controls	95	78**	100
PC % controls	107	107*	115**
Striatum			
HNE % controls	112	96	98
3-NT % controls	95	97	88
PC % controls	93	107	94
Hippocampus			
3-NT % controls	99	88.3**	96.5
PC % controls	99	88***	97
Spearmint extract containing 5% rosmarinic acid			
Cortex			
HNE % controls	100	88***	93.5
3-NT % controls	98	100	90.2***
PC % controls	103	115	108
Striatum			
HNE % controls	87	99	92
3-NT % controls	88	126	138
PC % controls	129*	132*	124
Hippocampus			
3-NT % controls	106.5	89.2*	103.2
PC % controls	106.5	89*	103

mice which received vehicle. The mice that received 1.6 and 32 mg/kg CA were not significantly different than the SAMP8 mice that received vehicle.

3.2.1.2. Object recognition. The one-way ANOVA for total time spent exploring the two similar objects on day one was significant $F(4,44) = 3.63$, $P < 0.01$. Dunnett's post hoc test indicated that there were no differences in exploration time between the SAM Veh group and any of the other groups The one-way ANOVA for time spent exploring the novel object was not significant $F(4, 44) = 2.249$; *p NS* (Fig. 4b) following administration of CA10.

3.2.1.3. Lever press. The two-way repeated measures ANOVA, treatment x day, for number of rewarded lever presses produced a significant effect for treatment $F(4253) = 6.74$, $P < 0.001$ and day $F(5253) = 7.53$, $P < 0.001$ (Fig. 4c) for mice administered CA10. The interaction treatment x day was not significant $F(20,253) = 1.83$, $P > 0.05$. Tukey's post hoc analysis indicated that on days 3, 4, 5 and 6, mice, which received 32 mg/kg and 16 mg/kg CA from CA10 sought significantly more rewards than the mice which received vehicle.

3.2.2. Triglyceride levels

The one-way ANOVA for triglyceride levels indicated a significant effect $F(4,44) = 2.93$, $P < 0.02$ following administration of CA10. Dunnet's post hoc test indicated that there was not a significant difference between any of the groups that received CA10 or the SAM50% and the SAMP8 mice that received vehicle (Fig. 4d).

3.2.3. Oxidative stress

The Mann-Whitney *U* test indicated that CA10 significantly decreased HNE in the cortex at 32 mg/kg CA, 3-NT in the cortex at 16 mg/kg CA and significantly increased protein carbonyls at 16 and 32 mg/kg CA in comparison to SAMP8 vehicle treated controls. CA10 had no effect on HNE, 3-NT or protein carbonyls in the striatum. Finally, CA10 significantly decreased protein carbonyls and 3-NT at 16 mg/kg CA in the hippocampus compared to vehicle treated SAMP8 controls.

3.3. Spearmint extract containing 5% rosmarinic acid (RA)

3.3.1. Behavioral testing

3.3.1.1. T-maze. The one-way ANOVA for trials to criterion on the T-maze acquisition test showed a significant treatment effect $F(4, 52)$ 6.38, $P < 0.001$ (Fig. 5a) following administration of RA in spearmint Extract. Dunnett's post hoc test indicated that the SAMP8 mice that received 32 and 16 mg/kg RA took significantly fewer trials to reach criterion than the SAMP8 mice that received vehicle. The one-way ANOVA for escape latencies on the first trial was not significant $F(4,50) = 0.36$, *p NS*. The one-way ANOVA for number of direction errors was not significant $F(4, 50) = 1.08$, *p NS*. The one-way ANOVA for trials to criterion on the T-maze retention test showed a significant treatment effect $F(4,50) = 12.77$, $P < 0.001$ (Fig. 5d). Dunnett's post hoc analysis indicated that the SAMP8 mice that received 32, 16 and 1.6 mg/kg RA took significantly fewer trials to reach criterion than the SAMP8 mice that received vehicle.

3.3.1.2. Object recognition. The one-way ANOVA for time spent exploring two like objects on day one was not significant $F(4,47) = 1.71$, *p NS*. The one-way ANOVA for time spent exploring the novel object on the 24 h retention test produced a significant treatment effect $F(4,47) = 2.79$; $P < 0.03$ (Fig. 6b) following administration of RA in spearmint Extract. Dunnett's post hoc test indicated that the mice which received 32 and 16 mg/kg RA spent significantly greater amount of time investigating the novel object than the SAMP8 mice which received vehicle.

