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Enhancing Spatial Memory Effects of Black Seed, Frankincense, and Ginger Extracts Using Albino Mice

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Abstract: Over the years, there has been an escalating interest in the use of herbal medicine worldwide. The use of traditional medicine has provided valuable formulas on the selection, preparation and application of herbal remedies. In this study, the effects of ginger, frankincense, and black seed methanolic extracts, on hippocampal synaptic transmission, plasticity and spatial memory were investigated using albino mice. Five groups of mice (each n = 8) were used, Group I. control, received 1% Tween 80; Group II, control received gum acacia 5%; Group III, received black seed extract; Group IV, received frankincense extract; Group V, received ginger extract. Group VI, received the mixture extract. Sub-acute administration was applied (1, 5 and 24 hrs before scoring). The levels of glutamate and GABA were measured by using HPLC. Western blot was performed to evaluate synaptic proteins expression. The extract increased the glutamate levels without any changes in GABA levels; it also enhanced the hippocampal long-term potentiation. In addition, extracts effectively enhanced the spatial recognition memory (hippocampal-dependenttask) in mice using Y-maze test. The amelioration of LTP and spatial memory in mice caused by extracts treatment was accompanied by improvement in AMPA -mediated synaptic transmission in the hippocampus. Interestingly, the protein levels of GluR1 AMPA subunit and NR1 NMDA subunit expressions were significantly increased in mice hippocampus. Memory enhancing is combined with increased glutamate level. These results provide a support for the potential therapy of ginger, frankincense, and black seed extracts for enhancing cognition.

Key words: Ginger, Frankincense, Black seed, Learning and memory.

1. Introduction

Herbal treatments are popular in developed world [1]. Several specific herbal extracts have been demonstrated to be efficacious for specific conditions [2]. Herbal medicines usually contain pharmacological active constituents [3]. Consumption of herbal medicines appears to be increasing for a number of world region. Herbal medicines contribute to human fight against diseases and maintenance of health [4].

Black seed (*Nigella sativa* L., Ranunculaceae) is an annual plant; it is distributed in the Mediterranean region [5]; black seed is traditionally used as a spice as well as a folk medicine as a natural remedy for various diseases [5]. The biological activities of the essential oil of black seeds comprise antibacterial [5, 6] and deodorizing activities [5]; it was reported that it exhibits anti-tumor [7], anti-inflammatory [8], antioxidant [9], anti-histaminic, and has immune boosting agent. Chronic oral administration of black Seed enhanced the consolidation and recall capability of stored information and spatial memory in diabetic animals [10] and improved memory in scopolamine induced amnesia in rats [11].

Ginger rhizome (Zingiber officinale) has been used for its health benefits and is a favorite medicinal as well as culinary herb [12]; it has been used for

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centuries to treat dementia. Dried ginger shows a combination of muscarinic, possible Ca++ antagonist and butyrylcholinesterase inhibitory activities. This indicates its benefit in dementia, and Alzheimer's disease [13]. It was reported that ginger extract has antioxidant effects [14]; also it acts as an antihistamine, produces anti-inflammatory properties, and can be used to treat rheumatoid arthritis, osteoarthritis, and various other muscular disorders [12]. Ginger rhizomes extract could enhance memory and protect against brain damage [15].

Frankincense is the resin of Boswellia species (***frankincense***, olibanum). It is used as incense in religious and cultural ceremonies since the beginning of written history [16]. Frankincense is widely used in the treatment of inflammatory conditions [17, 18], as well as in some cancerous diseases [19, 20], wound healing, and for its antimicrobial activity [21]. Recently, studies focused on the immunomodulatory properties of the resin; boswellic acids were considered to be the main active ingredients of the resin [22-24]. Frankincense resin is a traditional remedy for memory loss [25, 26]. It exhibits antidepressive-like effect in the Forced Swim Test (FST) in mice [27]. Frankincense medicinal properties are recognized, in the treatment of some cancerous diseases [20, 28]; furthermore, it was reported that cultured human bladder and breast cancer cells are more sensitive to frankincense essential oils prepared from both *Boswellia carteri* and *Boswellia sacra*, with suppressed proliferation and increased apoptosis [29, 30].

Regarding skincare, Frankincense is particularly helpful for older skins, and has a definite tonic effect, helping to restore some tone to slack looking facial skin, and slowing down the appearance of wrinkles [31].

Traditional and Tibb Nabawy books mentioned the remarkable and efficacious benefits of using these three remedies, Ginger, Frankincense and Black seed, to improve quality of human life, physically and spiritually, and to enhance memory and learning ability.

Aim of the work

Based on the antioxidant [32] and cognitive enhancing effects [33] from previous studies of ginger, black seed extracts [34, 35] and Frankincense extracts [36], an abundance of research has focused on the development of cognitive enhancers from medicinal plants reputed for antioxidant and cognitive enhancing effects. However, the precise underlying mechanism and possible active ingredient responsible for the cognitive enhancing effect of ginger, black seed and Frankincense still require further investigation. The present electrophysiological studies aims to investigate the screening and mechanism of ginger, black seed and frankincense extract on the enhancement of cognitive function in rodents.

The research is aimed to determine the effect of extracts/extract mixture of frankincense, black seed and ginger on enhancing the memory using albino mice.

2. Materials and Methods

2.1 Animal

The experiments were carried out on male mice (25-40 gm). Mice were bred in the animal house of Faculty of Pharmacy, University of Tripoli. Standard mice food pellet diet and water were available. The animals were kept at constant room temperature (20-25 °C), and on a 12h dark/light cycle. Animals were kept in laboratory throughout the period of the experiment.

2.2 Drugs and Administration

Methanolic extracts of ginger, black seed, and frankincense were administered at a dose 500 mg/kg. All were administered intra-peritoneal; both of ginger and black seed are prepared as suspension in 1% Tween 80 (1% T80), while frankincense was prepared as suspension in 5% acacia gum [37]. It was injected in a volume equivalent to 1ml/kg [38], and were prepared freshly prior to use.

