Original Research Article

Ayurvedic formulations amalaki rasayana and rasa sindoor improve age-associated memory deficits in mice by modulating dendritic spine densities

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1. Introduction

Ageing is a physiologically complex dynamic process that is yet to be fully understood [1]. Studies have shown that certain pathologies, such as brain injuries and depression, make the brain more susceptible to the ageing process [2,3]. An intriguing study involving 4263 people, in the age range of 18–95 years old, reported that proteins varying with age undergo a shift at ages 34, 60, and 78 years [4]. Interestingly, cognition declines during the young to middle age transition [5–7]. Intriguingly, ageing is the most significant risk factor for neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease [8,9]. Therefore, it is likely that lifestyle interventions beginning at an early age can help delay the ageing process, thereby reducing cognitive deficits associated with advanced age and susceptibility to neurodegenerative disorders. Such interventions are expected to improve or maintain cellular homeostasis and either prevent or reverse ageing-related cellular damages. For example, stimulation of autophagy by pharmacological interventions such as rapamycin or fasting could...
enhance protein clearance and turnover [10–12] and administration of anti-oxidative compounds [13] could delay the cellular ageing process. In humans, intermittent fasting interventions were shown to ameliorate obesity, insulin resistance, dyslipidaemia, hypertension, and inflammation [14]. Senolytics are compounds with the potential to selectively eliminate senescent cells and promote health in ageing individuals. The application of senolytics targeting various molecular pathways is known to slow down the ageing process and increase lifespan [15,16].

Ayurveda—the traditional Indian medical system—also has a branch practice named Rasayana that is dedicated to rejuvenation and healthy ageing [17]. For example, amalaki rasayana (AR) and rasa sindoor (RS)—two of the formulations described in the rasayana—are known to alleviate age-related decline of physiological functions, bring about healthy ageing [18,19], and reduce arthritis-associated knee pain [20,21]. While the exact mechanism of action of rasayanas is not well understood, studies have shown that AR, for example, improves telomerase activity and telomere length [20,21]. Moreover, both AR and RS are known to enhance levels of cAMP-response element-binding protein (CBP/p300), resulting in better stress tolerance and suppression of neurodegeneration in fly and mouse models of neurodegeneration [22–25]. Therefore, we hypothesised that these Ayurvedic formulations might be neuroprotective and thus might improve cognitive functions of aged animals. Using wild-type mice, we demonstrate that AR or RS administration improves cognition and memory in middle-aged wild-type mice, possibly by altering the dendritic spine densities.

2. Material and methods

2.1. Chemicals and reagents used

AR and RS (procured from Arya Vaidya Sala, Kotakkal, India), α-amylase (Cat. No. 28588), periodic acid (Cat. No. 19184), haematoxylin (Cat. No. 48441) (all from Sisco Research Laboratories Pvt. Ltd., India), Schiff reagent (Cat. No. 141407), Thomas Baker Chemicals Pvt. Ltd. India), donkey serum (Cat. No. S30), DPX (Cat. No. 61803502501730) (all from Merck, India), DAB chromogen kit (Cat. No. 1610500011730; Bangalore Genei Pvt. Limited, India), anti-glial fibrillary acidic protein (GFAP) antibody (1:500 for immunohistochemistry (IHC); Cat. No. ab7260), anti-ionised calcium-binding adapter molecule 1 (Iba1) antibody (1:500 for IHC; Cat. No. ab5076) (both from Abcam, USA), all secondary antibodies were procured from Jackson ImmunoResearch Laboratories, Inc., USA and used in 1:400 dilution for IHC.

2.2. Mouse models and ethical approval

We used male and female mice (C57BL/6j strain) for our study. All mouse colonies were maintained in the institutional animal housing facility at 22 ± 1 °C in a 12:12-h light/dark cycle with access to food and water ad libitum. All treatments and behavioural experiments were conducted in strict accordance with the approved protocol and guidelines of the institutional animal ethics committee.

