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Exploring the combined protective effect of *Rosa damascena* and *Commiphora wightii* in alleviating aluminum-induced neurotoxicity in *Drosophila melanogaster*: Insights from behavioural, synaptic, mitochondrial, and oxidative dysfunction

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ABSTRACT

Introduction: Metals are crucial for cellular physiology; however, their excessive accumulation triggers neuronal damage and leads to the progression of diverse neurodegenerative disorders, notably Alzheimer's disease (AD). Accumulating evidence explored several plants and their active metabolites with established antioxidant properties as critical therapeutic interventions to allay metal-mediated neurotoxicity. In this avenue, both *Rosa damascena* and *Commiphora wightii* have demonstrated significant antioxidant and neuroprotective behaviours. Thus, the present research investigated the effects of *R. damascena, C wightii,* and their significant phytoconstituents, per se and in combinations, in the aluminum chloride ($AlCl_3$)-induced neuronal damage in Drosophila.

Methods: Following dose optimization via 7-day toxicity and 14-day survival assays, flies (50 per vial) were exposed to $AlCl_3$ and various treatments for 7 consecutive days. On 7th day, the oxidative stress markers, the level of neurotransmitters and mitochondrial complexes were assessed following locomotion and memory assessment.

Results: Results demonstrated that *R. damascena* and *C. wightii* per se enhanced motor activity and exhibited antioxidant potential in fruit flies, and their combination showed more significant improvement ($P<0.001$). Similarly, a noteworthy modulation in the mitochondrial complexes and neurotransmitter levels was also observed. Moreover, their phytoconstituents combination, i.e. geraniol and guggulsterone, respectively, was also found effective in mitigating the neurotoxic effect induced by $AlCl_3$ overexposure, demonstrating a significant improvement in neuroprotective effect in flies.

Conclusion: The overall outcomes suggest the potential of *R. damascena* and *C. wightii* or their combination as therapeutic agents for aluminum-induced neuronal toxicity and related neurodegenerative conditions.

Implication for health policy/practice/research/medical education:
This research paper highlights the neuroprotective effect of *Rosa damascena, Commiphora wightii,* and their significant phytoconstituents, i.e. geraniol and guggulsterone, alone or in combinations, against aluminum chloride ($AlCl_3$)-induced neuronal damage in fruit flies. A combination of *R. damascena* and *C. wightii* plant extract significantly improves behavioural, synaptic, mitochondrial, and oxidative dysfunction.

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Introduction

Metals are crucial for life, but typically in minimal amounts; however, prolonged exposure to heavy metals like copper, chromium, arsenic, lead, mercury, and aluminum over time constitutes a silent yet potent insult to the central nervous system (1). These metals, beyond their well-known systemic toxicities, trigger a diverse array of pathological cascades within the brain, including oxidative

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damage, DNA fragmentation, mitochondrial dysfunction, protein misfolding, autophagic modulations, synaptic dysfunctions, and neuronal death, ultimately contributing to a spectrum of neurological disorders. (2,3). Several experimental and human studies affirm the implication of metal-induced neurotoxicity in the pathogenesis of diverse neurodegenerative ailments, including Alzheimer's disease (AD) (4). Nonetheless, aluminum exposure poses a substantial threat to neurological health, inducing a complex cascade of cellular dysfunctions contributing to an array of neurological disorders, highlighting the need for targeted preventative and therapeutic strategies (5,6).

Currently, the landscape of toxicological research is undergoing a substantial shift, with *Drosophila melanogaster* gaining wide recognition and also being widely acknowledged for exploring the therapeutic potential of the diverse array of compounds and natural products. *D. melanogaster* (fruit fly) proves to be a predominantly advantageous alternative animal model, and its remarkable degree of genetic homology, with approximately 75% of human disease-associated genes, provides a powerful platform for translational research (7). In addition, the shared repertoire of neurotransmitters between humans and fruit flies further affirms its relevance in studying neurodegenerative disorders, particularly AD (8). Furthermore, the practical benefits of Drosophila, including short lifespan, high fecundity, and low maintenance requirements, facilitate large-scale and high-throughput experiments, offering statistically robust data (7). These characteristics are essential for exploring the nuanced effects of environmental toxins and screening potential therapeutic interventions.

Recently, intensive research has highlighted the therapeutic prominence of natural products as viable alternatives in augmenting neuronal survival against metal-mediated neurotoxicity by acting as metal chelators and scavengers of reactive oxygen species (ROS) (9,10). The Damask rose, *Rosa damascena*, has long been recognized for its diverse medicinal properties for ages, and accumulating evidence has unveiled its significant neuroprotective potential in augmenting neurogenesis, diminishing oxidative stress, mitigating neuroinflammation, and elevating synaptic plasticity (11). Among the array of bioactive components within *R. damascena*, geraniol, one of the abundant monoterpenes, has also shown noteworthy multifaceted therapeutic action, including protection of neurons from neurotoxin injuries, suppression of neuroinflammation, and augmentation of memory, learning, and cognitive function via impeding acetylcholinesterase (AChE) activity (12). Similarly, *Commiphora wightii*, commonly known as guggul, is a well-established hypolipidemic agent (13) and is widely being explored for its potential cognitive and antioxidant benefits as well (14). Further, the dual therapeutic promise (cognitive and antioxidant benefits) stems mainly from *C. wightii* resin, particularly the bioactive compound guggulsterone. However, guggulsterone also extends its pharmacological efficacies beyond lipid regulation by preserving neuronal integrity and improving learning and cognition in several experimental models via mitigating oxidative stress, neuroinflammation and synaptic dysfunction (15). Consequently, *C. wightii* and its phytoconstituent present a unique therapeutic profile, simultaneously addressing cardiovascular risk factors and offering neuroprotective benefits.

The polygenic and multifactorial pathogenesis associated with metal-induced neurotoxicity underscores the necessity for therapeutic interventions capable of simultaneous modulation of multiple pathways. Consequently, a combination of plant extracts may offer a promising rational strategy for augmenting neuroprotection, thereby improving therapeutic effectiveness, lessening side effects, and promoting additive or synergistic benefits (16). Hence, the combination of both plant extracts is highly likely to exert its neuroprotective effects through diverse and complementary mechanisms via anti-inflammatory, antioxidant, AChE inhibition, metabolic regulation, and lipid modulation. Thus, the current investigation explores the combined neuroprotective effects of *R. damascena* and *C. wightii* and their key components on oxidative harm, neurotransmitter levels, and mitochondrial function in a fruit fly against aluminum-induced neurotoxicity.