3.3.1.3. Lever press. The two-way repeated measures ANOVA, treatment x day, for number of rewarded lever presses produced a significant effect for treatment $F(4257) = 6.18$, $P < 0.001$ and day $F(5257) = 40.98$, $P < 0.001$ (Fig. 6d). The interaction treatment × day was not significant $F(20,257) = 2.44$, $P > 0.05$. Tukey's post hoc analysis indicated there was no significant difference between the SAMP8 mice that received RA from spearmint Extract and the SAMP8 mice that received vehicle (Fig. 6c).

3.3.2. Triglyceride levels

The one-way ANOVA for triglyceride levels after treatment produced a significant effect $F(4, 47) = 5.06$, $P < 0.001$ following administration of RA from spearmint extract. Dunnett's post hoc analysis indicated that the 50% SAM control mice had significantly higher triglyceride levels compared to the SAMP8 mice that received vehicle (Fig. 6d).

3.3.3. Oxidative stress

Mann-Whitney *U* test indicated that RA in spearmint Extract significantly decreased HNE in the cortex of mice that received 16 and 32 mg/kg and decreased 3-NT at 32 mg/kg. RA had no effect on protein carbonyls in the cortex. In addition, RA had no effect on HNE or 3-NT within the striatum. Mice that received RA had significantly higher protein carbonyl levels at 1.6 and 16 mg/kg compared to the vehicle treated control SAMP8 mice within the striatum. RA significantly reduced levels of 3-NT and protein carbonyls in the hippocampus at 16 mg/kg.