2.3. Drug administration

Mice were fed orally with AR (1025 mg/kg per day) or RS (41 mg/kg per day) prepared in gelatin blocks (12% gelatin w/v in sterile distilled water) flavoured with cocoa (5 gm/ltr). The dosage was equivalent to those reported for human use [24,26] and converted to mouse dose using body surface area conversion as described earlier [27]. Jelly blocks lacking the Rasayanas, called control jelly (CJ), served as a vehicle. AR (5 mg/mL) and RS (0.2 mg/mL) containing jelly were prepared similarly; all jelly blocks (CJ, AR, and RS) were cooled and stored at 4 °C until use. Animals were force-fed these formulations by replacing food and water daily for 12 h at night with CJ, AR, or RS jelly, while control animals received regular chow with water. The next morning, unused jellies were removed, and chow and water were provided ad libitum for the next 12 h. We started the treatment at 2 months of age and continued it for the next 6 months.

2.4. Behavioural assays

Apparatuses used in our study had white floors to provide optimum contrast with respect to the mice used in all behavioural experiments. All apparatuses were kept on the floor. A monocrome camera was fitted on the ceiling of the behavioural testing room to get a top view so that the whole apparatus for the paradigm being tested was visible in a single field. Signal obtained from the camera was recorded and processed using ANY-maze software (Stoelting Co., Wood Dale, USA) to obtain various parameters in the behavioural testing. Illumination was kept constant throughout the behavioural testing room at 45–50 Lux so that the mice being tested could not see the light sources directly. All tests were performed between 8:00 am and 6:00 pm to minimize the effect of the circadian rhythm.

2.5. Radial arm maze (RAM) test

We used an eight-armed RAM (arms numbered from 1 to 8) in our study to test short-term (working) and long-term (reference) spatiotemporal memories of mice from control and treatment groups essentially as described previously [28]. The spatial cues were placed on the walls of the behavioural testing room to help mice orient themselves in space and help them identify and learn the positions of the arms. Four of the arms contained small food pellets (baited arms—1, 2, 4, and 7), and the other four arms did not contain any food (unbaited arms—3, 5, 6, and 8) (as shown in Fig. 1A). Mice were partially starved before being tested in RAM. Visits to the unbaited arms were scored as reference memory errors. The first visit to a baited arm was considered a correct choice. However, subsequent visits to the same arm or visits to the baited arm and not eating the food pellet were scored as working memory errors. The experiment was repeated for 16 consecutive days, with one trial per day for each mouse. The errors were pooled from four trials for statistical analyses and plotting the data.

2.6. Novel object recognition (NOR) task

NOR task was performed essentially as described earlier with minor modifications [29,30]. Briefly, the test apparatus consisted of a 40 cm × 40 cm arena with 40 cm high opaque walls. The NOR task consisted of two familiarisation trials with similar objects (denoted as familiar objects) and a third trial where one of the familiar objects was replaced with a novel object of similar size but of different shapes and colours. All trials were conducted for 10 min. The position of the objects was constant across all trials and was placed at 7 cm from two adjacent corners. Mice were gently placed in the testing apparatus, always facing away from the objects, and the time spent by mice exploring the novel and familiar objects was quantified, analysed, and plotted to quantify episodic memory deficits. The discrimination index, an indicator of the ability of a mouse to distinguish a novel object from a familiar object, was calculated using the following formula:

\[
\text{Discrimination Index} = \frac{t_{\text{novel}} - t_{\text{familiar}}}{t_{\text{novel}} + t_{\text{familiar}}}
\]
Discrimination index = (Exploration time with novel object)/(Total exploration time with the known and novel objects).

2.7. Open field test (OFT)

The OFT was performed to assess the anxiety of open spaces in mice as described previously [31] with minor modifications. The OFT apparatus consisted of an open arena of 40 cm × 40 cm, enclosed by 40 cm high walls. The arena was virtually divided into two zones using ANY-maze software; an outer zone that extended 7 cm inwards from the walls and an inner zone consisted of the remaining central part. Mice were gently taken from holding cages, put in the apparatus always facing the same wall, and their activity in the arena was recorded for 10 min. This was repeated once a day for three consecutive days, the first two days being training trials and the third day being scored as the test trial. Only the third trial was used for analysing the distance travelled and time spent by the mouse in the inner zone to assess anxiety.