Materials and Methods

Chemicals

All solvents of analytical grade were used, and their purity was checked using blank runs. Geraniol was purchased from CDH Chemicals, India, Guggulsterone from Sigma-Aldrich, USA, and an extract of *C. wightii* from Vital Herbs (consisting of 2.5% guggulsterone), New Delhi. Aluminum chloride, acetylthiocholine iodide, n-butanol, and all the required chemicals used in the experimental study were purchased from Loba Chemie Pvt Ltd., India.

Extraction of Rosa damascena

Fresh flowers of *R. damascena*, collected from the University's botanical garden, were air-dried and pulverized at room temperature. Then 100 g of dried powder was extracted with 250 mL of hydro-alcoholic solution (70:30) using maceration for 3 days, as described previously (17). Nevertheless, for the current investigation, hydro-alcoholic extracts are favoured for *R. damascena* flowers because they efficiently capture a broad spectrum of bioactive compounds, ranging from water-soluble flavonoids and phenolics to more alcohol-soluble terpenes. This comprehensive extraction maximizes the plant's therapeutic potential, yielding a rich extract with proven antioxidant, anti-inflammatory, and other beneficial properties, while utilizing a safe and widely accepted solvent system. The extract revealed 3.54 ± 0.23 % w/w geraniol content following high-pressure thin layer chromatography (HPTLC) analysis (17).

Selection of the animal model

For the proposed studies, the Oregon R+ strain was collected from the Drosophila Stock Centre, Department of Biochemistry, Dault Ram College, University of Delhi, New Delhi, India. *Drosophila melanogaster* of both genders (1–3 days old) were cultured on corn meal medium in a controlled environment of 22–24 °C temperature and 60–70% relative humidity, along with a 12 h dark/light cycle. Due to its rapid life cycle, high reproductive rate, low cost, and ease of genetic manipulation, Drosophila serves as an excellent model for neurological studies. Crucially, about 75% of human disease-related genes have counterparts in *Drosophila*, making it highly relevant for studying human conditions. While the fly's nervous system is far less complex than a human's (around 100,000 neurons compared to 86 billion), it still exhibits complex behaviours like learning and memory, and fundamental molecular pathways are highly conserved (7).

Toxicity assessment

A 7-day acute toxicity study was done following the method described by Etuh et al to determine the median lethal concentration (LC_{50}) (18). Thirty young fruit flies (1-5 days old) each were anesthetized under ice, and all four test compounds, i.e. *R. damascena, C. wightii*, geraniol, and guggulsterone, were exposed to various concentrations ranging from 1.56 to 50 mg/mL along with a control group in replicated three times. The mortality rate was counted every 24-hour interval for seven successive days. The survival percentage was plotted against the concentration, and the LC_{50} value was calculated.

Survival assay

The survival assay of each test compound was accomplished according to the method defined by Abolaji et al for 14 days using three different concentrations (19). Thirty flies of either sex (1-5 days old) were treated with three different doses and evaluated after determining the LC_{50}. One-tenth of the LC_{50} was selected as the highest dose for the survival assay. The experiment was replicated thrice, and the number of live and dead flies was recorded daily for 14 days. The concentration that showed the highest survival rate till day 14 was selected for further experiments.

Treatment schedule

$AlCl_3$ (40mM) was used to induce neurotoxicity in experimental animals, and the dose was selected based on a study by Inneh and Eiya (20)**.** Flies were divided into nine groups, Group I, served as control group, while all other groups received $AlCl_3$ (40 mM); Group II was exposed to only $AlCl_3$ (40 mM); Group III *R. damascena* (50 µg/mL); Group IV received *C. wightii* (75 µg/mL); Group V received combination of *R. damascena* (50µg/mL) and *C. wightii* (75 µg/mL); Group VI geraniol (15 µg/mL); Group VII guggulsterone (5 µg/mL); Group VIII combination of geraniol (15 µg/mL) and guggulsterone (5 µg/mL); and in Group IX flies were exposed to standard drug, donepezil (4 µg/mL). After 7 days of drug administration, behavioural changes, oxidative stress markers, levels of neurotransmitters, and mitochondrial complexes were assessed, as shown in Figure 1.

Evaluation of oxidative stress markers and AChE level

The legs and wings of all flies were amputated using a sharp blade after being anesthetized in ice; each fly was weighed, homogenized in 0.1 M potassium phosphate buffer, and centrifuged at 4000 rpm for 10 minutes at 4 °C. The supernatant thus attained was used for a further assessment of oxidative stress markers and AChE

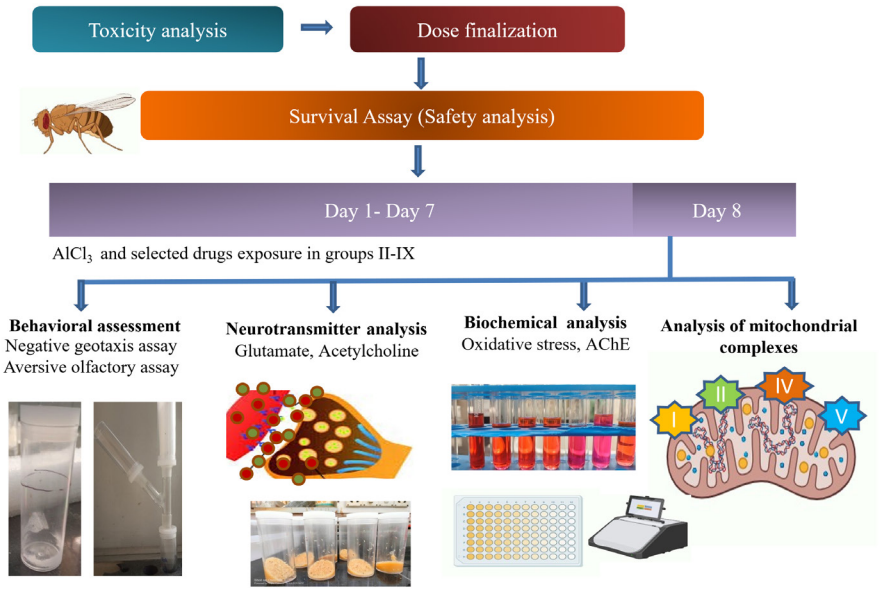

Figure 1. Experimental workflow.

level using a UV-Visible spectrophotometer, following assessment of protein level using the Lowry method (21).