2.3 Y-maze Test

Y-maze consisted of 3 arms at a 120-degree inclination from each other. Each arm measured 7.5 cm x 38 cm x 12 cm (w x l x h). The maze was constructed of opaque beige plastic. The arms were elevated a further 2 cm to prevent animals from climbing up the walls and escaping the maze. The arms were randomly labeled as arms A, B, and C. Extra-maze cues were provided by a cylinder covered in a checkerboard pattern (Arm A), a triangle (Arm B), and a 3-dot domino pattern (Arm C). All extra-maze cues elevated about 15 cm above the arm back wall.

The maze was thoroughly cleaned with a 35% (v/v) alcohol solution between subjects to eliminate odor cues, and it was located in a dimly illuminated room with a white noise background. All sessions were recorded using a camera located above the center of the maze. All animals were subjected to a training (exploration) and testing sessions. During the training session, one of the arms was closed. Thus, subjects could only navigate the remaining two arms, the arm in which they were started in the maze (Start Arm) and another arm (Other arm). The identity of the Start and Other arms were randomized within groups but remained constant within subjects. Subjects were released into the Start arm and allowed to explore the Start and Other arm for 15 min, upon which time they were removed from the maze and returned to their home cages. Three hours later, all animals were returned to the maze and allowed to explore all three arms for 6 min. Because of rodents' natural curiosity, it was expected that they would spend a large proportion of the test session exploring the previously occluded arm (Novel arm).

Upon completion of the test period, all animals were returned to their home cages. Recordings of the test session were scored by three independent observers who were blind to the group and identity of the Novel arm for all subjects. All scores were averaged across observers with the constraint that if a given observer's score deviated by more than 10 s or 1 entry from the ratings of the other two observers, that observer's score was not included in the average. This criterion is very stringent, but it ensures high interpreter reliability.

All scores were obtained from at least two independent observers. Measures for the start and other arm were averaged for all variables, and they will be referred as the familiar arms. Number of alternations and dwell time into each arm were recorded, dwell time was analyzed by contrasting the proportion of the total time spent in the maze's arms that subjects spent in the novel and familiar arms [39].

2.4 Acute Hippocampal Slice Preparations

Acute hippocampal slices were prepared using standard experimental procedure, previously described [40, 41]. Mice were decapitated under CO_2 anesthesia. After rapid dissection, the brains were immediately placed in ice-cold oxygenated (95% O_2, 5% CO_2), using dissection buffer consisting of (in mM): NaCl 85, KCl 2.5, $MgSO_4$ 4.0, $CaCl_2$ 0.5, NaH_2PO_4 1.25, $NaHCO_3$ 25, glucose 25, sucrose 75, kynurenic acid 2.0, ascorbate 0.5). Coronal hippocampal slices of 350 μm thickness were obtained using a vibratome series 1000 tissue, sectioning system (Technical products international Inc., St. Louis, MO, USA).These slices were incubated for 2 hours at 30 °C in holding chamber of artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26, dextrose 11.0), and continuously bubbled with 95% O_2/ 5% CO_2.

2.5 Extracellular Field Recordings

For electrophysiological recording, a single slice was transferred to a submerge-type recording chamber and held between two nylon nets. The submersion chamber was continuously perfused with oxygenated ACSF (30 °C) at a flow rate of 2–3 ml/min. A platinum bipolar electrode was placed in the stratum radiatum to deliver test pulses (0.033 Hz) to the

Schaffer-collateral commissural pathway of the hippocampal CA1 region. Glass microelectrodes (1–4 MΩ) were pulled from 1.5-mm-outer diameter glass tubing with the use of a micropipette puller (Narishigie scientific instruments Lab, Tokyo) and filled with (ACSF). The glass electrodes used for recording field excitatory postsynaptic potentials (fEPSPs) from the CA1. Recordings of field EPSPs (fEPSPs) were made in the middle of the stratum radiatum with an Axoclamp 2B (Axon Instruments, Foster City, CA) [42].

In experiments to examine the effects of kinase inhibitor on basal synaptic transmission and Long-term potentiation (LTP), the slices were pretreated with KP-15792 (50μm) or control vehicle for two hours and experimental levels of LTP were measured on slices prepared from the same hippocampus. Following stable baseline recordings of at least 20 minutes, theta burst stimulation consisted of one episode of the following: 10 bursts of stimuli, each of four pulses at 100 Hz; interburst interval, 200 msec was applied to the Schaeffer-collaterals and fEPSPs were measured in *stratum radiatum*. LTP was measured as the percentage of the baseline fEPSP slope. During the 20- minute baseline and 1 hour following the tetanus, fEPSP peak amplitude and slope were analyzed online using Win- LTP acquisition software [43]. The data are presented as means ±SEM. For plasticity experiments, significance was determined using 2-way repeated measures ANOVA, and a two-tailed unpaired *t*-test was used to determine significance for all basal measures.

2.6 Western Blot Analysis

For western blotting [44], hippocampal cell lysates were resolved by 10% SDS (sodium dodecyl sulfate) SDS-PAGE (polyacrylamide gel electrophoresis) and blotted to PVDF (polyvinylidene difluoride) membranes (immobilon- P; Millipore, Bedford, MA). Membranes were blocked with 5% non-fat dry milk in PBS (phosphate buffered saline) containing 0.01% Tween 20 for one hour and then incubated with anti-PSD 95 (anti-post synaptic density 95) antibodies (1:1000; Cell Signaling Technology), anti-GluR1 (1:1000; Cell Signaling Technology), and anti- β-actin (1:1000; Cell Signaling Technology) overnight at 4 °C. Membranes were then probed with corresponding anti-rabbit or anti-mouse horseradish peroxidase conjugated second antibodies for one hour (1:5000; Cell Signaling Technology, 1:5000; Cell Signaling Technology) and developed using the enhanced chemiluminescence (SuperSignal West Femto ECL reagent (Pierce Biochem). All immunoreactive bands were scanned with a desktop scanner and were subsequently quantified by densitometric analyses using Quantity 1 analysis software (Bio-Rad, Hercules, CA, USA). The densities of each band which represented individual animals were normalized to β-actin and then compared with control levels for both control and treated groups. The data represent the mean ± SEM. Significance was determined using a two-tailed Student's *t*-test.