2.8. Elevated plus maze (EPM) test

The EPM test was performed to assess the anxiety of elevated heights in mice. The EPM apparatus consisted of two open arms and two arms enclosed by 40 cm high walls, all placed at a height of 50 cm from the ground. Each open and closed arm was 5 cm wide and 50 cm long. The open and closed arms intersected each other to make a shape of ‘+’ at the centre of the apparatus. The test was performed essentially as described earlier [31,32]. Briefly, each mouse was placed in the apparatus at the intersection of arms, always facing the open arms. Distance travelled and the time spent in open arms was plotted and analysed for assessing anxiety. The test was performed once a day for three consecutive days, with the first two being training and the third being the test trial. Data from the third test trial was used for analysing anxiety in animals.

2.9. Tail suspension test (TST)

TST was performed to assess the depressive phenotype in mice, essentially as already described [31,33]. We hung the mice from their tails using adhesive tape to a stationary horizontal pole. The head of the mouse being tested was kept 15–20 cm above the ground level, and its movement was videotaped for 6 min. For scoring the depressive phenotype, time spent by each mouse in showing escape-related behaviour (mobile time)—running like movements of one or both hind limbs and the attempts to grasp any possible support by forelimbs while bending their bodies was quantified. We used XNote stopwatch (http://www.xnotet stopwatch.com/) to keep track of time during scoring. We bound the start/stop of the XNote stopwatch to a specific key to avoid any bias. The immobile time (total duration minus the mobile time), which directly correlated to depressive phenotype, was quantified, analysed, and plotted.

2.10. Golgi-Cox staining

For staining dendritic spines, Golgi-Cox staining was performed essentially as described earlier [34] with minor modifications. Normal saline-perfused mouse brains were incubated in filtered Golgi-Cox stain for 15 days in the dark at room temperature, and the stain was changed every alternate day. After 15 days of incubation in the stain, the brains were transferred to a 30% sucrose solution and incubated at 4 °C in the dark. After 24 h, the sucrose solution was replaced, and the brains were incubated at 4 °C for four more days. Then, 100-μm thick coronal sections were cut using a vibrating microtome (VT 1000S, Leica Biosystems, USA) in 30% sucrose solution. Sections were transferred to glass dishes using a paintbrush, and processing was done in a fume hood under low-light conditions. Sections were washed twice in water for 1 min each and then for 5 min in 50% ethanol, followed by incubation in a 3:1 ammonia solution for 15 min. Sections were washed twice with water and incubated in 5% (w/v) sodium thiosulphate for 15 min. Sections were dehydrated in graded ethanol and mounted on glass slides using DPX. Slides were air-dried in a dark place at room temperature for at least 24 h before imaging.

2.11. Periodic acid- Schiff (PAS) staining

For staining corpora amylacea, we performed PAS staining, essentially as described earlier [35]. Briefly, paraffin-embedded brain blocks were cut into 5-μm thick sections. The sections were rehydrated in serial dilutions of ethanol and were incubated in 1 mg/mL α-amylase (in PBS) for 5 min at 37 °C. Then, the sections were incubated in periodic acid (2% w/v) for 20 min at room temperature, followed by incubation in Schiff’s reagent for 30 min. Slides were washed under running tap water for 10 min, counterstained with haematoxylin, dehydrated, and mounted in DPX.
2.12. Immunohistochemical staining

Immunohistochemistry was performed as described previously [36]. For this, 5-μm thick brain sections were cut from paraffin-embedded brain blocks. Sections were rehydrated in the graded dilutions of ethanol. Antigen retrieval was done by boiling the slides in a Tris–EDTA buffer (10 mM tris base, 0.5 mM EDTA, pH 9.0) for 30 min. The sections were permeabilized in 0.2% Triton X-100 in 1×TBST (tris buffered saline containing 0.025% Triton X-100). Primary antibodies were diluted in 10% (v/v) donkey serum blocking, applied to the sections, and incubated at 4°C overnight. Sections were then incubated in HRP-conjugated secondary antibodies, and the signal was developed using a DAB kit according to the manufacturer’s recommendations. Sections were dehydrated in graded ethanol solutions and mounted in DPX.

2.13. Image acquisition and analysis

Brightfield images for immunohistochemical and PAS-stained sections were acquired on Nikon Eclipse Ci microscope using 40× oil objective. For quantification of the signal intensities, ImagePro 6.0 (Media Cybernetics) and ImageJ [37] software were used. Quantification of astrocyte and microglial activation was performed as described earlier [38,39]. For quantifying the arborisation of GFAP stained cells, we used the Simple Neurite Tracer plugin in ImageJ [40]. The same plugin was used to measure the maximum caliper’s radius, an indicator of the spread of the branching of a cell.