Lipid peroxidation, i.e. the level of malondialdehyde (MDA), was determined by incubating 0.25 mL of sample with 0.25 mL of Tris-HCl buffer at 37 °C for 2 hours with constant shaking. After two hours, the reaction was stopped by adding 0.5 mL of ice-cold 10% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 3000 rpm for 10 minutes, and to 0.5 mL of the supernatant, 0.5 mL of 0.67% (w/v) thiobarbituric acid (TBA) was added and heated at 100 °C for 10 minutes, until the pink color appeared. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm against a blank solution. Results were expressed as nmol/mg protein, applying a molar extinction coefficient of 1.53×10^5 $M^{-1}cm^{-1}$ for the MDA-TBA adduct (22).

Nitrite content, an indirect measure of nitric oxide (NO) production, was determined using the Griess reagent method. The supernatant was mixed with an equal volume of Griess reagent (0.1% sulphanilamide in 2.5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). After incubating at room temperature for 10 minutes, the absorbance was measured at 540 nm against a blank sample and nitrite content is expressed as µmol/mg protein, calculated using a sodium nitrite standard curve (23).

Superoxide dismutase (SOD) activity was determined by measuring its inhibition of nitro blue tetrazolium (NBT) reduction. A reaction mixture was prepared containing 0.15 mL of hydroxylamine hydrochloride, 75 µL of NBT, 1.9 mL of distilled water, 0.15 mL of Triton-X100, and 0.1 mL of the supernatant. The absorbance of this mixture was measured at 560 nm and SOD activity was reported as units/min/mg protein, with one unit defined as the amount of enzyme that reduces the reaction rate by 50% (24).

The enzymatic activity of catalase was determined by combining 0.1 mL supernatant and 1.9 mL phosphate buffer (50mM, pH 7.4). with 1 mL of 30mM hydrogen peroxide, and the decrease in absorbance was monitored spectrophotometrically at 240 nm for 2 minutes. The results were expressed as micromoles of H_2O_2 decomposed per minute per milligram of protein, utilizing a molar extinction coefficient of 71 $M^{-1}cm^{-1}$ (25).

For the assessment of reduced glutathione (GSH), the processed sample (0.5 mL) was mixed with an equal volume of 10% TCA and centrifuged at 2000 rpm for 10 minutes at 4 °C. For the assay, 0.1 mL of the resulting supernatant was combined with 1.15 mL of phosphate buffer (pH 8.4) and 0.25 mL of 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB). After 5 minutes, the absorbance was measured at 412 nm, where the yellow derivative formed by the reaction of GSH with DTNB absorbs. Results were determined applying a molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ and expressed as nmol/mg protein, were (26).

AChE activity was determined by monitoring absorbance changes at 412 nm. The assay mixture consisted of 0.1 mL supernatant, 0.025 mL DTNB, and 0.65 mL of phosphate buffer (pH 8.4). An initial absorbance reading was taken, and subsequently, 5 µL of acetylthiocholine iodide solution was added, when the absorbance stopped increasing, and the absorbance was measured again at 412 nm. AChE activity, expressed as nmol of substrate hydrolyzed/min/mg protein, was calculated from the change in absorbance and applying a molar extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ (27).

Estimation of neurotransmitters

The level of glutamate was estimated using HPTLC-densitometry, referring to the procedure of Sancheti et al. Following homogenization using 0.1 N HCl and the supernatants (20 µL) obtained were spotted on the silica plates and developed in butanol/glacial acetic acid/water (22:3:5 v/v/v). Spots were then visualized with 0.2% ninhydrin and scanned at 550 nm. Glutamate content was then determined by densitometry, using a standard curve of glutamate (28). Furthermore, the content of acetylcholine (ACh) was determined spectrophotometrically following the method described by Augustinsson. The assay involves adding 2 mL of alkaline hydroxylamine, 1 mL of HCl, and 1mL of $FeCl_3$ solutions to 0.1 mL of the sample. After vigorous shaking, the color absorbance was measured at 540 nm, and the concentration of ACh was determined following a standard curve of absorbance versus known ACh concentrations. The results were expressed in µmoles of ACh/mg protein (29).

Mitochondrial isolation and detection of mitochondrial complexes

Mitochondria were isolated from the fruit flies according to the method described by Berman and Hastings in1999. Flies were dissected, rinsed in ice-cold isotonic saline, and homogenised in ice-cold extraction buffer, and the homogenate was centrifuged at 5000 rpm for 15 minutes (4 °C). The supernatant was then centrifuged at 14000 rpm for 15 minutes (4 °C) to yield crude mitochondrial pellets. These pellets were washed with ice-cold extraction buffer by centrifugation at 10000 rpm for 15 minutes (4 °C). The final mitochondrial pellets were resuspended in resuspension buffer for subsequent analysis of mitochondrial electron transport chain complex activities (30).

Mitochondrial NADH dehydrogenase (Complex I) activity was assessed using King and Howard's method. The reaction mixture contained 0.35 mL of glycyl-glycine buffer (0.2M, pH 8.5), 0.10 mL NADH (6Mm), 0.1 mL oxidized cytochrome c (1mM), and 2.4 mL water. After adding the 0.05 mL of prepared sample, a reduction in Cytochrome c was monitored spectrophotometrically at 550 nm for 3 minutes. Results were then expressed as nmol NADH oxidized/min/mg protein, calculated using a molar extinction coefficient of 19.6 $mM^{-1}cm^{-1}$

(31). For complex II, the reaction mixture consisted of 1.5 mL sodium phosphate buffer (0.0M, pH 7.8), 0.3 mL of 1%bovine serum albumin (BSA), 0.2 mL succinate (0.6 M), and 0.4 mL potassium ferricyanide (0.03M). The reaction was initiated by adding the 0.05 mL mitochondrial preparation, and a decrease in absorbance at 420 nm for 3 minutes due to ferricyanide reduction was measured spectrophotometrically. Results were expressed as nmol succinate oxidized/min/mg protein, following the extinction coefficient of 2.08 $mM^{-1}cm^{-1}$ (32).