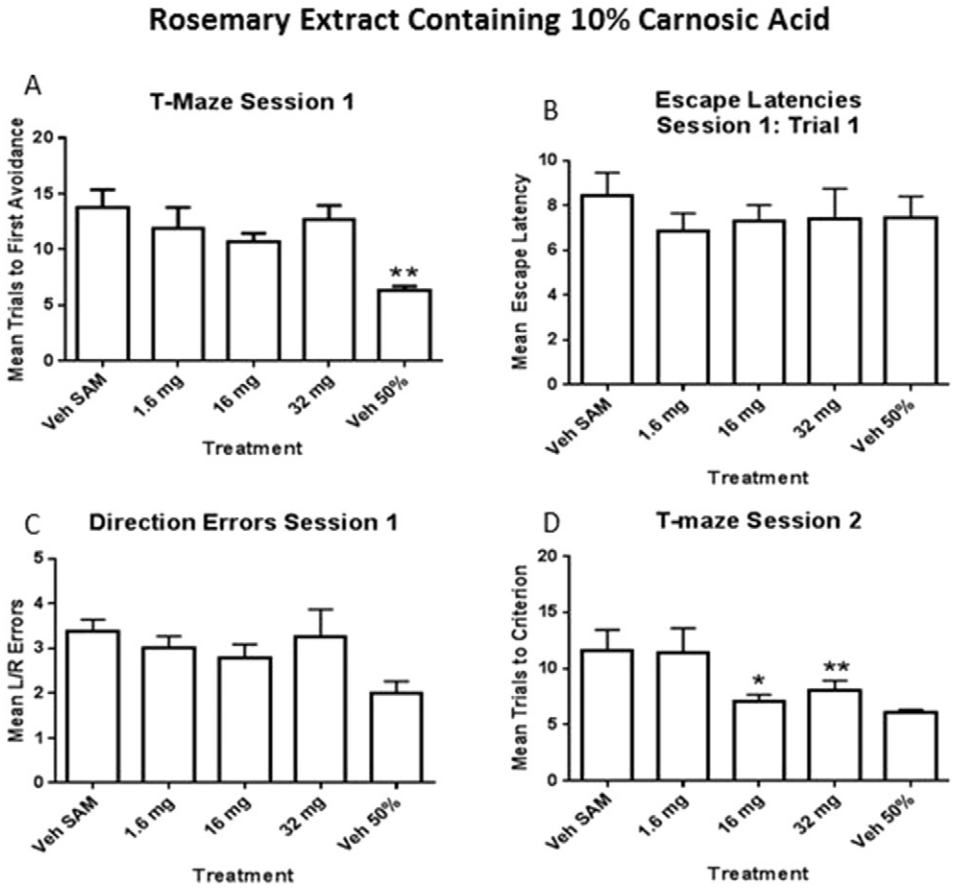

Fig. 3. Effects of rosemary extract containing 10% carnosic acid (CA10) on T-maze foot shock avoidance. Rosemary extract containing CA10 had no effect on T-maze performance in session 1 (a). CA10 improved T-maze performance in session 2 at 16 mg/kg (d). There was no difference in escape latencies during Trial 1 session 1 (b). There was a difference in number of direction errors with any of the extract doses compared to the Veh SAM during session 1. There was a difference between Veh SAM and Veh 50% mice which took significantly fewer trials to learn which direction (L/R) compared to the Veh SAM mice (c). The * indicates $P < 0.05$ and the ** indicates $P < 0.01$ compared to the vehicle treated SAMP8 mice. Values represent means + SEM.

4. Discussion

In the current study all the extracts tested, spearmint extract containing RA (5%) and rosemary extract containing two levels of CA (60% and 10%) improved learning and memory. While both the 10 and 60% CA extract treatments significantly improved T-maze session two and level press test results, only the 60% CA extract treatment showed significant improvements in session 2 of the T-maze and object recognition. There was no difference in latency to escape the shock on the first trial of T-maze indicating that the extract did not affect motivation to escape a noxious stimulus. There no difference on day one of object recognition in exploration time indicating each group was equally habituated to the initial object when presented in the 24 h retention test. The spearmint extract with RA improved acquisition in the T-maze and memory in object recognition memory. There was no difference in latency to escape the shock on the first trial of T-maze indicating that the extract had no effect on motivation to escape a noxious stimulus. There no difference on day one of object recognition in exploration time indicating each group was equally habituated to the initial object when presented in the 24 h retention test. There was no difference in triglyceride level in the SAMP8 mice that received vehicle and the SAMP8 mice that received an extract. These findings indicate that the extracts were not improving memory by lowering triglycerides in the SAMP8 mice. We have previously reported that elevated triglycerides can impair memory in mice [22]. All three compounds decreased HNE in the cortex and protein carbonyls were reduced in the hippocampus following the administration of the 10% CA and the 5% RA extracts. None of the compounds had an effect on body weight or triglyceride levels. These studies suggest that the current novel extractions of carnosic and rosmarinic acid from rosemary and spearmint are natural products that are beneficial at preventing learning and memory deficits associated a mouse model of accelerated aging.

Both extracts worked in a dose-response fashion on memory. This is not surprising as most memory enhancing compounds demonstrate hormesis [31]. Hormesis is the phenomena where there is an optimal dose for memory enhancement of memory whereas doses that are too high may have a negative effect. This was previously reported in water maze testing with RA and in its anxiolytic ability as tested in an elevated plus maze [21,31]. This study used a "stress" condition in a normal healthy, non-aging mouse model and found a cognitive-enhancing capability. RA was also shown to work in models of Aβ toxicity where it protected against toxicity in mice injected with Aβ [32]. In the current study, we found that spearmint with RA prevented memory loss in a mouse model that naturally overproduces Aβ.

The findings in the current study are similar to previous findings following antioxidant supplementation in SAMP8 mice. Alpha lipoic acid improved learning and memory following only one-week of treatment in 12 month old SAMP8 mice and after just 2 weeks in 18 month old SAMP8 mice [9,32]. The antioxidant docosahexaenic acid, found in fish oil, also improved learning and memory in SAMP8 mice [33]. Supplementation of mulberry extract, rich in the antioxidant anthocyanins, resulted in improved avoidance learning and memory, reduced cholesterol and reduced indices of oxidative stress in SAMP8 mice [34]. In addition, these findings are encouraging as several of the above mentioned molecules/extracts have demonstrated positive effects in follow-up human clinical trials for cognition, indicating the