2.7 Electrochemical Detection of Hippocampal Glutamate and Gamma-amino Butyric Acid Levels

Tissue preparation: Animals were sacrificed by cervical dislocation in the morning before 11.00 AM in order to avoid any diurnal variations of the endogenous amines, enzymes and other antioxidant molecules. For the analysis of neurotransmitters, whole brains were dissected out within thirty seconds, rinsed in ice cold normal saline, blotted dry on ash-free filter paper and each left and right cortex were dissected out separately. The hippocampus were weighed and homogenized by sonication in 1 ml of chilled homogenization buffer (0.1M citric acid, 0.1M sodium dihydrogen phosphate monohydrate, 5.6 mM octane sulfonic acid, 10 μm EDTA in 10% (v/v) methanol solution, pH 2.8 with 4M NAOH). After homogenization samples were centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant stored at −80 °C until final HPLC analysis.

Measurement of Glutamate and GABA*:* The amino acids were estimated by employing HPLC-electrochemistry detection as described by [45]. Briefly, the method utilizes the chemical properties of primary amines to react with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide to produce 1-cyanobenz-isoindole (CBI) derivatives that can be detected by either electrochemical or fluorescent detectors. After derivatization of both standards and tissue samples, 20 µl of injection of each was injected in the HPLC system.

HPLC Equipment*:* The HPLC system (shimadzu) consisted of shimadzu SCL 10-Avp system controller, LC-10AS pump, LECD-6A electrochemical detector, degasser, Rheodyne injector with 20 µl injection loop, and C18, ion pair, analytical column (4.6 mm x 250 mm; Ultrasphere IP; Beckman, USA), with a particle size of 5 µm and pore size of 80 Å. The flow rate was 0.65 ml/min and the electro-detection was performed at + 0.8 V for the analyses of glutamate and GABA.

Derivatization protocol and HPLC procedure: The derivatization of GABA and glutamate was done according to the method published by Clark et al., [45] with some modifications. Briefly, 10µl of either standard mix or sample supernatant, 90 µl of borate buffer (0.1 M, pH 9.5), 10 µl of potassium cyanide (10 mM) and 10 µl of NDA (6 mM) were added to a single reaction tube, vortex mixed and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20 µl of the derivative was injected into the appropriate HPLC system. The composition of mobile phase was 0.1M di-sodium hydrogen orthophosphate/50 µM EDTA (pH 5.6, 1 M OPA) and HPLC grade methanol (35:65). Mobile phase was filtered through 0.45 µm and vacuum degassed prior to use. The samples were eluted isocratically over a 30 min runtime at a flow rate of 0.65 ml/min after a 20 µl injection. A standard solution containing glutamate and GABA was run immediately prior to and following sample injections. Results are presented as pmol/mg tissue.

3. Results and Discussion

3.1 Extract Mixture Treatment Enhances Spatial Working Memory Performances in Mice

In this study, we investigated the effect of extracts/extract mixture treatment on the spatial memory in mice. The time spent into each maze's arm by all mice groups was measured. We measured the time spent into each maze's arm (as opposed to dwelling in the center of the apparatus) by all mice groups. Dwell time into novel arm was significantly different in vehicle-treated mice and extracts/extract mixture. For example, a one-way ANOVA conducted on the proportion of time spent in the Novel arm, F (2, 11) = 7.43, MSE = 157.68, $p < 0.05$, followed by a series of planned comparisons, revealed that ginger treated mice dwelled in the Novel arm significantly more than control mice, $p < 0.05$. Black seed-treated mice dwell time in the Novel arm was significantly higher than that of vehicle-treated mice F (1, 11) = 5.42, $p < 0.01$. Furthermore, the Frankincense-treated mice's dwell time did not differ from that of control mice, F (1, 11) < 1.0 (Fig.1.A). The extract mixture shows significantly highest dwelling time compared with control F (1, 11) = 6.21, $p < 0.05$. The possibility that the extent to which exploratory behavior may be compromised due to increased anxiety in a novel environment especially in Frankincense treated group, was analyzed by looking at the number of alternations animals made within the maze; this provides a rough measure of general exploratory behavior. Number of alternations was analyzed with a one-way analysis of variance (ANOVA), F (2, 10) = 26.80, MSE = 28.77, $p< 0.001$ (Fig.1.B). Extract mixture treated mice produced more alternations than the vehicle-treated mice, Fs (1, 10) = 47.69, $p< 0.001$. However, the control mice and Frankincense treated mice did not differ in this measure, F (1, 10) < 0.10. Thus, although Frankincense mice showed same activity and general exploratory behavior as vehicle-treated mice, anxiety was not a concern when comparing the two mice groups.

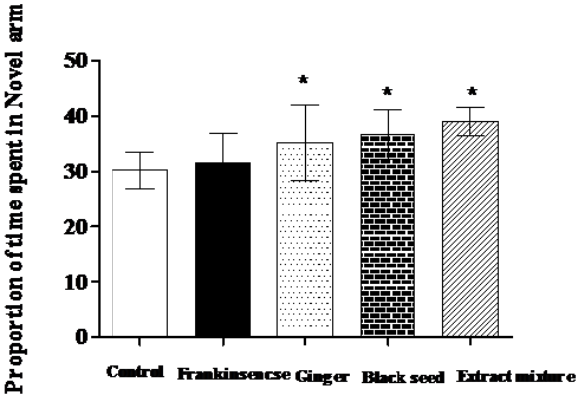

Fig. 1(A) Bar chart shows the significant increase in the time spent by extract mixture-treated mice in exploring the novel arm in the y-maze than in the vehicle-control treated mice p < 0.01 or in black seed-treated micep < 0.05 or in ginger-treated mice p < 0.05. Frankincense- treatment did not affect the time spent in exploring the novel arm compared to control mice.

