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A traditional poly-herbal formulation improves cognitive function in C57BL/6 mice

Una formulación tradicional de poli-hierbas mejora la función cognitiva en ratones C57BL / 6

Gulam Mohammed Husain[1]
Mohd Nadeem[1]
Ghazala Javed[2]
Mohd Urooj[1]
Mahe Alam[2]
Muskula Anudeep Reddy[1]
Munawwar Husain Kazmi[1]

[1]Pharmacology Research Laboratory, National Research Institute of Unani Medicine for Skin Disorders (under CCRUM), Hyderabad, India.

[2]Central Council for Research in Unani Medicine, Under Ministry of AYUSH, Government of India, New Delhi, India.

Correspondence

Dr. Gulam Mohammed Husain
Email: gmhusain@gmail.com

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Conflict of interest

The authors declare no conflict of interest.

Abstract

Introduction: Khamira Gawzaban Ambari Jadwar Ood Saleeb Wala (KGAJOS) is a polyherbal compound Unani Pharmacopoeial formulation described in traditional Unani texts as *Muqawwi-e-Aza-e-Raeesa* (tonic for brain, heart, liver and stomach). KGAJOS is reported to possess anxiolytic and antidepressant activity in mice. Though it is used clinically for various neurological conditions, preclinical efficacy of this formulation in learning and memory enhancement / improvement is not established.

Method: KGAJOS was evaluated for cognitive function improvement activity using Morris water maze test in C57BL/6 mice. Piracetam was used as positive control for comparison. Anymaze video tracking software was used for tracking the path of mice in pool as per standard protocol.

Results: During probe trial in Morris water maze test, a significant increase in time spent in platform quadrant was observed at 1000 and 1500 mg/kg bw of KGAJOS ($p<0.01$ and 0.001, respectively) as well as in piracetam group ($p<0.01$) compared to vehicle control. Latency to reach the platform quadrant (escape latency) was significantly reduced ($p<0.001$) in piracetam and KGAJOS group at 1000 and 1500 mg/kg bw compared to vehicle control. No change in time spent in platform quadrant and escape latency was observed at 500 mg/kg bw of KGAJOS.

Conclusions: Morris water maze experiment conducted in mice revealed improved learning and memory function of KGAJOS at the dose levels of 1000 and 1500 mg/kg bw whereas 500 mg/kg bw was not found to be effective. Observed efficacy of KGAJOS confirmed the traditional claims and usage of this formulation in conditions associated with cognition and memory.

Keywords: Unani; Morris water maze; Polyherbal formulation; Cognition; Memory; Learning.

Resumen

Introducción: Khamira Gawzaban Ambari Jadwar Ood Saleeb Wala (KGAJOS) es una formulación de Unani compuesto de poliherbal descrito como tónico para el cerebro, corazón, hígado y estómago. Este estudio se realizó para evaluar la eficacia preclínica de KGAJOS en el aprendizaje y la memoria.

Método: Se evaluó la actividad de mejora de la función cognitiva de KGAJOS utilizando la prueba de laberinto de agua de Morris en ratones C57BL / 6. Se utilizó piracetam como control positivo. Se utilizó el software de seguimiento de video Anymaze para rastrear la ruta.

Resultados: Durante la prueba de la sonda, se observó un aumento significativo en el tiempo empleado en el cuadrante de la plataforma a 1000 y 1500 mg / kg de peso corporal de KGAJOS ($p <0,01$ y 0,001, respectivamente) y en el grupo de piracetam ($p <0,01$) en comparación con el control. La latencia para alcanzar el cuadrante de la plataforma (latencia de escape) se redujo significativamente ($p <0,001$) en el grupo de piracetam y KGAJOS a 1000 y 1500 mg / kg de peso corporal en comparación con el control.

Conclusiones: El experimento del laberinto de agua de Morris reveló una mejora en la función de aprendizaje y memoria con 1000 y 1500 mg / kg de peso corporal de KGAJOS, mientras que 500 mg / kg de peso corporal no fue efectivo. La eficacia observada de KGAJOS confirmó las afirmaciones tradicionales y el uso de esta formulación en condiciones asociadas con la cognición y la memoria.

Palabras clave: Unani; Morris laberinto de agua; Formulación polivérica; Cognición; Memoria; Aprendizaje.