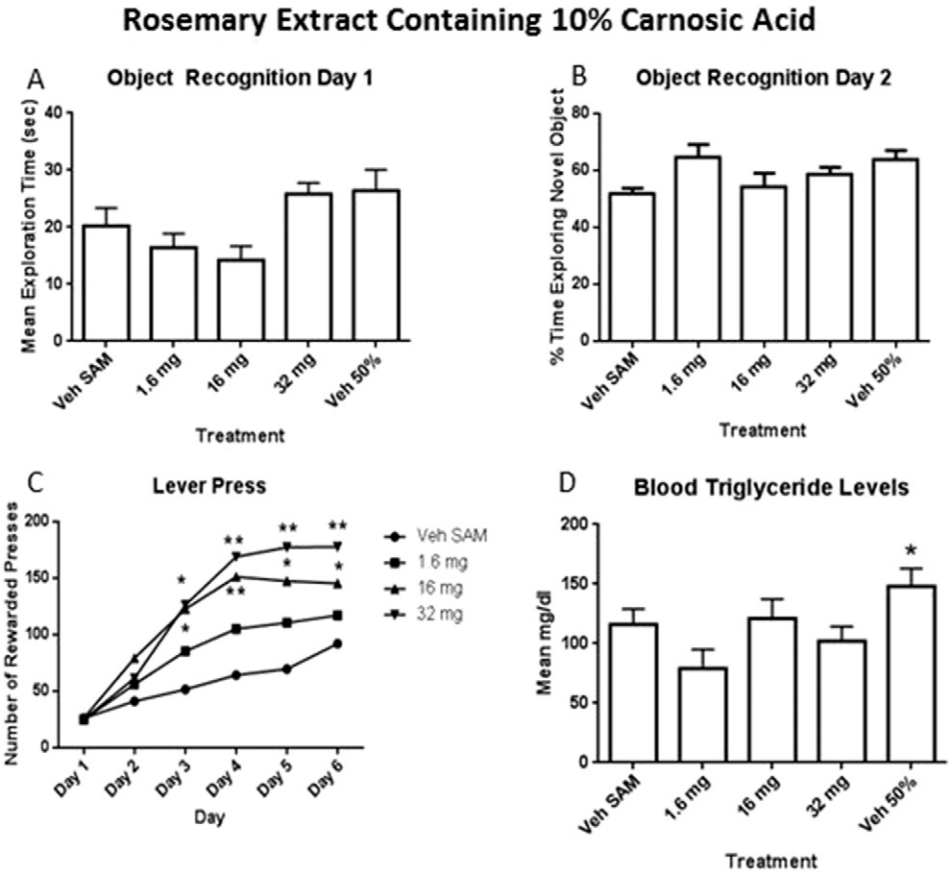

Fig. 4. Effects of CA10 on novel object recognition and operant lever press. CA10 had no effect on exploration time during day 1 of novel object recognition (a) or 24 h retention in novel object recognition (b). CA10 however, improved operant learning at 16 (days 4 & 5) and 32 mg/kg (days 3, 4 & 5) CA10 in the lever press (FR1) compared to the Veh SAM (c). The * indicates $P < 0.05$ and the ** indicates $P < 0.01$ compared to the vehicle treated SAMP8 mice. Values represent means + SEM. The * indicates $P < 0.05$ and the * only the means are represented in the lever press for graph readability. * indicates $P < 0.01$ compared to the vehicle treated SAMP8 mice. Values represent means + SEM.

predictive and translatable nature of this model from rodent to human [35,36].

Protein and lipid oxidation occurs in SAMP8 mouse brains with age [10,32,37]. Proteins and lipids play important roles in the normal structure and function of cells [38]. Abnormal cell function and eventual cell death can occur with oxidative modification of proteins in cells [39]. In the present study, sensitive immunochemical methods were used to determine if treatment with the antioxidants had any effect on protein carbonyl levels. These results indicated a decrease in protein oxidation in the hippocampus (following administration of rosemary extract with 10% CA or the spearmint extract with 5% RA) and decreased lipid oxidation in the cortex (following administration of all extracts). Both the hippocampus and cortex have been found to be important areas for T-maze learning and memory [37,38]. In addition, studies have found that the hippocampus is important for memory in object recognition when using a 24 h retention delay [40]. These data demonstrate that both rosemary extract with CA and spearmint extract with RA can help in reversing oxidative changes that occur with aging and cognitive decline in SAMP8 mice.

Many antioxidants do not cross the blood-brain-barrier (BBB); however, RA has been detected in the brain after intraperitoneal injection [41]. Administration of RA in cultured neurons indicates that it is capable of blocking neuroinflammatory cytokines in the BBB [42]. Inflammation is thought to play a key role in the BBB breakdown with age [42]. Here, we see improved learning and memory suggesting that peripheral administration had a positive effect on brain function.