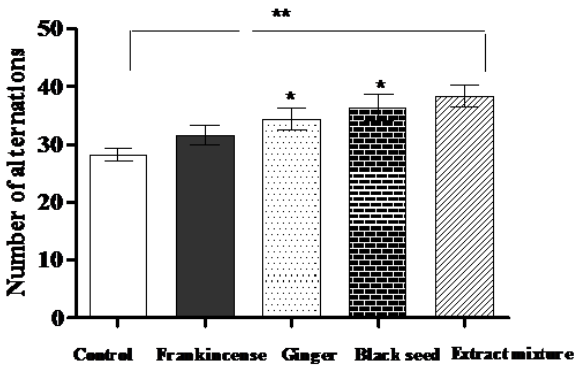

Fig. 1(B) Bar plot depicts the significant increase in activity and general exploratory behavior in extract mixture mice relative to the control mice *p* < 0.001. Frankincense treatment did not have any differential effect on the activity and general exploratory behavior compared to control mice.

3.2 Treatment with Extract Mixture Enhances LTP in Mice

Since LTP is a form of synaptic plasticity that is widely accepted now as a cellular correlate of memory processing, we next examined LTP in the Shaffer collateral synapses of hippocampal slices of vehicle-treated and different extracts treated mice. Long-term potentiation was induced with theta burst stimulation (TBS) protocol that involves 5 trains of 10 bursts of 4 pulses at 100 HZ; with interburst interval 200 msec. A two-way repeated-measures ANOVA showed that LTP, measured 55-60 min following TBS, was significantly induced in 5 slices from ginger-treated mice, (Fig.2.A ; $p < 0.001$; n = 5) with an average of 165.3 % ± 3.510 % , compared with control mice in which LTP was induced in five of five slices with an average of (146.7 % ± 1.419 %; $p < 0.001$; n = 5). However, LTP significantly enhanced in extract

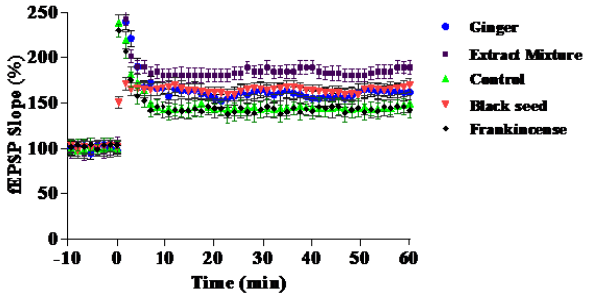

Fig. 2(A) LTP average percent induced by ginger, black seed, frankincense. LTP was induced by theta burst stimulation (TBS) and measured at 55-60 min after TBS. Long-term potentiation was significantly enhanced in acute hippocampal slices from Ginger, black seed and extract mixture-treated mice, normalized fEPSPs slopes of 60 min post-TBS averaged 146.7 % ± 1.419 % for control-treated mice , 165.3 % ± 3.510 % for Ginger treated mice slices, p < 0.001; n = 5) and 167.3 % ± 5.510 % for black seed treated mice slices, p < 0.001; n = 5). Extract mixture treatment significantly improved LTP in mice, (LTP averaged 189.4% ± 3.408 % p < 0.0001; n = 5). Frankincense-treatment did not affect the fEPSPs slopes average 151.7 % ± 1.218 % in mice slices compared to control to any significant extent.

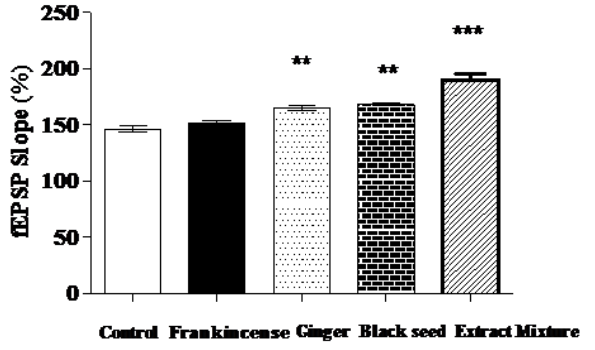

Fig. 2(B) Bar plot showing drastic increase of LTP. Bar plot showing drastic increase of LTP in extracts-treated mice compared to vehicle- treated mice. Long-term potentiation recovered to a potentiated level in extract mixture treated mice slices, whereas LTP was not affected by Frankincense treatment.

mixture-treated mice with an average of 189.4% ± 3.408 % (Fig.2.B $p < 0.0001$; n = 5). In addition, LTP average percent induced in the Frankincense-treated mice was not significantly different from that induced in control mice (151.7 % ± 1.218 %; n = 5). Treatment with black seed increases LTP average in (167.3 % ± 5.510 %; n = 5). These results demonstrate that treatment with extract mixture enhances LTP in mice, and thereby enhances spatial memory performances in mice.