Introduction

Khamira Gawzaban Ambari Jadwar Ood Saleeb Wala (KGAJOS) is a compound Unani Pharmacopoeial formulation. KGAJOS is considered as *Muqawwi-e-Aza-e-Raeesa* (tonic for brain, heart, liver and stomach)[1] and is used in epilepsy, *ummus-subiyan* (infantile epilepsy) and *ikhtenaqur-reham* (hysteria)[2]. KGAJOS is reported to possess anxiolytic[3] and antidepressant activity in mice[4]. Most of the herbal ingredients of KGAJOS have been reported to possess cognitive improvement potential[5-17]. However, no data is available regarding efficacy of this valuable Unani formulation on cognitive function. The present study is designed to evaluate efficacy of KGAJOS on learning and memory function in mice.

Materials and Methods

KGAJOS was evaluated for cognitive function improvement activity using Morris water maze (MWM) test in C57BL/6 mice. Piracetam was used as positive control for comparison. Anymaze video tracking software was used for tracking the path of mice in pool as per standard protocol.

Preparation of the formulation

Test formulation (i.e., KGAJOS) was prepared in the GMP certified Pharmacy Section of National Research Institute of Unani Medicine for Skin Disorders, Hyderabad as per the composition and classical methodology mentioned in National Formulary of Unani Medicine (NFUM) Part-V[2]. The composition of KGAJOS is given in Table 1:

Table 1. Composition of KGAJOS.

S.No.	Ingredients (Unani Name)	Scientific Name	Quantity
1.	Abresham Muqarraz	Cocoon of *Bombyx mori* L.	25 g
2.	Badranjboya	*Melissa officinalis* L.	175 g
3.	Burada Sandal Safaid	*Santalum álbum* L.	125 g
4.	Berg Gaozaban	*Borago officinalis* L. (Leaf)	150 g
5.	Behman Surkh (Neem Kofta)	*Salvia haematodes* W.	100 g
6.	Tukhm Balango	*Lallemantia royleana* Benth.	125 g
7.	Tudri Surkh	*Cheiranthus cheiri* L.	50 g
8.	Kishneez Khushk (Dhania)	*Coriandrum sativum* L.	150 g
9.	Gul Khatmi	*Althaea officinalis* L.	50 g
10.	Gul Gaozaban	*Borago officinalis* L. (Flower)	50 g
11.	Shakar Safaid	White sugar	10 Kg
12.	Sat Leemu	*Citrus aurantifolia* (Christm.)	20 g
13.	Natroon Banjawi	Sodium benzoate (as preservative)	6 g
14.	Ambar Ashhab	*Ambra grasea*	2.645 g
15.	Warq-e-Nuqra	Silver leaves	33 g
16.	Warq-e-Tila	Gold leaves	55 Nos.
17.	Jadwar Saeeda	*Delphinium denudatum* Wall. ex Hook & T	106 g
18.	Ood Saleeb Saeeda	*Paeonia emodi* Wall. ex Royle	137 g

Experimental mice

C57BL/6 Mice (25-30 g, 8-9 weeks old) used for the present study were procured from Hylasco Bio-Technology (India) Pvt. Ltd., Hyderabad (A Charles River Licensee). Mice were group housed in polysulfone cages in the temperature-controlled room maintained at the temperature of 22°C ± 3°C and relative humidity of 30-70%, with a 12:12 h light/dark illumination cycle. CPCSEA guidelines of laboratory animal care was followed throughout the experiment[18]. Study protocol was presented before the Institutional

Animals Ethics Committee and approved *vide* protocol no. CRIUM/IAEC/2018/01/P03 dated 21.07.2018. Mice were maintained on standard diet (SDS diet, England) and water *ad libitum*, unless mentioned otherwise. Only male mice were used in order to avoid the influence of the estrus cycle on drug metabolism and/or efficacy. Mice were acclimatized to the laboratory conditions for one week before using them for experiment.