Although the current study found positive effects on markers of oxidative stress, there were some markers that went the opposite direct. This is similar to some other reports showing on negative effects of polyphenols on oxidative stress. Polyphenols, such a quercetin, have been found to be detrimental to neurons at high doses [43]. Another possible mechanism of action by which polyphenols may act on learning and memory is through inducing cellular stress responses that result in the up regulation of protective genes [44–46]. The present study, we found some negative effect on oxidative stress markers suggestion that not of its all actions of the compounds are through decreases in the markers of oxidative stress explored here.

Dietary supplements and additives increase in number each year. The benefits of these supplements are controversial [47]. Nutritional antioxidants rich in polyphenolics may help lower the incidence of disease, such as certain cancers, cardiovascular disease and have reported anti-aging properties [48]. There are many reports of positive beneficial results in the animal literature [11,49,50]. Previous work in humans has not always been as positive [5,51]. However, there have been some positive results with cancer using selenium especially in men [52] and recently studies utilizing extra virgin olive oil and vitamin E have had positive results on markers of inflammation [53,54]. The current study using both rosemary extract with CA and spearmint extract with RA suggests that when provided at the appropriate dose these molecules have the potential to delay or prevent age-related memory impairments.

In the current study, the natural antioxidants, rosemary extract with CA and spearmint extract with RA, were orally supplemented to determine if they had beneficial effects on learning and memory in the SAMP8 mouse model of the cognitive dysfunction. The extracts are natural products that when extracted from the rosemary and spearmint result in positive effects on memory in a mouse model that develops age-related memory decline. To our knowledge this is the first study

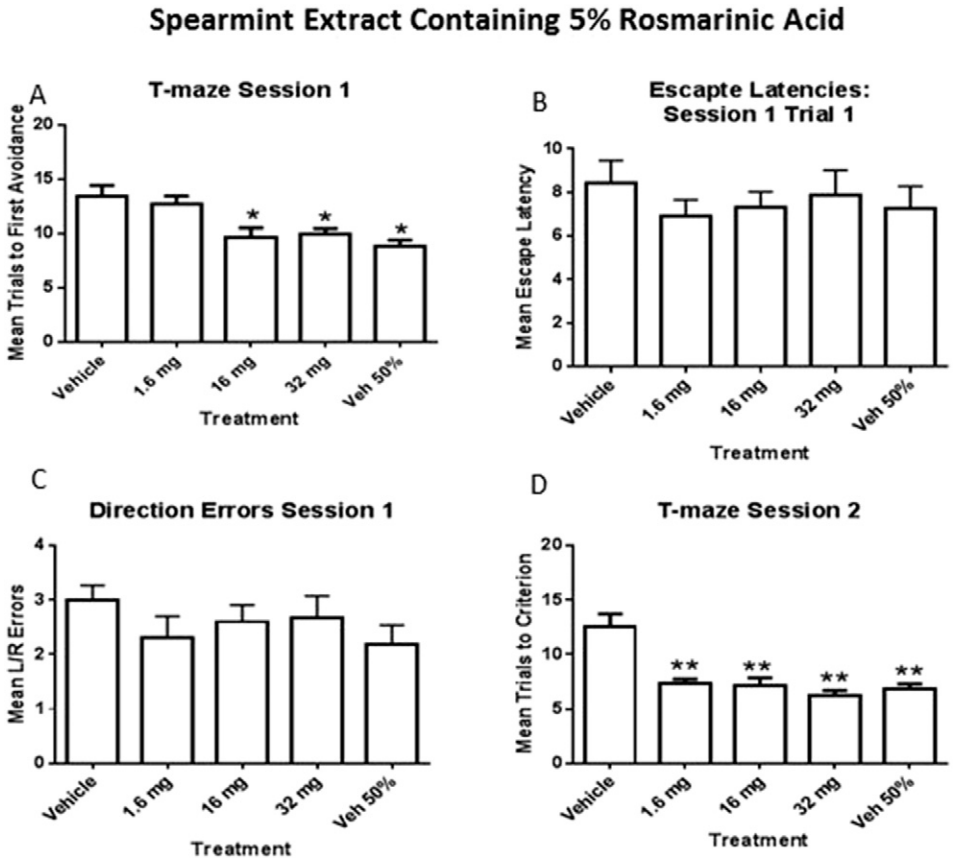

Fig. 5. Effects of spearmint extract containing 5% rosmarinic acid (RA) on T-maze foot shock avoidance. RA improved T-maze performance in session 1 at 16 and 32 mg/kg (a). RA had no effect on escape latencies during trial 1 session 1 or number of L/R direction errors during session 1 (b and c). RA improved performance in session 2 at 1.6, 16 and 32 mg/kg RA (d). The * indicates $P < 0.05$ and the ** indicates $P < 0.01$ compared to the vehicle treated SAMP8 mice. Values represent means + SEM.