3.3 Effect of Frankincense, Ginger, Black Seed and Extract Mixture on Endogenous Glutamate in Mice Hippocampal Slices Using HPLC

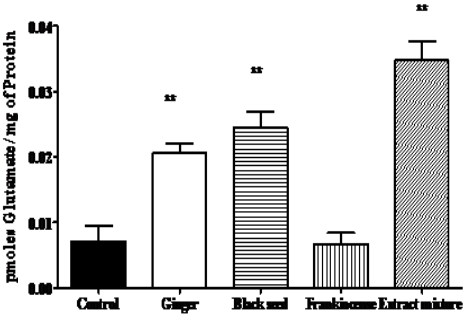

Fig. 3 Data show significant difference between the extracts and the control, extract mixtures shows higher level of glutamate. Data are mean ± SEM of three to five experiments run in triplicate. ($P < 0.01$) one-way ANOVA followed by Dunnett *post hoc* test.**

3.4 Effect of Frankincense, Ginger, Black Seed and Extract Mixture on Endogenous GABA in Mice Hippocampal Slices

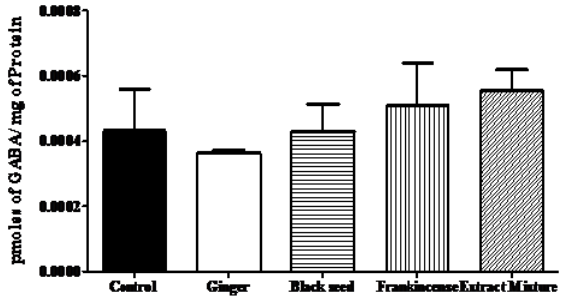

Fig. 4 There was no significance different between the extracts and the control ($P > 0.05$). Data are mean ± SEM of three to five experiments run in triplicate. One-way ANOVA followed by Dunnett post hoc test.

3.5 Effects of Extracts and Extract Mixture on the Expression of AMPA and NMDARs Subunits and Their Associated Proteins in Whole Hippocampus Lysate

Enhancing in the expression of synaptic glutamate receptor subunits could contribute to the enhancement in synaptic transmission and plasticity. In addition, could be due to enhancing of protein synthesis. Therefore, we performed western-blot experiments to quantify the protein expression levels of the most abundantly expressed AMPARs subunit, GluR1 AMPARs subunit, NR1 NMDARS subunits. We found that the protein level of GluR1 and NR1, were increased in the Ginger, black seed and in extract mixture in the whole hippocampi fractions. For GluR1 (Fig.5.A.B), 100.0 ± 4.807 and 100.0 ± 3.208 for controls and g115.6 ± 2.852, b115.6 ± 2.901 and E123.6 ± 3.621 for NR1, and 126.9 ± 3.850, 133.5 ± 3.470, 142.9 ± 6.434 for Glur1; respectively, *$p < 0.01$; **$p < 0.001$ n = 6). There was a slight but a non-significant increase in NR1 level in the Frankincense treated whole hippocampus lysate, but no change in GluR1 level (Fig. 5.A). 100.0 ± 4.807 and 100.0 ± 3.208 for control and 107.2 ± 4.927 for NR1 $p < 0.05$, n = 6) and 99.44 ± 3.739 for GluR1.

We also quantified the protein level of certain synaptic proteins that regulate AMPA and NMDARs kinetics and postsynaptic expression. Stargazin is AMPARs auxiliary subunit that regulates AMPARs expression at synapses and enhances their conductance [46, 47]. Post synaptic density (PSD-95) is a synaptic scaffolding protein that promotes the surface expression of AMPA and NMDARs. Moreover, PSD-95 was reported to modulate NMDARs channel properties; it reduces the desensitization of NMDARs responses and enhances NMDAR channel opening [48-50]. There was a significant increase in the protein level of Stargazin and PSD-95 in Frankincense, Ginger, black seed and in extract mixture in the whole hippocampus fractions (Fig 5.C.D). 100.0 ± 4.118 and 100.0 ± 4.807 for controls and 106.8 ± 3.230, 115.8 ± 3.340,