Cytochrome oxidase (Complex IV) activity in brain mitochondria was assessed following the method described by Sottocasa et al. First, oxidized cytochrome C was reduced using sodium borohydride and neutralized to pH 7.0 with 0.1 M HCl. Reduced cytochrome C (0.3 mM) was then combined with 0.075M phosphate buffer, and 10 µL of the mitochondrial sample was added. The oxidation of cytochrome c was monitored spectrophotometrically by measuring the reduction in absorbance at 550 nm over a 3-minute period. The results were reported as nmol of cytochrome C oxidized per minute per milligram of protein, using a molar extinction coefficient of 19.6 mM^{-1} cm^{-1} (33).

For Complex V activity, the reaction was started by mixing 100 µL of the mitochondrial preparation with 1 mL of ATPase buffer (containing 5 mM ATP, 50 mM Tris, and 5 mM MgCl2, pH 7.5) and incubating at 37 °C for 10 minutes. The reaction was halted by adding 1 mL of 10% (w/v) TCA. After centrifugation at 4500 rpm for 20 minutes, the supernatant was diluted with water. Following a 10-minute incubation at room temperature, the amount of inorganic phosphate produced was measured at 660 nm. The results were expressed as nmol ATP hydrolyzed per minute per milligram of protein (34).

Negative geotaxis behaviour assay

The locomotive activity of flies was assessed after a 7-day treatment period. Briefly, flies were gently affixed to the bottom of a marked glass column. The number of surviving flies ascending above the 6 cm spot within 6 seconds was noted. This procedure was performed three times with a 1-minute interval between trials. Locomotor performance was expressed as the percentage of flies that successfully completed the climbing task (35).

Aversive olfactory behaviour assay

The Aversive olfactory behaviour assay is done by Y-maze, a behavioural test used to assess olfactory responses in drosophila. A Y-shaped maze presents flies with a choice between two arms containing an odorant (acetic acid) and a solvent control. Flies are introduced into the start arm, and their choices are recorded. The olfactory index, calculated as the difference in fly numbers between the odour and solvent arms divided by the total number of flies, quantifies the preference for the odorant. This assay provides valuable insights into chemosensation, learning, and memory in Drosophila (36). The olfactory index was determined with the formula: (flies in odor tube - flies in solvent tube) / total flies.

Statistical analysis

Data were reported as mean ± SD (n = 3 vials, with each vial containing 50 flies) and analysed using one-way ANOVA with Bonferroni's multiple comparisons test using GraphPad Prism Version. 10.4.2. $P < 0.05$ was considered significant.

Results

Determination of LC_{50} of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents

The mortality in fruit flies was determined daily for 7 consecutive days in 6 different concentration groups of *R. damascena*, *C. wightii*¸ geraniol, and guggulsterone. LC_{50} value after 7 days was found to be 1.7039 mg/mL for *R. damascena*, 3.1858 mg/mL for *C. wightii*¸ 0.6580 mg/mL for geraniol, and 0.1602 mg/mL for guggulsterone, as shown in Figure S1.

Effect of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents on the survival rate of flies

A survival assay was conducted using three doses of each test compound selected based on the LC_{50} values determined in the 7-day acute toxicity study. Figure 2 illustrates the survival rates of flies that were administered with *R. damascena, C. wightii,* and their major phytoconstituents at different doses. No statistically significant ($P > 0.05$) alteration in survival was detected among flies intervented with various doses of *R. damascena, C. wightii*, geraniol, and guggulsterone compared to the control group. Based on these findings, the following doses were selected for further studies: 50 µg/mL for *R. damascena,* 75 µg/mL for *C. wightii,* 15 µg/mL for geraniol, and 5 µg/mL for guggulsterone.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on MDA level

The amount of lipid peroxidation in all treatment groups after $AlCl_3$ exposure to fruit flies is represented in Figure 3a. The results affirmed a noteworthy upregulation in the MDA levels in the $AlCl_3$-treated group to 6.966 ± 0.047 nmol MDA/mg protein, compared to the control group (2.810 ± 0.115 nmol MDA/mg protein, $P < 0.001$), signifying oxidative damage. However, treatment with *R. damascena* (3.529 ± 0.118 nmol MDA/mg protein), *C. wightii* (3.273 ± 0.068 nmol MDA/mg protein), geraniol (3.309 ± 0.063 nmol MDA/mg protein), and guggulsterone (4.041 ± 0.023 nmol MDA/mg protein) showed a more substantial decrease in the level of MDA either per se and in combination. Moreover, the combination of *R. damascena* and *C. wightii* (2.441 ± 0.176 nmol MDA/mg protein) exhibited the most pronounced reduction

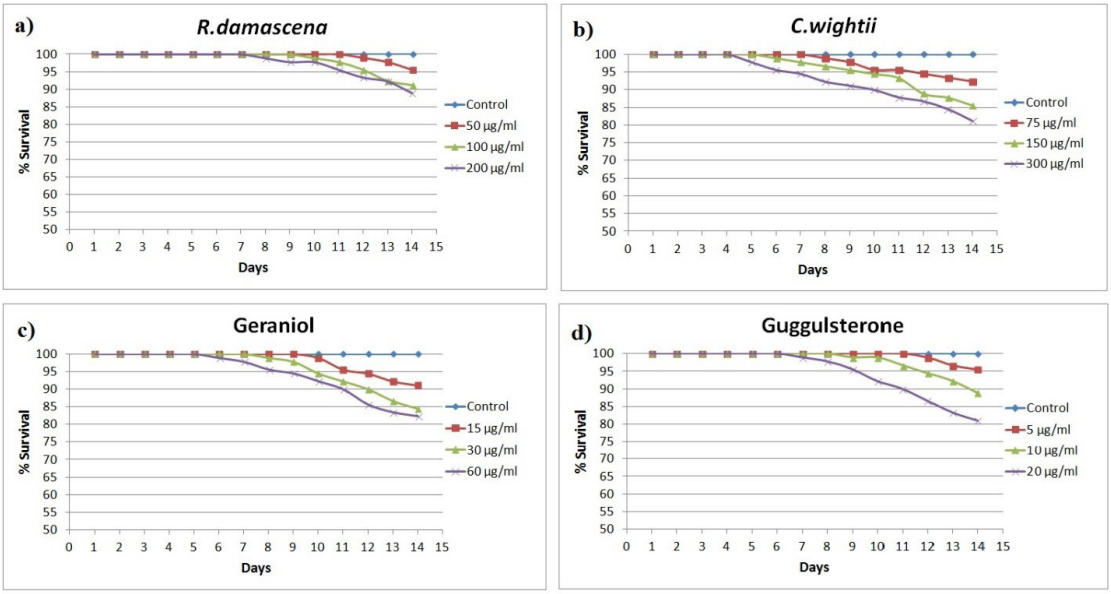

Figure 2. The 14-day survival assay of various doses of *Rosa damascena* (a), *Commiphora wightii* (b), geraniol (c), and guggulsterone (d).