2.14. Statistical analysis

All results are expressed as mean ± standard error of the mean in the histograms. Box plots show median and first and third quartiles; the lower and upper whiskers represent the lowest and highest values. Ordinary one-way analysis of variance and Student’s t-test was applied for calculating the statistical significance of the differences. GraphPad Prism software (GraphStats Technologies Pvt. Ltd., India) was used for the statistical analysis.

3. Results

3.1. Ageing mice treated with AR or RS show improvement in short-term and long-term spatiotemporal memories

Since RS was shown to improve memory and energy metabolism of the central nervous system in mouse models of Alzheimer’s disease [42], and since AR reduces age-related problems in humans [14], we wanted to check if Ayurvedic formulations would improve the memory deficits known in the aged mice [43]. We tested the effect of AR and RS on short- (working) and long-term (reference) spatiotemporal memories in middle-aged mice using the RAM test (Fig. 1A). Total repeated visits made to the baited arms after eating the food pellet in respective arms and baited arms but not eating the food pellet were scored as errors in working spatiotemporal memory. We found that mice fed with AR or RS made significantly fewer working spatiotemporal memory errors than those made by control or CJ-fed mice (Fig. 1B). For reference memory, total visits that the mouse made to the unbaited arms were scored as reference memory errors. Mice fed with AR or RS showed a significantly lower number of reference memory errors as compared to those made by control or CJ-fed mice (Fig. 1C).

Next, we wanted to check if AR or RS treatments would improve episodic memory deficits associated with age [44]. For this, we performed NOR task in middle-aged mice (Fig. 2A). NOR exploits the inherent exploratory nature of mice for novel objects. Mice with a normal memory will tend to explore the novel object for a longer time as compared to the time spent exploring a familiar object. We found that AR or RS treatment led to mice spending a significantly longer duration exploring the novel object as compared to the familiar object (Fig. 2B and C). We also quantified the discrimination index, calculated as the ratio of time spent with a novel object to the total exploratory time with novel and familiar objects combined, as an indicator of how well the mice can discriminate the familiar and novel objects. The scores closer to 1 indicate better discrimination. Moreover, the better the memory, the higher would be the discrimination index score obtained by the mouse. We found that mice fed with AR or RS had a significantly higher discrimination index as compared with that of control mice (Fig. 2D). However, the difference in the discrimination index between the CJ-treated group and the AR or RS treated mice was not statistically significant, although the plot showed a trend indicative of the beneficial effect of the two Rasayanas compared to the control jelly. Since gelatin is a proteinaceous material, it is less likely that the control jelly could have had a beneficial effect. One possibility could be the animal-to-animal variations, and the difference could be statistically significant if we increase the number of animals. Nonetheless, our observations are strong enough to conclude that the AR and RS treatments significantly improved episodic memory in middle-aged mice as compared to the untreated control group.

3.2. Treatment with AR or RS does not significantly affect anxiety and depressive phenotype in mice

Anxiety and depression are well established in middle-aged mice [45,46]. To assess whether AR or RS impacts these, we quantified anxiety and depression in middle-aged mice from control and treatment groups. For this, we tested two different types of anxious behaviour: the anxiety of open spaces and elevated places using OFT and EPM test, respectively. Mice show an inherent aversion to open spaces and elevated heights. However, after repeated exposure to the same apparatus, mice would show exploratory behaviour and will start exploring the central zone in OFT (Fig. 3A) and open arms in EPM (Fig. 3E). Mice with anxiety would have a reduced tendency for these exploratory behaviours. In OFT, we quantified and plotted the total distance travelled in the apparatus (Fig. 3B), distance in the central zone (Fig. 3C), and the time spent in the central zone (Fig. 3D) from the third trial. The mice fed with AR or RS did not show a significant difference either in the distance travelled or the time in the central zone of OFT as compared to those from control or CJ-fed mice (Fig. 3C and D). Using EPM test, we quantified and plotted the total distance travelled in the apparatus (Fig. 3F), total distance travelled in the open arms (Fig. 3G), and the time spent in the open arms (Fig. 3H) from the third trial. Mice fed with AR or RS did not show a significant
difference either in the distance travelled or the time spent in the open arms as compared to those from the control or CJ-fed mice (Fig. 3G and H).