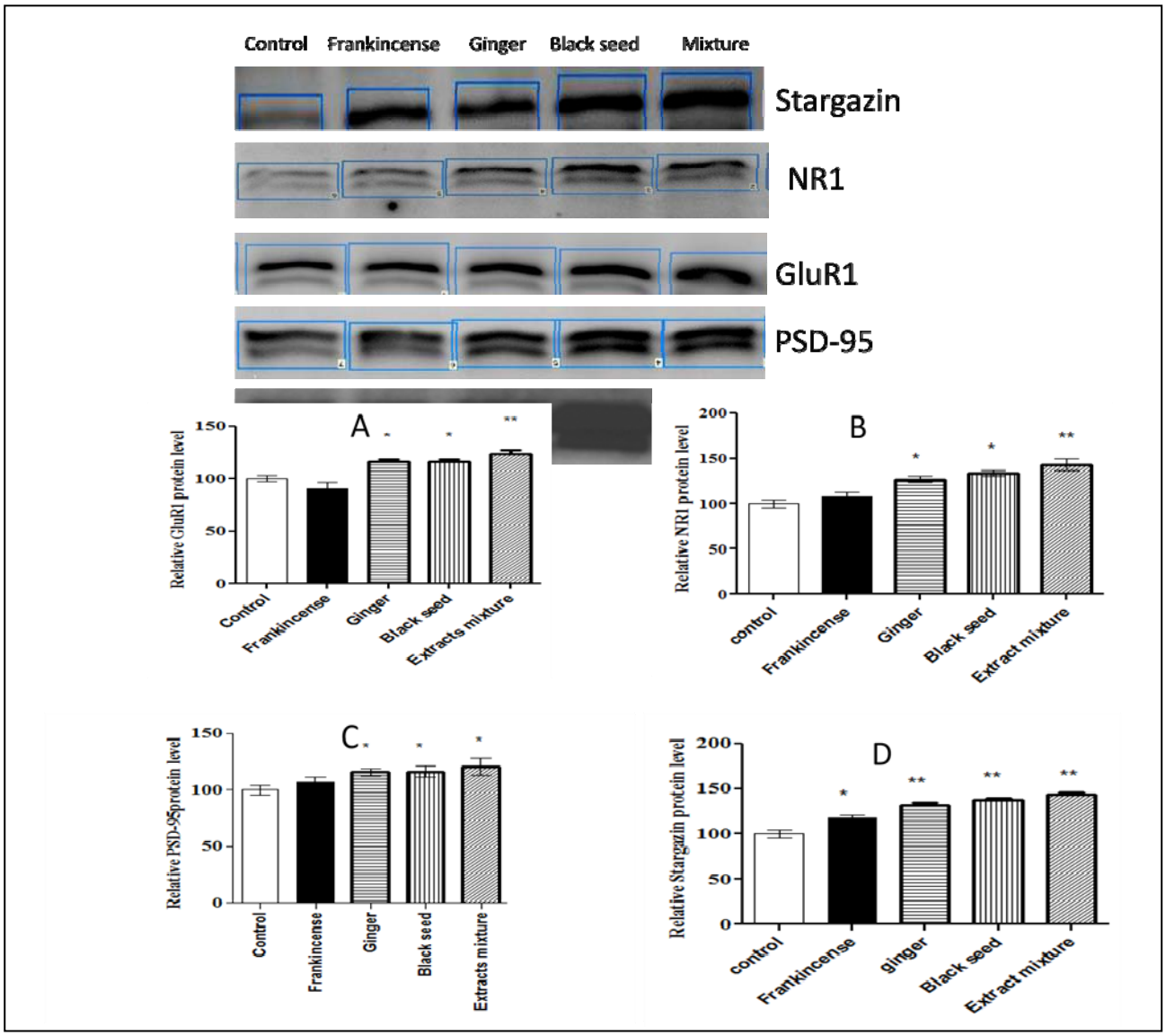

Fig. 5 Western blot analyses were performed to quantify the protein levels of (A) GluR1, (B) NR1, (C) stargazin and (D) PSD-95 protein levels in the control and extracts/extract mixture treated rodents in the whole hippocampus lysate. Protein levels are normalized to β-actin and expressed as a percentage of the control group which was set as 100%. Representative bands are shown above the bar graphs. (n = 6, **$p < 0.001$, *$p < 0.01$ two-tailed, unpaired Student's *t*-test).

116.3 ± 4.872 and 120.4 ± 7.182 for PSD-95, and, 117.7 ± 3.025, 131.1 ± 3.181, 137.8 ± 1.941, 143.7 ± 2.029 respectively for stargazing; *$p < 0.01$ and **$p < 0.001$ n = 6).

4. Discussion

It is widely accepted that memory formation is dependent on changes in synaptic efficiency that permit strengthening of associations between neurons; synaptic plasticity during memory formation is necessary for storage of information [51]. When presynaptic activity correlates with postsynaptic firing, synaptic modifications that strengthen connections will be able to generate memories [52]. Learning and memory are the most important executive functions performed by the human brain, the loss of which is a prominent feature in dementia. Disturbances, which cause oxidative stress and elevated cortisol levels, can lead to neurodegeneration that may subsequently induce a fall in cognitive ability [53].

Spatial memory involves memory for spatial information by which the brain functions in recognizing, codifying, storing, and recovering information about objects or routes [54]. A previous study indicated that spatial working memory assessed in the Y-maze model in mice [39, 55]. In this study we investigate the effect of extracts / extract mixture of Ginger, Frankincense, and Black Seed using Y-maze. Our finding indicates the improvement of spatial memory using ginger, black seed, frankincense extracts and mixture treated mice as an evidence of highest dwelling time into novel arm as compared with vehicle – treated mice. It is obvious that mixture treated animal was able to use spatial cues to discriminate the novel from the previously visited arm. In addition, mixture extract treated mice produced more alternations in rough measure of general exploratory behavior than the vehicle-treated mice. Therefore, we suggest that the extract mixture treatment with ginger, frankincense and black seed enhances spatial working memory performances in mice.

Long-term potentiation (LTP) is a form of synaptic plasticity that is widely accepted now as a cellular correlate of memory processing [56]. Long-term potentiation is the most easily demonstrable in the hippocampus; it is an area of the brain known to be fundamentally important in memory acquisition [57]. In our behavioral test, the enhancement of memory was shown using Y maze. Next we investigated whether administration of extract of each alone or extracts mixture affects hippocampal LTP, which is the prevalent cellular correlate of memory. Our data revealed that there were significant enhancements of LTP by ginger, black Seed and mixture extracts treated mice in the CA1 hippocampal region. The results of our study indicate that treatment with extract mixture is the most effective in enhancing the LTP observed. Moreover, no significant difference in LTP average percent was noted when frankincense was administered alone to mice compared with control.

Ethanolic extract of black seed contained various types of bioactive. Bioactive have estrogenic, neuroprotective, antioxidant and anti-inflammatory effects; these are due to thymoquinone, terpinen, p-cymen, carvacrol, thymol, flavonoids and unsaturated fatty acids in the form of linoleic acid [53]. Black seed extract has a potent anti-inflammatory effect and enhanced remyelination in the hippocampus [58]; its active constituent thymoquinone (TQ) is the largest component of volatile oil, which is also found in fixed oil [59].

Black seed has potent antioxidative effects, it can protect the brain from the oxidative stress following lipid peroxidation [10]; also Terpinen is a good inhibitor of lipid peroxidation [60, 61]. Thymoquinone (TQ) as an antioxidant; it inhibits oxidative stress in the hippocampus and improve spatial memory, via attenuation of lipid peroxidation [62]. Black seed active constituent (TQ) restored antioxidant enzymes as glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase in streptozotocin-(STZ-) induced cognitive impairment [63]. In addition, thymoquine (TQ) and thymohydroquinone (THQ) are usually present in the form of glycosidically bound aglycones, which easily cross the blood-brain barrier, hence, possibly related to its neuroprotective effects [60]. Thymoquine appears to be the major neuroprotective constituent present in black seed oil. The other bioactive compounds, that is, thymol and carvacrol, also attenuated scopolamine-induced cognitive impairments in rats [64]. It was found that nutraceutical containing thymol and p-cymene has been patented for cognitive enhancement properties [65]. It has been stated that black seed including thymoquinone therapy causes morphologic improvement on neurodegeneration in hippocampus after chronic toluene exposure in rat [66].

Acetylcholine (ACh) as a neurotransmitter plays a role in facilitating learning and memory; therefore, its decreased release will result in memory impairment.