Dose selection and study design

Therapeutic dose of KGAJOS in human is reported as 05 g per day. Therapeutic Equivalent Dose (TED) in mouse is about 1000 mg/kg bw per day as per body surface area conversion method[19]. Accordingly, present study was performed at three dose levels of KGAJOS i.e., 500, 1,000 and 1,500 mg/kg bw/day to observe any dose dependent activity. A total of 40 male mice were randomly divided into five groups containing 08 mice in each (n=8). Group-I served as vehicle control. Group-II served as positive control and was administered with piracetam (400 mg/kg bw, i.p.)[20]. Group III-V were treated with three dose levels of KGAJOS (i.e., 500, 1,000 and 1,500 mg/kg bw/day) for seven consecutive days before recording the Morris water maze performance and treatment was continued throughout the procedure till probe trial. KGAJOS was administered as an aqueous suspension which was freshly prepared each day and orally administered using stainless steel gavage by calculating the dose for individual mouse as per body weight. Mice of piracetam group were subjected to behavioural recording 60 min after piracetam injection (i.p.) while MWM performance of vehicle and KGAJOS treated mice was observed 90 min after oral gavage administartion[20].

Morris water maze test

Morris water maze (MWM) method consisted of six-day trials of mice in circular pool. The basic paradigm requires an animal to swim in a pool until it finds a hidden escape platform. The mice learn to find the platform using extra-maze cues and, after several training trials, are able to swim directly to platform from any starting location. Memory for the platform location is assessed by examining swimming pattern with the platform removed from the maze[21].

A metallic circular pool with a diameter of 150 cm and a depth of 50 cm and wall height 20 cm above the water level was used. Non-toxic white tempera paint was added to make the water opaque so that black mouse will be captured by software on an otherwise white background[22,23]. A circular platform of about 10 cm diameter was hidden 2 cm below the water level. Pool was filled with water until the platform was 2 cm above the water surface. The water was kept at about 23°C during the experiment. Room was arranged such that the mouse being tested cannot see the experimenter during testing and no change in arrangement of instrument and other objects was allowed throughout the experiment duration. High contrast spatial cues were placed in the room and on the interior of the pool at a location which were above the water surface.

The pool was calibrated using computerized software (Anymaze, Stoelting, USA) so that the camera can create physical distance information from captured pixel-based information. Pool was virtually divided into 4 quadrants and platform zone was specified as a variable zone which can change with each trial. 5 platform subzones were specified *viz.* one in each quadrant, and one in the centre of the pool. Calibration was saved and used for the remaining test days. The maximum trial duration was set as 60 sec. If the mouse finds the platform before this time, software was programmed to stop the trial. Program protocol was specified to begin tracking automatically, when the experimenter exits the testing area. Time spent in each quadrant and time to reach the platform quadrant was tracked using Anymaze video tracking system.

Procedure: Mice were trained for five consecutive days, and each mouse was subjected to five trials per day[21], followed by a probe test on Day 6.

Day 1 (Visible Platform): Five trials were conducted with an inter-trial interval about 5-10 minutes. The platform location and starting direction was different with each trial.

Testing procedure: To begin testing, mouse was lifted from the home cage by the base of the tail. The mouse was gently placed into the water, facing the edge of the pool. If the mouse finds the platform

before the 60 seconds cut-off, mouse was allowed to stay on the platform for 5 seconds and then returned to home cage. If any mouse did not find the platform within 60 seconds, mouse was guided to the platform and allowed to stay there for 20 seconds before returning to home cage. Each subsequent trial was initiated with a different platform location and starting direction. When testing was complete, mice were returned to their housing facility. Mice were dried off properly with towel and normothermia was assured prior to returning to the cage.

Days 2-5 (Hidden Platform): Five trials were conducted with an inter-trial interval of about 5-10 min. Platform location was programmed to remain in the same position throughout all trials and days, but the starting direction was different with each trial, each day.

Testing procedure: Platform was submerged in water by adding additional water. Platform was not visible from the surface of the water. Rest of the procedure was same as for Day-1.

Day 6 (Probe Trial): Only single probe trial was conducted on Day-6 with platform removed from the pool, and one starting direction for all mice. The starting direction farthest from the platform quadrant used on days 2-5 was used. Maximum trial duration was kept as 60 seconds. For day 6, escape latency (time to reach platform quadrant), and time spent in the platform quadrant for each mouse was recorded. A higher percentage of time spent in the platform quadrant is interpreted as a higher level of memory retention[21,24,25].

Statistical Analyses

Data from the experiments was expressed as mean ± standard error of mean (SEM). The mean difference between the control and treatment groups was analysed by one-way Analysis of Variance using Graph Pad prism (version 5) Graph Pad Software, Inc., CA, USA. p value < 0.05 was considered as statistically significant.