For assessing the depressive phenotype, we employed TST to control, CJ-, AR-, and RS-fed mice. Mice hung from their tails would tend to escape and show escape-related behaviour. Conversely, mice with a depressive phenotype would have a lower tendency to show escape-related behaviour and an increased immobile time. The immobile time directly correlates with the depressive phenotype. Treatment of mice with AR or RS did not significantly affect the immobile time compared to those of control or CJ-fed mice (Fig. 3I).

3.3. Treatment with AR or RS increases the dendritic spine density in mice

Dendritic spines are the small button-like protrusions present on the dendrites and are the physical sites of synapse formation [47]. The dendritic spines are highly dynamic structures, and their size and number change with the synaptic strength [48,49]. Moreover, they are remodelled during learning and memory [49]. Since both AR and RS improved memory, we sought to find if the treatments had any effect on the dendritic spine density or morphology in mouse brains. For this, we used Golgi-Cox staining to stain the neurons (Fig. 4A). We quantified the length and width of dendritic spines and the number of dendritic spines per unit length (spine density) on the primary apical dendrites of hippocampal pyramidal neurons. We found that AR or RS treatment increased the dendritic spine density significantly as compared to those of control or CJ-fed groups (Fig. 4B). AR- or RS-treated mice also showed longer dendritic spines as compared to those of control or CJ-fed mice (Fig. 4C). However, AR or RS treatment did not significantly affect the dendritic spine width as compared to those of control or CJ-fed mice (Fig. 4D).

3.4. Treatment with AR or RS does not affect the formation of corpora amylacea in mice brain

Corpora amylacea are the granular structures composed mainly of glucose polymers and some proteins that accumulate in the brain during normal ageing and neurodegenerative conditions like Alzheimer’s disease, vascular dementia, and certain epilepsies [50,51]. Their presence in the ageing brain may indicate their formation as an ageing by-product. Furthermore, they may be neuroprotective by sequestering toxic proteins during pathological conditions like Alzheimer’s disease [52]. Since corpora amylacea are conceived as containers of cellular waste, we wanted to test if AR or RS treatment has any effect on their number in middle-aged mice. For this, we stained brain sections with PAS stain, imaged using a brightfield microscope, and quantified the \( \alpha \)-amylase-resistant corpora amylacea (Fig. 5A). The treatment of mice with AR or RS did not significantly change the average size of corpora amylacea compared to those in control or CJ-fed mice for any of the areas scored (Fig. 5B).

3.5. AR or RS treatment has no effect on gliosis in middle-aged mice

Age-associated inflammation contributes to various age-related problems, including psychological and cognitive decline, morbidity, and mortality [53,54]. Intriguingly, dendritic spine number and...
Fig. 3. *Amalaki rasayana* or *rasa sindoor* treatment does not significantly affect anxiety and depressive behaviours in the middle-aged mice. (A) The heat map diagram showing the average occupancy duration in the open field apparatus by the middle-aged mice from the control or Rasayana treated groups, as indicated. In the heat map, the blue and yellow colours represent, respectively, the minimum and maximum occupancy time displayed by the animals and 'CZ' represents 'central zone' in the open field apparatus (A). (B, C, D) Scattered plots showing the total distance travelled by mice in the open field apparatus (B), the distance travelled in the central zone (C), and time spent in the central zone (D), as indicated. (E) The heat map diagram showing the average occupancy rate in the elevated plus maze (EPM) apparatus by the middle-aged mice from the control or Rasayana treated groups, as indicated. (F, G, H) Scattered plots showing the total distance travelled by mice in the elevated plus maze apparatus (F), the distance in the open arms (G), and time spent in the open arms (H), as indicated. (I) Scatter plot showing the immobile time in seconds (S) by the middle-aged mice from the control or Rasayana treated groups, as indicated.
Fig. 4. *Amalaki rasayana* or *rasa sindoor* treatment increases dendritic spine density and average spine length in the middle-aged mice. (A) Representative microscopic images of Golgi-impregnated thick brain sections from middle-aged mice from the control or *Rasayana* treated groups showing spines on the primary dendrites of the hippocampal pyramidal neurons, as indicated. Magnified views of the section, identified in the red colour rectangle (upper panel), are shown in the lower panel. (B) Bar diagram showing the mean spine density (numbers of spines per 10-μm dendritic segments) in the hippocampal pyramidal neurons from the control and rasayana treated groups. (C, D) As indicated, the cumulative frequency percentage plots show the difference in length (C) and width (D) of dendritic spines of pyramidal neurons from the different groups. Here, ‘C’ refers to ‘control’, ‘CJ’ refers to ‘control jelly’, ‘AR’ refers to ‘Amalaki rasayana’, and ‘RS’ refers to ‘rasa sindoor’. Ordinary one-way ANOVA was performed to assess the statistical significance of the difference in means of various groups, where \( p < 0.05 \) and \( **p < 0.01 \) when compared to untreated control mice (N = 5 in each group, scale bar 20 μm).