($P < 0.001$), lowering MDA levels below even the control group.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on nitrite level

Nitrite, an essential indicator of oxidative stress, was elevated significantly ($P < 0.001$) after $AlCl_3$ exposure in fruit flies, with the level reaching 3.423 ± 0.256 µmoles nitrite/mg protein. However, treatment with *R. damascena* (1.923 ± 0.061 µmoles nitrite/mg protein), *C. wightii* (2.032 ± 0.084 µmoles nitrite/mg protein), and their phytoconstituents, i.e. geraniol (2.185 ± 0.067 µmoles nitrite/mg protein) and guggulsterone (2.459 ± 0.055 µmoles nitrite/mg protein), demonstrated significant reduction in the nitrate level, with F (8, 18) = 65.33, $P < 0.001$, as shown in Figure 3b, highlighting antioxidant potential. Nevertheless, the combination of both *R. damascena* and *C. wightii* affirmed a more extensive decrease in nitrite levels (1.376 ± 0.066 µmoles nitrite/mg protein) than their individual intervention.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on SOD level

The level of SOD in all groups is described in Figure 3c. It was observed that, compared to the control group (2.110 ± 0.040 units/min/mg protein), the vehicle-treated group demonstrated a notable decline in SOD enzymatic activity (0.687 ± 0.038 units/min/mg protein, $P < 0.001$), reflecting compromised antioxidant defence. However, treatment with *R. damascena* (1.976 ± 0.045 units/min/mg protein) and *C. wightii* (1.814 ± 0.039 units/min/mg protein) notably improves SOD activity. Similarly, administration of geraniol and guggulsterone elevates SOD level to 1.729 ± 0.040 units/min/mg protein and 1.390 ± 0.107 units/min/mg protein, respectively. Furthermore, the combination of both plant extracts demonstrated a more significant elevation of SOD level (2.165 ± 0.042 units/min/mg protein), similar to standard drugs (2.158 ± 0.040 units/min/mg protein).

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on catalase level

The effect of *R. damascena, C. wightii*, and their major phytoconstituents on catalase level was illustrated in Figure 3d. $AlCl_3$ exposure was found to significantly reduce the catalase enzymatic activity from 3.275 ± 0.160 µmoles H_2O_2 decomposed/min/mg protein in the control to 1.790 ± 0.013 µmoles H_2O_2 decomposed/min/mg protein in the vehicle group. Results affirmed that herbal intervention per se and, in combination, significantly improves the enzymatic activity of catalase following $AlCl_3$ exposure with F (8, 18) = 54.70, $P < 0.001$. However, the combination group of *R. damascena* and *C. wightii* (3.228 ± 0.059 µmoles H_2O_2 decomposed/min/mg protein); and geraniol and guggulsterone (3.044 ± 0.056 µmoles H_2O_2 decomposed/min/mg protein), demonstrated a comparatively more significant increase in enzymatic activity. The donepezil-treated group increased the catalase level to 3.414 ± 0.101 µmoles H_2O_2 decomposed/min/mg protein.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on GSH level

Results affirmed that exposure to $AlCl_3$ in *D. melanogaster*

reduces the GSH concentration (17.091 ± 0.315 nmol GSH/mg protein) compared to the control group (30.345 ± 0.451 nmol GSH/mg protein, $P < 0.001$). However, the treatment with *R. damascena* (29.258 ± 0.455 nmol GSH/mg protein), *C. wightii* (28.839 ± 0.293 nmol GSH/mg protein), and their major phytoconstituents, i.e., geraniol (27.512 ± 1.132 nmol GSH/mg protein) and guggulsterone (29.120 ± 0.303 nmol GSH/mg protein), significantly elevates their level, as revealed in Figure 3e, with F (8, 18) = 120.3, $P < 0.001$. Interestingly, the combination of *R. damascena* and *C. wightii* demonstrated the utmost enhancement in GSH level (31.202 ± 0.407 nmol GSH/mg protein), similar to donepezil (31.148 ± 0.511 nmol GSH/mg protein).

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on AChE level

The effects of *R. damascena*, *C. wightii,* and their major phytoconstituents on the AChE level are demonstrated in Figure 3f. A momentous augmentation in the AChE level was found in the $AlCl_3$ group (217.907 ± 1.243 nmol AChE decomposed/min/mg protein), compared to the control (148.854 ± 0.519 nmol AChE decomposed/min/mg protein). However, treatment with *R. damascena* (164.182 ± 1.314 nmol AChE decomposed/min/mg protein), *C. wightii* (174.183 ± 1.054 nmol AChE decomposed/min/mg protein), and their combination (152.637 ± 1.684 nmol AChE decomposed/min/mg protein) effectively lowered AChE activity, with the combination reaching near-control levels. Similarly, geraniol (161.477 ± 1.263 nmol AChE decomposed/min/mg protein), guggulsterone (176.119 ± 2.044 nmol AChE decomposed/min/mg protein), and their co-administration (159.415 ± 3.341 nmol AChE decomposed/min/mg protein) also showed significant reductions in the AChE level. Nevertheless, the donepezil-treated