Results

During probe trial (i.e., day-6), average time spent by mice in target platform quadrant of MWM is depicted in Figure 1. There was a significant increase (p<0.01) in time spent in platform quadrant in piracetam treated group compared to vehicle control group. No significant difference was observed at KGAJOS 500 mg/kg bw compared to vehicle. KGAJOS significantly increased the time spent in the target quadrant at 1000 and 1500 mg/kg bw as compared to vehicle control (p<0.01 and 0.001, respectively). Latency to reach the platform quadrant (escape latency) was significantly reduced (p<0.001) in piracetam and KGAJOS group at 1000 and 1500 mg/kg bw compared to vehicle control (Figure 2). No change in escape latency was observed at 500 mg/kg bw of KGAJOS. Further, average distance travelled by mice in platform quadrant is significantly higher (p<0.001) in piracetam group compared to vehicle control. KGAJOS significantly increased distance travelled by mice in platform quadrant at 1000 mg/kg (p<0.01) and 1500 mg/kg (p<0.001) while no difference was observed at 500 mg/kg bw compared to vehicle (Figure 3).

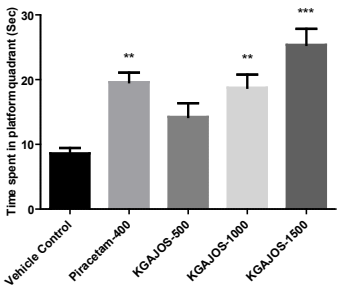

Figure 1. Time spent in platform quadrant in MWM test in seconds; data presented as mean ± SEM (n=8); One-way ANOVA; **=p<0.01 *vs.* Vehicle Control; ***=p<0.001 *vs.* Vehicle Control

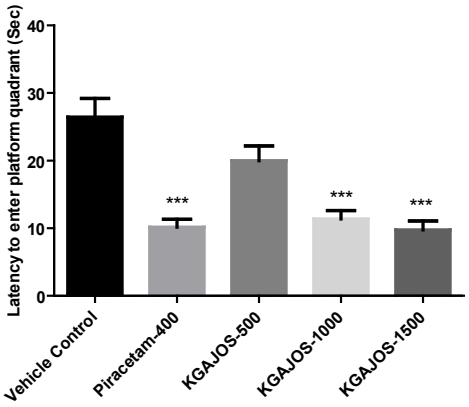

Figure 2. Latency to enter platform quadrant (escape latency) in MWM test in seconds; data presented as mean ± SEM (n=8); One-way ANOVA; ***=p<0.001 *vs.* Vehicle Control

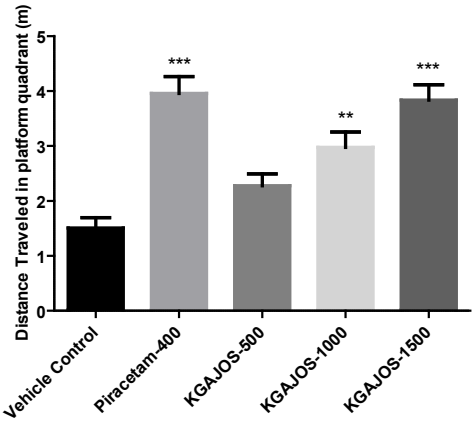

Figure 3. Distance travelled in meters in the target platform quadrant in MWM test; data presented as mean ± SEM (n=8); One-way ANOVA; **=p<0.01 *vs.* Vehicle Control; ***=p<0.001 *vs.* Vehicle Control

Discussion

Morris Water Maze was first established by neuroscientist Richard G. Morris in 1981 in order to test hippocampal-dependent learning, including acquisition of spatial memory and long-term spatial memory[26]. MWM test has become one of the most frequently used research tools in various aspects of learning and memory[27]. The robustness/simplicity and reliability make this paradigm as one of the 'gold standards' of behavioural neuroscience. The main advantage is the differentiation between the spatial (hidden-platform) and non-spatial (visible platform) conditions[24-26,28]. In addition, the MWM testing environment reduces odour trail interference[29]. Therefore, considering these advantages, MWM test is extensively used in the study of the neurobiology and neuropharmacology of spatial learning and memory.