corpora amylacea were shown to correlate with neuroinflammation in animal models [55,56]. Therefore, we wanted to assess the contribution of inflammation in AR- or RS-fed mice in improving memory and dendritic spines. For this, we stained sagittal brain sections from the control and treatment groups for markers of astrocytes and microglia, respectively—GFAP (Fig. 6A) and Iba1 (Fig. 7A). Glial activation leads to an increased arborisation of astrocytes and microglia. To assess the activation of astrocytes, we quantified the total length, the number of branches as a function of the distance from the nucleus, and the maximum calliper’s distance (the largest distance between two extremes of the astrocytic branches) of the GFAP-stained astrocytes. We did not find a significant difference in the total length, branching, or the maximum calliper’s distance in AR- or RS-fed mice as compared to control or CJ-fed mice (Fig. 6B–D). To evaluate the effect of the treatments on microglial activation, we quantified the total area of Iba1 staining in each microscopic field (Fig. 7B) and the average area of Iba1-stained microglial cells (Fig. 7C). We did not find a significant difference in the total area occupied by the immunoreactive cells in each microscopic field and the average area of each cell from the AR- or RS-fed groups as compared to those from the control or CJ-fed groups, indicating that the AR or RS treatment did not significantly affect the activation of microglia for this age group (Fig. 7B and C).

4. Discussion

The process of ageing can be defined as the progressive failure of the body to maintain adaptive homeostasis. Intriguingly, a decline in the homeostatic processes begins at the cellular level [57]. These include the defective signalling pathways for protection against stressors such as heat shock, hypoxia, oxidative stress, and abnormal/unfolded proteins. These cellular deficits may eventually affect the organismal function, resulting in the onset of age-associated phenotypic alterations. Several studies have shown that the ageing process begins during the transition from young to middle age [5–7]. Thus, any effort to prevent or delay the process should begin during this transition stage. Indeed, the *Rasayana* branch of *Ayurveda* advocates rejuvenation as the process to bring about healthy ageing [17], and AR and RS are two such *Rasayanas* that are known to confer neuroprotection in humans [20,21] and in animal models of neurodegenerative diseases [17,19].
demonstrate here that the prolonged administration of AR or RS led to a significant improvement of episodic and working spatiotemporal memories in middle-aged mice. However, only the treatment with AR led to a significant improvement in the reference spatiotemporal memory in these mice. Thus, the two Rasayanas appear to have a differential effect on the brain, and thus, the observed difference in the cognitive functions is likely due to specific effect on different brain regions [58, 59]. It may be noted here that while AR is a completely herbal derivative, RS is an organometallic derivative of mercury, and therefore these two formulations could have some shared and distinct influences on physiology. Alternatively, the metabolites of the two Rasayanas could have some distinct identities and modes of action.

One of the intriguing observations of this study is that the Rasayanas used did not show a significant effect on anxiety and depressive behaviour exhibited by middle-aged mice. This is despite the positive effect of the Rasayanas on the spatiotemporal and reference memory in the same group of animals, suggesting that the Rasayanas may not have a similar effect on all cognitive abilities. An alternate possibility could be that memory deficit sets in earlier in the lifespan as compared to anxiety and depression behaviour which probably begin during the middle-age [45]. Thus, more extended treatment with Rasayanas, beyond the 6-to-8-month window, could have also altered the anxiety and depression behaviour. Since the underlying mechanisms governing the distinct cognitive abilities remain elusive and since the exact

![Fig. 5](image1.png)