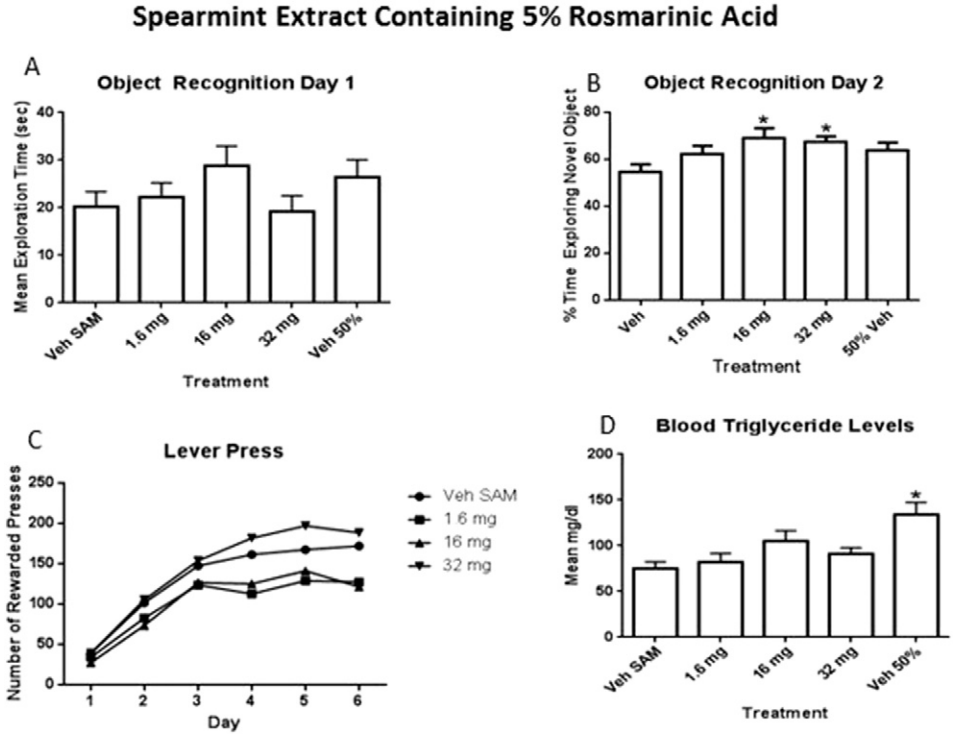

Fig. 6. Effects of RA on novel object recognition and operant lever press. RA had no effect on exploration on day 1 with the 2 like objects in novel object recognition (a). RA improved novel object recognition memory at 16 and 32 mg/kg RA on the 24 h test with on similar and one novel object recognition (b). Spearmint extract containing RA had no effect on operant learning (FR1) (c). The * indicates $P < 0.05$ and the ** indicates $P < 0.01$ compared to the SAMP8 vehicle control. Values represent means + SEM. Only the means are represented in the lever press for graph readability.

showing prevention of cognitive decline following administration of either rosemary extract with CA or spearmint extract with RA in a mouse model of cognitive decline due to accelerated aging. These findings suggest that rosemary extract with CA and spearmint extract with RA are potential natural nutritional interventions for age-associated cognitive decline.

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Conflict of interest

M. A. C., K. A. H., B. J. L., and S. F. were employed by the sponsor company and manufacturers of the ingredients at the time of the study. However, a third party study site conducted the study on behalf of the sponsoring company.

The authors' contributions are as follows: S. A. F. contributed to experimental design, data analysis, interpretation of data and wrote first draft of paper; M. L. N. was responsible collection data and data analysis; M. A. C. was responsible for concept and experimental design; K. A. H. was responsible for concept and experimental design; B. J. L. was responsible for extract development; S. F. was responsible for extract development; A. W. was responsible for biochemical measures and data analysis; D. A. B. was responsible for biochemical data analysis and interpretation; J. E. M. contributed to experimental design and interpretation of data.

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