Pharmacological studies demonstrated that black seed is involved in AChE inhibition activity, leading to increase ACh levels and retaining its effects in the encoding of new memories [53]. The active constituents, Thymol and carvacrol and their derivatives thymoquinone and thymohydroquinone, terpinen, p-Cymen, inhibit the activity of acetylcholinesterase AChE [60, 61]. Oral administration of black seed oil produces AChE inhibition as donepezil; it decreases malondialdehyde (MDA) and brain tumor necrosis factor-alpha (TNF-α) content as well as increasing glutathione brain contents. Also, it reverses the amnesic effect of scopolamine-induced deficit of spatial and nonspatial working memory impairment [11].

It is known that thyroxin plays an important role in growth, development, and function of the brain. Learning and memory impairments in hypothyroidism could be reversed by alcoholic extract of black seed; this may due to its antioxidant effects [67].

Evidence from animal studies indicated that ovarian hormone severely influence brain regions which have crucial role in learning and memory, such as the hippocampus [68, 69]; this effect is due to their neurotrophic and neuroprotective actions [70, 71]. Investigations indicated that steroid hormones produce structural synaptic remodeling including increasing the number of dendritic spines and synapses and promoting the magnitude of LTP in hippocampal CA1 [72, 73]. Dai et al. [70] showed that treatment with estrogen promotes LTP in hippocampal CA1 following a global ischemia; also clinical and experimental evidence showed that treatment with estrogen ameliorate performance of learning and memory tasks both in animal models and in humans [68, 74]. It was demonstrated that sex steroid hormones, such as the potent estrogen 17β-estradiol (E_2), affect hippocampal morphology, plasticity, and memory in male and female rodents [75]; also they regulate learning and memory in male and female rodents that is influenced by estrogen receptor expression [76-78].

This estrogenic effect induces synaptic potentiation [79] and alterations in CA1 spine density and LTP with memory formation [75]; also its effect on hippocampal physiology is due to its ability to potentiate NMDA-dependent LTP in female CA3-CA1 synapses [80-82]; LTP results in a persistent increase in synaptic excitability thought to underlie memory formation [75].

Estrogenic effect enhances LTP, this may result from estrogenic effect on intracellular calcium or on activation of classical and nonclassical ER mechanisms [83-85]. In the brain, especially in the hippocampus, the estrogen beta receptor RE-β expression is more than RE-α although RE-α and RE-β are both expressed there. Memory function regulation including cholinergic system is through RE-α, while RE-ß modulates RE-α activity [75]. Black seed extract has an estrogenic effect; it increased rat 17-β estradiol serum levels [86]; Flavonoids content provide an estrogenic effect [87]. Also, Linoleic acid as an estrogen receptor ligand and may induce the up-regulation of estrogen receptor mRNAs [88].

Frankincense facilitated spatial learning and memory in the Morris water maze [89, 90]. It was indicated that alcoholic extract of frankincense has the potential to improve memory retrieval in lipopolysaccharide (LPS) treated rats, possibly via an anti-neuroinflammatory activity [26]. It is known that neuroinflammation impairs memory processing during consolidation and retrieval stages and induce severe cognitive disturbances [91, 92]. The effect of frankincense on memory may be due to functional changes in the brain [93] and structural alterations in the brain neuronal circuits [93, 94]. Frankincense showed an increase in the number of neurons and dendritic spines in hippocampal region CA1 in rats [95].

Studies indicated that the hydro-alcoholic extract of frankincense decreased the levels of TNF-α in the hippocampus of rats treated with lipopolysaccharide,

which increased the levels of TNF-α in the hippocampus, leading to induce neuroinflammation that impair memory retrieval [26, 96, 97]. It is probable that boswellic acids of frankincense might prevent initiation of neuroinflammation [26]. It also prevents hypothyroidism-induced spatial memory impairment in rats [98]. Boswellic acids were shown to inhibit the synthesis of pro-inflammatory enzyme, 5-lipooxygenase (5-LO), leading to inhibition the formation of inflammatory leukotrienes. Incensole acetate, another active component of frankincense, inhibit activation of nuclear factor-kappa B (NF-κB) [99].

Acetyl-11-keto-β-boswellic acid (AKBA) is an active triterpenoid compound from frankincense extract and exhibited potential neuroprotective activity, which is used as antioxidant and anti-inflammatory agents [100]. AKBA counteracted oxidative stress indicated as restoring of intracellular reactive oxygen species content, lipid peroxidation, and oxidative DNA damage [101]. Wei et al found that AKBA modulates its antioxidant and anti-inflammatory pathway through increasing nuclear erythroid 2-related factor 2 (Nrf2) and hem oxygenase-1 (HO-1) expression, and also via declining phosphorylation of inhibitor of nuclear factor-kappa B alpha (IkBα) and p65 [36]. Frankincense acts by inhibition of leukotriene synthesis, of cyclooxygenase ½ and 5-lipooxygenase, of oxidative stress [20]; acetyl-11-keto-β boswellic acid (AKBA) is the most important inhibitor of 5-lipoxygenase which is responsible for inflammation [102].

Incensole acetate, which is an active constituent in frankincense, has been reported to reduce the amount of degenerating neurons within the hippocampus; it inhibits transforming growth factor β-activated kinase/transforming growth factor β-activated kinase-binding protein(TAK/TAB)-mediated IκB kinase (IKK) activation loop phosphorylation, resulting in the inhibition of cytokine and lipopolysaccharide-mediated NF-κB activation [103]. Therefore, incensole acetate and its derivatives were reported to be responsible for its anti-inflammatory effects [104]. Anti-inflammatory properties of frankincense were suggested to be involved in the neuroprotective effects [95, 105]. There is evidence that acetylcholine has a role in suppression of cytokine release through 'cholinergic anti-inflammatory pathway' [106]. Frankincense may improve the cholinergic system of the brain [93]. Therefore, owing to an evident effect of frankincense on decreasing an important pro-inflammatory cytokine (TNF-α) in the hippocampus of rats; it appears that frankincense was able to improve cognitive functions probably due to anti-neuroinflammatory property [26].