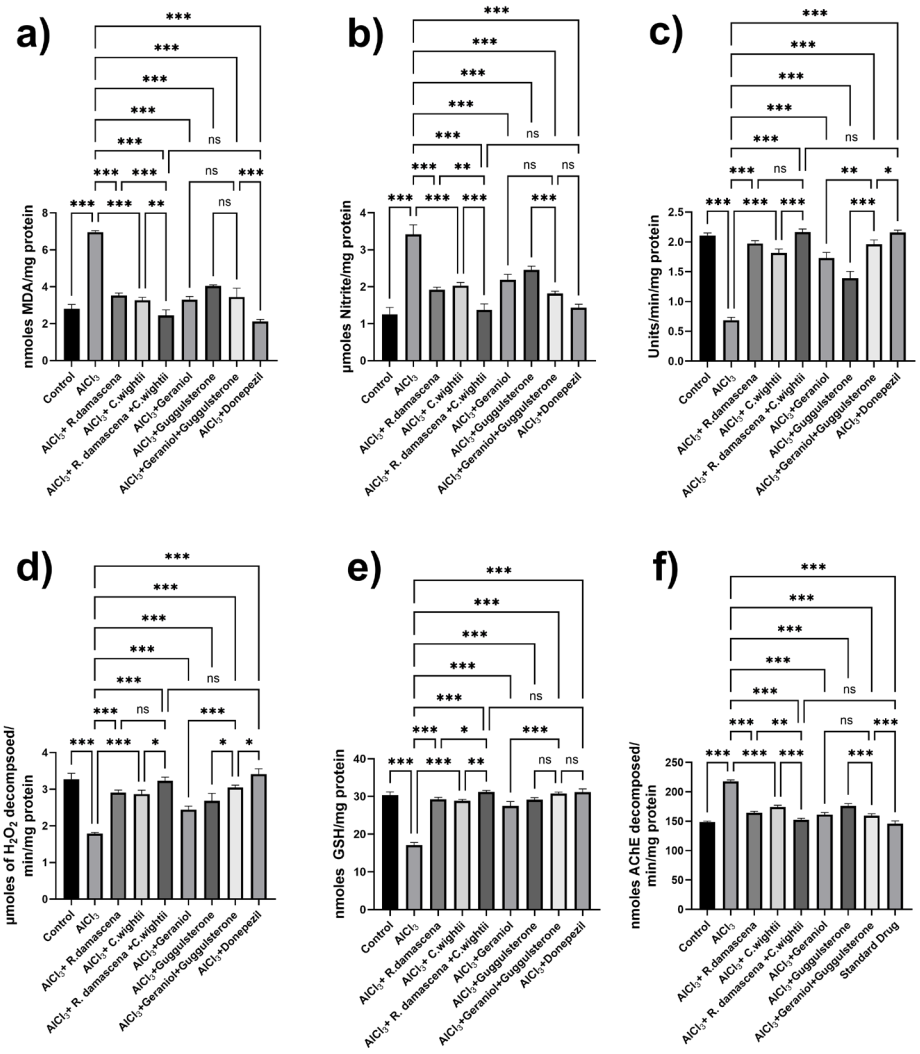

Figure 3. Effects of *Rosa damascena*, *Commiphora wightii*, and their major phytoconstituents per se and in combination on (a) malondialdehyde (MDA) levels, (b) nitrite levels, (c) superoxide dismutase (SOD) activity, (d) catalase activity, (e) reduced glutathione (GSH) levels, and (f) acetylcholinesterase (AChE) activity. Data were reported as Mean ± SD (n=3 vials, with each vial containing 50 flies) and analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. ns signifies $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

groups exhibited the most significant reduction in AChE level (145.857 ± 3.590 nmol AChE decomposed/min/mg protein), reaching below baseline.

Effects of *Rosa damascena, Commiphora wightii*, and their significant phytoconstituents per se and in combination on mitochondrial complexes

The level of mitochondrial complexes, i.e. Complex I, Complex II, Complex IV, and Complex V, was found to be extensively improved in the drug treatment groups, as depicted in Figure 4. $AlCl_3$ exposure significantly decreased Complex I (NADH oxidase) activity to 39.007 ± 0.384 nmol NADH oxidized/min/mg protein, Complex II (succinate oxidase) activity to 125.110 ± 1.628 nmol succinate oxidized/min/mg protein, Complex IV (cytochrome C oxidase) activity to 90.044 ± 2.379 nmol cytochrome C oxidized/min/mg protein, and Complex V (ATP hydrolysis) activity to 55.845 ± 1.301 nmol ATP hydrolyzed/min/mg protein compared to the control group, ($P < 0.001$). All the tested treatment groups exhibited significant improvement in the enzymatic activity of all the mitochondrial complexes with $P < 0.001$ compared to the