In the present experiment, mice treated with 1000 and 1500 mg/kg bw of KGAJOS and piracetam (400 mg/kg bw) spent much longer time in platform quadrant compared to vehicle control group, suggesting that these mice gathered the clue from spatial arrangement in the room and pool regarding the possible location of the platform and explored the same area of pool (quadrant) for the platform repeatedly which is also supported by longer distance travelled in platform quadrant by mice of these groups compared to vehicle group. Further, mice treated with 1000 and 1500 mg/kg bw of KGAJOS and piracetam took significantly less time to reach the platform quadrant compared to vehicle treated mice as they slowly learn the escape platform location using objects or symbols placed outside the maze as cues, and progressively swim to the platform in a shorter time[29,30], which is again an indicator of improvement in spatial memory.

Most of the herbal ingredients of KGAJOS or their phytoconstituents have been extensively reported to exhibit improvement in cognitive function. Cocoon of *Bombyx mori* which is composed of two proteins i.e., fibroin (72-81%) and silk gum or sericin (19-28 %)[1]. The silk fibroin protein enzymatic hydrolysate is reported to significantly improve memory in healthy adults at the daily doses of 280 mg and above for three weeks[2]. Oral administration of *Melissa officinalis* (lemon balm) extract (200 mg/kg) significantly augmented learning and memory of naïve rats and significantly ameliorate scopolamine-induced learning deficit[3]. Similarly, repeated administration of a 50% ethanol extract of *M. officinalis* leaves (200 mg/kg, p.o. for 28 days) showed an improvement in long-term memory in rats and a decrease of Acetylcholinesterase (AChE) mRNA level by 52% in the cortex and a strong inhibition of BACE1 mRNA transcription (64% in the frontal cortex; 50% in the hippocampus) was also observed[4]. A randomised, placebo-controlled, double-blind, balanced-crossover study investigated the acute effects of standardised extract of *M. officinalis* on cognition and mood. There was improvement in Accuracy of Attention following 600 mg of *M. officinalis* and time- and dose-specific reductions in both Secondary Memory and Working Memory factors[5]. Protective effects of *Borago officinalis* extract has been reported on Amyloid β (Aβ)-induced memory impairment in rats[6]. It is also reported that that Aβ (25-35) can effectively inhibit long term potentiation in the granular cells of the dentate gyrus in hippocampus, and *B. officinalis* supplementation reverse the synaptic plasticity in dentate gyrus following Aβ treatment and may lead to an improvement of Alzheimer's disease -induced cognitive dysfunction[7]. Hydro-alcoholic extract of *Salvia haematodes* root possesses protective effect on cognitive functions in scopolamine-induced amnesia in rats and may prove to be a useful memory restorative agent in the management of cognitive dysfunctions[8]. Dose-dependent improvement in memory scores was reported in young as well as aged mice following dietary administration of *Coriandrum sativum* leaves for 45 days. It also reversed the memory deficits induced by scopolamine and diazepam. Brain cholinesterase activity was also considerably reduced[9]. Oral administration of *C. sativum* seed extract for 12 weeks is reported to ameliorate age induced memory deteriorations in the senescence-accelerated SAMP8 mouse model[10]. Phytochemical investigation of *Delphinium denudatum* resulted in the isolation of isotalatazidine hydrate in crystalline form which is a potent dual cholinesterase inhibitor and may be used as a target drug in Alzheimer diseases[11]. Norditerpenoid alkaloids of *D. denudatum* were also reported as cholinesterase inhibitors[12]. Oral administration of ethanolic root extract of *Paeonia emodi* (300 and 600 mg/kg) is reported to effectively improved impaired learning and memory performance in Morris water maze in pentylenetetrazole kindled mice[13].

Efficacy of KGAJOS observed in MWM test confirmed the traditional claims and usage of this formulation. The observed efficacy of KGAJOS in MWM test is in agreement with the reported pharmacological activities of individual phytoconstituents of KGAJOS and their synergistic effect on memory and cognition.

Conclusion

Morris water maze experiment conducted in mice revealed improvement inlearning and memory function at the dose levels of 1000 and 1500 mg/kg bw of KGAJOS whereas 500 mg/kg bw was not found to be effective. Observed efficacy of KGAJOS confirmed the traditional claims and usage of this formulation in conditions associated with cognition and memory.

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