There is an evidence that antioxidants may improve cognitive performance in healthy elderly subjects [107, 108]; therefore, it was concluded that the antioxidant, anti-inflammatory and anti-acetylcholinesterase activities of frankincense, may have direct effect on improvement of cognitive functions [93].

A previous study showed that Frankincense enhanced power of learning at post-learning stage, short-term memory, and long-term memory [109]. Administration of aqueous extract of frankincense facilitated the learning and spatial memory formation in rats [110]. Frankincense extract up-regulated expression of brain-derived neurotrophic factor (BDNF) transcripts that may has a role in memory formation [111]. There is evidence supporting the cognitive-enhancing efficacy of genus Boswellia. Potential beneficial actions may be attributed to neurotrophic factors BDNF up-regulation [112].

The main pharmacological properties of ginger include anti-inflammatory, antihyperglycemic, antiarthritic, antiemetic and neuroprotective actions; also it was found that ginger administration significantly improved the ability of mice to recognize novel objects, indicating improvements in learning and memory [113]. Ginger is considered as a potential cognitive enhancer [33], improve memory and protect against brain damage in rats. It was reported that

ginger induces vasodilation, leading to enhancement of cerebral blood flow that will improve spatial memory [15]. Ginger extract could increase the neurons density in hippocampus and improve the spatial memory [15, 114].

The mechanisms of ginger-mediated cognitive enhancement, is through nerve growth factor (NGF)-induced signaling pathways. Ginger administration led to elevated NGF levels in mouse hippocampus and resulted in phosphorylation of extracellular-signal-regulated kinase (ERK) and cyclic AMP response element-binding protein (CREB); ginger-triggered activation of ERK and CREB in the hippocampus was inhibited with specific NGF antibody. Therefore it was concluded that ginger has a synaptogenic effect via NGF-induced ERK/CREB activation, resulting in memory enhancement [113].

Cerebral cortex and hippocampus areas contribute an important role on working memory [33, 115]. Cognitive enhancing effects of ginger might be associated with alteration of the monoamine system and the cholinergic system in various brain areas, including the prefrontal cortex and hippocampus [33]. Ginger enhances the level of norepinephrine, epinephrine, dopamine and serotonin contents in the cerebral cortex and hippocampus [116], where dopamine, and norepinephrine play a key role in numeric working memory. Acetylcholine and serotonin in the hippocampus were simultaneously activated during spatial working memory tasks [33, 117].

Ginger active component, 6-gingerol, inhibited cholinesterase activity; this effect will increase acetylcholine (ACh), which plays an important role in learning and memory [13]. There is evidence that antioxidants could also improve cognitive performance in healthy elderly subjects [107, 108]; therefore, it can be concluded that antioxidant effects of ginger may lead to cognitive enhancing effects [15, 118].

It was demonstrated that ginger could decrease oxidative stress by increasing the activity of SOD in cerebral cortex, hippocampus, and striatum, and increasing the activities of CAT and GSH-Px in cerebral cortex and hippocampus, leading to a decrease of lipid peroxidation level in all areas mentioned above. Therefore, the neuroprotective effect of ginger extract might be related to its antioxidant effect [15].

Flavonoid as an active constituents is present in black seed [53], in Frankincense [119] and in ginger [120]. Flavonoid strengthens hippocampal signaling and induces changes in the strength of hippocampal synaptic connections that underlie learning [121]. Flavonoids have been shown to modulate critical neuronal signaling pathways involved in processes of memory, and that will affect synaptic plasticity and long-term potentiation (LTP) mechanisms, and provide a basis for memory [62]. Flavonoids are able to reverse age-related spatial memory and spatial learning impairment [122]. An increase in hippocampal NMDA receptors containing the NR2B subunit was observed, suggesting an enhancement of glutamate signaling following flavonoid intervention [62].

In our study, glutamate levels were increased in mice hippocampal slices after treatment with ginger, black seed and the mixture extract, without any changes in GABA levels. It was found that mGluR1 and mGluR5 are critically involved in synaptic plasticity in the CA1 region and may enable functional information encoding through LTP [123]. In our study glutamate levels are increased by extract treatment, the extract active constituents may stimulate mGlu1 and 5. Previous studies have shown that activation of mGluR1 results in an increase in intracellular calcium concentration, depolarization of CA1 pyramidal neurons and an increased frequency of spontaneous inhibitory postsynaptic potentials [124]. While activation of mGluR5 results in suppression of the calcium - activated potassium current (I_{AHP}) and a potentiation of *N* - methyl - d - aspartate (NMDA) receptor currents [124-126]. These effects may have

distinct consequences for synaptic plasticity and memory processes; these different mechanisms may facilitate the mediation of LTP by these receptors [123]. In this study frankincense extract did not produce any changes in glutamate levels or LTP; it may need longer duration for administration to produce positive effect.

5. Conclusions

Black Seed, Frankincense, and Ginger extracts enhance the hippocampal long-term potentiation (LTP). In addition, the extracts effectively enhance the spatial recognition memory (hippocampal-dependent task) in mice using Y-maze test. The amelioration of LTP and spatial memory in mice caused by extracts treatment was accompanied by improvement in AMPA-mediated synaptic transmission in the hippocampus.

The glutamate levels in hippocampus were increased without any changes in GABA levels. The protein levels of GluR1 AMPA subunit and NR1 NMDA subunit expressions were significantly increased in mice hippocampus. In addition, memory enhancing is combined with increased glutamate level. These results provide support for the potential therapy of ginger, frankincense, and black seed extracts for enhancing cognition. Our findings suggest that Black Seed, Frankincense, and Ginger extracts enhance glutamatergic excitatory neurotransmission in the hippocampus, can be potential therapy for Alzheimer disease.

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