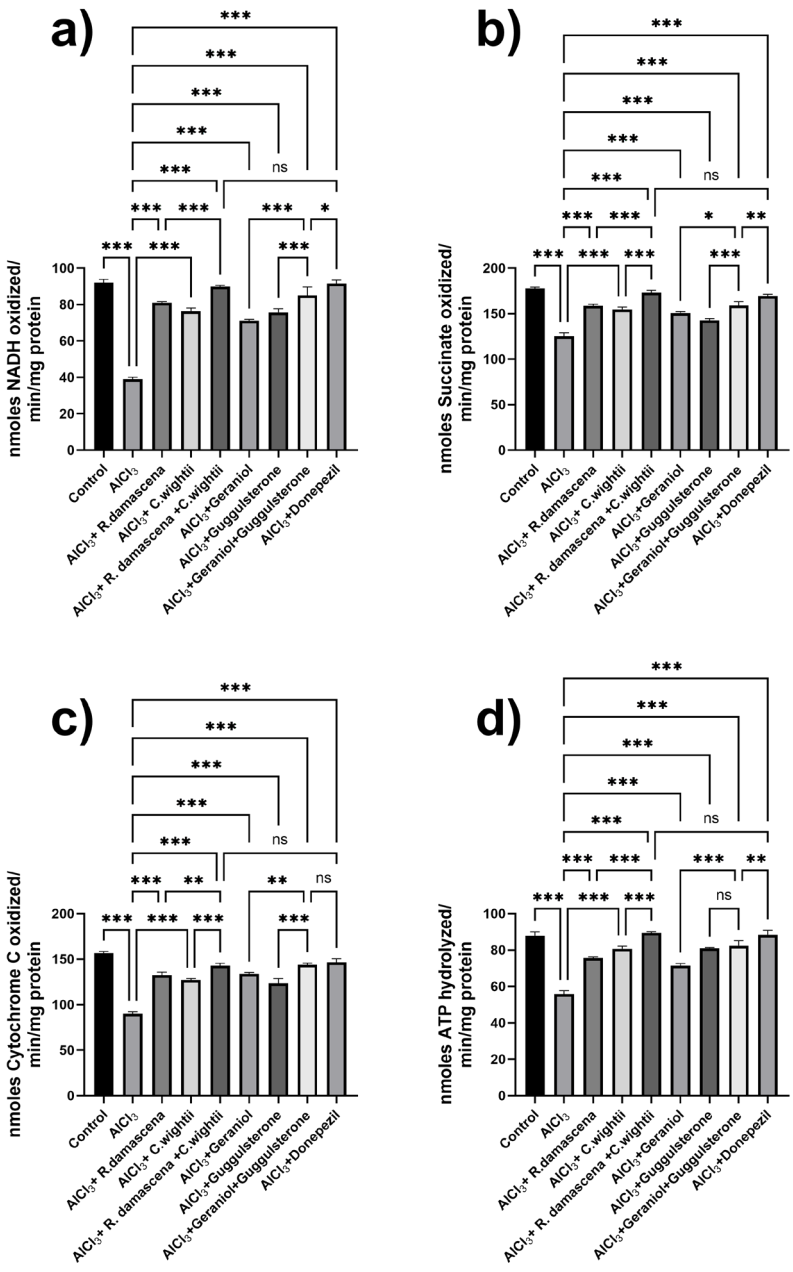

Figure 4. Effects of *Rosa damascena*, *Commiphora wightii*, and their major phytoconstituents per se and in combination on (a) Complex I (NADH oxidase) activity, (b) Complex II (Succinate oxidase) activity, (c) Complex IV (Cytochrome C oxidase) activity, and (d) Complex V (ATP Hydrolysis) activity. Data were reported as Mean ± SD (n=3 vials, with each vial containing 50 flies) and analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. ns signifies $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

vehicle-treated group. Flies treated with the combination of *R. damascena* and *C. wightii* demonstrated the most pronounced elevation in the mitochondrial activity with levels reaching to 89.815 ± 0.710 nmol NADH oxidized/min/mg protein, 172.905 ± 1.960 nmol succinate oxidized/min/mg protein, 142.875 ± 1.703 nmol cytochrome C oxidized/min/mg protein, and 89.484 ± 0.640 nmol ATP hydrolyzed/min/mg protein for Complex I, II, IV, and V, respectively, indicating the significant restoration of mitochondrial enzymatic activity following $AlCl_3$-mediated neurodegeneration.

Effects of *Rosa damascena, Commiphora wightii*, and their significant phytoconstituents per se and in combination on glutamate level

$AlCl_3$ exposure significantly elevated glutamate levels in fruit flies to 147.031 ± 3.146 ng glutamate/mg protein compared to the control group (78.593 ± 1.341 ng glutamate/mg protein, $P < 0.001$). However, treatment with herbal extracts and their bioactive compounds restored glutamate levels to normal, as depicted in Figure 5a, with F (8, 18) = 119.4, $P < 0.001$. Treatment with *R. damascena*, *C. wightii*, geraniol, and guggulsterone declined glutamate level to 80.532 ± 3.453 ng glutamate/mg protein, 92.795 ± 3.766 ng glutamate/mg protein, 97.052 ± 1.217 ng glutamate/mg protein, and 105.755 ± 1.734 ng glutamate/mg protein, respectively, affirming their ability to restore modulated glutamate neurotransmission. Notably, the combination of plant extract demonstrated a more significant modulation of glutamate levels (79.762 ± 2.340 ng glutamate/mg protein), similar to that observed after donepezil treatment (72.944 ± 0.874 ng glutamate/mg protein).

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on the level of ACh

As shown in Figure 5b, exposure to $AlCl_3$ drastically diminishes the level of ACh to 0.532 ± 0.095 µmoles of ACh/mg protein compared to the control group (2.586 ± 0.165 µmoles of ACh/mg protein, $P < 0.001$). Whereas treatments with *R. damascena*, *C. wightii,* and their major phytoconstituents considerably restore the level of ACh in $AlCl_3$-induced neurotoxicity in fruit flies with F (8, 18) = 157.9, $P < 0.001$. *R. damascena* and *C. wightii* increased ACh level to 2.171 ± 0.056 µmoles of ACh/mg protein and 1.886 ± 0.021 µmoles of ACh/mg protein, respectively; however, their combination improved the level to 2.494 ± 0.0036 µmoles of ACh/mg protein. Similarly, geraniol (1.944 ± 0.020 µmoles of ACh/mg protein), guggulsterone (1.839 ± 0.013 µmoles of ACh/mg protein), and their combination (2.057 ± 0.0441 µmoles of ACh/mg protein) also significantly restored ACh levels in flies after $AlCl_3$ exposure. Notably, the combination of both extracts demonstrated the most significant improvement in ACh level, similar to that of donepezil (2.625 ± 0.065 µmoles of ACh/mg protein).

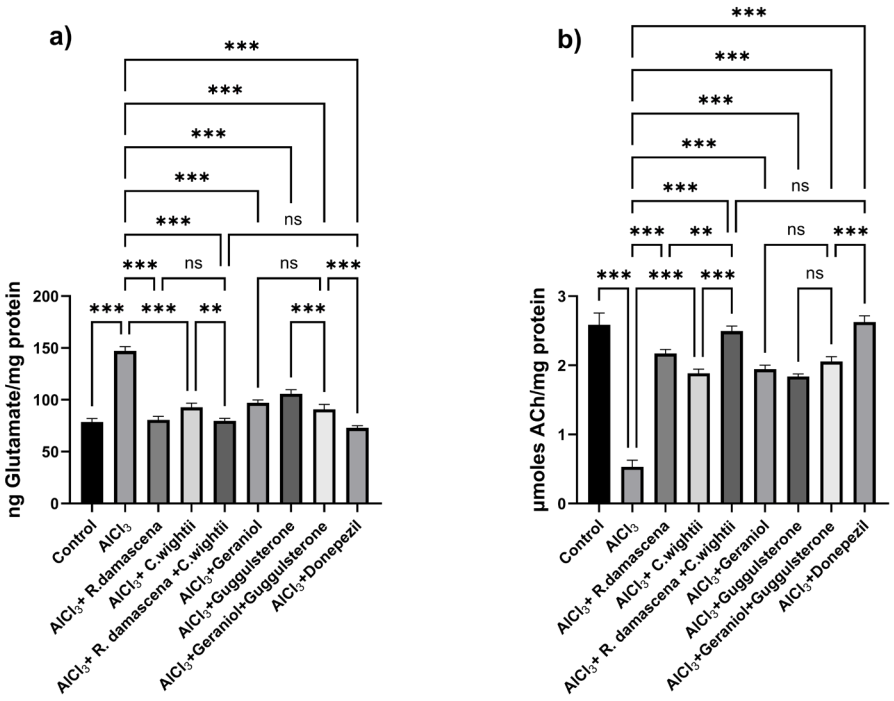

Figure 5. Effects of *Rosa damascena*, *Commiphora wightii*, and their major phytoconstituents per se and in combination on a) glutamate levels and (b) acetylcholine (ACh) levels. Data were reported as Mean ± SD (n=3 vials, with each vial containing 50 flies) and analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. ns signifies $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on Climbing assay