**Fig. 5.** *Amalaki rasayana* or *rasa sindoor* does not significantly affect corpora amylacea deposition in the middle-aged mice brains. (A) Representative microscopic images of brain sections of the middle-aged mice from the control or rasayana treated groups showing periodic acid-Schiff (PAS)-positive corpora amylaceae (CA) in the hippocampal areas, as indicated. The enlarged areas revealing the pink coloured corpora amylaceae are shown in the lower panels (black arrows). (B) Histograms representing the quantification of the size of corpora amylaceae as number of pixels of PAS staining in hippocampi of the control and Rasayana treated groups. The data is represented as the mean ± SEM from each group. Here, 'C' refers to 'control', 'CJ' refers to 'control jelly', 'AR' refers to 'Amalaki rasayana', and 'RS' refers to 'rasa sindoor'. Ordinary one-way ANOVA was performed to assess the statistical significance of the difference of the means, and no significant differences in the means were observed (N = 5 in each group, scale bar 20 μm).

![Fig. 6](image2.png)

**Fig. 6.** *Amalaki rasayana* or *rasa sindoor* does not significantly affect the activation of astrocytes. (A) Representative brightfield microscopic images of the brain sections showing the distribution of GFAP-positive astrocytes in the hippocampi of the control and Rasayana treated middle-aged mice, as indicated. (B, C, D) Histograms showing quantification GFAP-positive astrocytes. The total length of processes (B), the number of branches as a function of distance from the nucleus (C), and the maximum calliper's distance (D) of GFAP-stained cells as quantified using the densitometric analyses are plotted. The data is represented as the mean ± SEM from each group, and each dot represents the time or distance quantified for each mouse. Here, 'C' refers to 'control', 'CJ' refers to 'control jelly', 'AR' refers to 'Amalaki rasayana', and 'RS' refers to 'rasa sindoor'. Ordinary one-way ANOVA was used to analyse the statistical significance of the difference in the means, where no significant differences in the means were observed (N = 4, scale bar 50 μm).
molecular pathways through which the Rasayanas work are not yet worked out, it is imperative that further studies are warranted to understand the differential effect of Rasayanas on the cognitive abilities.

Dendritic spines are membranous protrusions of dendrites that receive inputs from excitatory axons. Although these spines are considered dynamic structures, chronic stress or abnormalities in the neurons are known to alter the spine densities or their maturation [60]. Indeed, a reduction in the number of spines is observed with advancing age [61], thus correlating with the decline in cognitive functions [62]. We show here that AR or RS treatment increased dendritic spine number per unit length after the treatments. While we have not yet understood the exact mechanism of action of these two Rasayanas, it is tempting to speculate that both AR and RS could have either suppressed the physiological stress in the neurons or altered the expression of proteins that modulate the dendritic spine formation and density. Nonetheless, the increased number of dendritic spines might be underlying the observed improvements in the episodic and working- and reference-spatiotemporal memories. Our findings are in concordance with the finding of previous studies, which show a direct correlation between an increase in the number of spines and better memory [63–66].

Ageing is often associated with cognitive decline and concomitant deficits in molecular pathways like heat shock, glycogen metabolism, oxidative stress, and neuroinflammation [67–70]. Intriguingly, when we analysed neuroinflammation and accumulation of corpora amylacea, two pathways known to be affected with aging, we did not find any difference in the activation of microglia and astrocytes or the number of corpora amylacea in the brains of the treated animals. One possibility could be that middle-aged mice used in this study did not show such a striking difference in the levels of such readouts (while older animals would have revealed), or that an increase in the duration of the treatment could have led to a better outcome. Clearly, further studies on older mice are required to dissect the molecular pathways through which these Rasayanas confer neuroprotection. In conclusion, the findings from this study indicate a protective role of Ayurvedic formulations on some of the age-associated anomalies in middle-aged mice. Moreover, we establish that age-associated cognitive decline that begins with the transition from young to middle age can be averted if these formulations are administered at a young age.

Author contributions

The conceptualization, methodology, and study design were done by SG and BV. Validation, formal analysis, and investigations were done by BV and PS. Data curation, writing – review and editing, and visualization were done by BV, PS, and SG. The original draft was written by BV. Provision of resources, supervision, project administration, and funding acquisition were done by SG. All the authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors have no competing interests.
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