Exposure to $AlCl_3$ has been observed to reduce the climbing assay to 30.0 ± 10.0 % compared to the control group (96.667 ± 5.092%, $P < 0.001$), as shown in Figure 6a. Whereas treatments with *R. damascena*, *C. wightii*, and their major phytoconstituents significantly elevate the climbing performance in $AlCl_3$-induced neuronal damage in fruit flies with F (8, 18) = 13.58, $P < 0.001$. *R. damascena* improved climbing index to 66.667 ± 5.092%, *C. wightii* to 76.667 ± 5.092 %, geraniol to 66.667 ± 10.184 %, and guggulsterone to 66.667 ± 15.031 %. However, the combination of *R. damascena* and *C. wightii* demonstrated the most significant improvement in negative geotaxis behaviour with 90.0 ± 5.774% climbing index, compared to the donepezil, which exhibited 83.333 ± 5.092 %.

Effects of *Rosa damascena, Commiphora wightii*, and their major phytoconstituents per se and in combination on olfactory index

As shown in Figure 6b, control flies demonstrated an aversion towards the odorant, with an olfactory index of −58.33 ± 10.41 %, whereas flies exposed to $AlCl_3$ were found to have attraction toward the same with 13.33 ± 10.41 % olfactory index ($P < 0.001$). Furthermore, treatments with *R. damascena*, *C. wightii*, and their major phytoconstituents per se and in combination significantly (F (8, 18) = 19.01, $P < 0.001$) restore the aversive olfactory response. Specifically, *R. damascena*, *C. wightii,* geraniol, and guggulsterone exhibited an olfactory index of −68.333 ± 7.515%, −76.667 ± 9.766%, −55.0 ± 10.408%, and −73.333 ± 12.649%, respectively. Interestingly, the co-administration of *R. damascena* and *C. wightii* also significantly improved aversive olfactory response by −86.667 ± 5.092%, comparatively more than the per se intervention.

Discussion

Dyshomeostasis of metal ions such as iron, copper, zinc and lead, which are essential for brain function, precipitates the cascade of deleterious events, significantly affecting neuronal viability and contributing to the persistent progression of neurodegeneration and associated diseases (37). Aluminium is a potent neurotoxin associated with the etiology of various neurological disorders, accumulates in the frontal cortex and hippocampus, contributing to neuroinflammation, oxidative stress, apoptotic neuronal loss, and cognitive impairment (38). Aluminium, after systemic absorption, specifically binds with the iron transporting protein, i.e. serum transferrin, due to similarity in ionic radii, which subsequently intercedes the aluminium transportation across the blood-brain barrier (BBB) (5,39). Extensive scientific studies have demonstrated that prolonged aluminium exposure can trigger a cascade of notorious effects within the brain, leading to considerable neurobehavioral, neurochemical, and neuropathological changes (6,40).

Nevertheless, aluminum has been consistently linked to the progression of AD (41,42). Several clinical studies found an elevated concentration of aluminum in the brain samples of AD patients, compared to non-AD

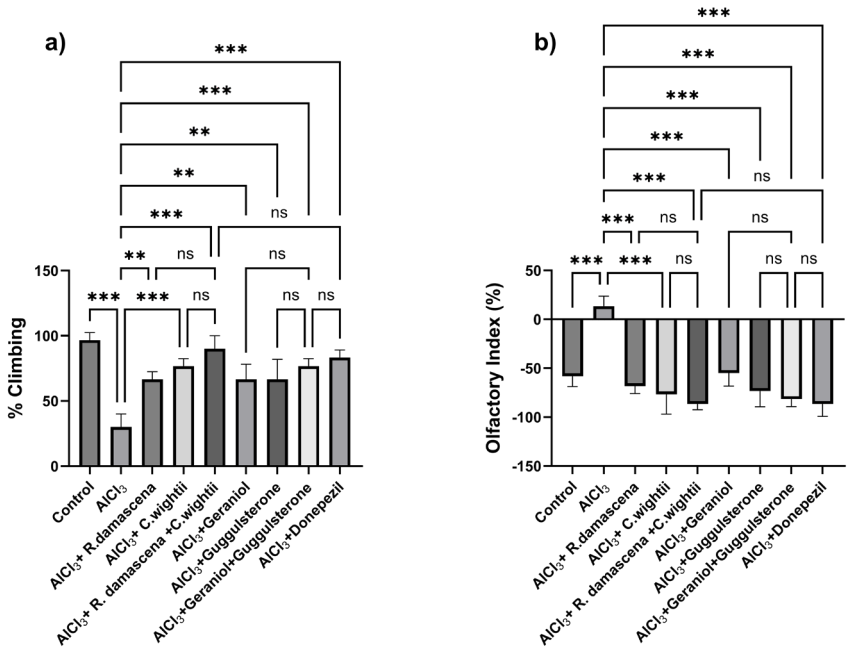

Figure 6 Effects of *Rosa damascena*, *Commiphora wightii*, and their major phytoconstituents per se and in combination on (a) climbing ability and (b) Y-maze olfactory index. Data were reported as Mean ± SD (n=3 vials, with each vial containing 50 flies) and analyzed using one-way ANOVA with Bonferroni's multiple comparisons test. ns signifies $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

people (43). Aluminum administration has also been found to elevate amyloid-β (Aβ) levels via upregulation in mRNA and protein expression of amyloid precursor protein (APP), presenilin, and secretase enzymes, which boosts amyloidogenesis (44). Furthermore, hyperphosphorylation of proteins, a hallmark of AD, is also observed after aluminum therapy, suggesting either increased phosphorylation or reduced dephosphorylation of these proteins in neurofibrillary tangles (NFTs) (45).

Consequently, $AlCl_3$ serves as a valuable and reliable instrument for investigating the intricate mechanisms underlying neurodegeneration and for evaluating the efficacy of potential therapeutic strategies. Its ability to consistently induce a spectrum of pathological events, such as oxidative damage, synaptic alterations, mitochondrial damage, and apoptosis in *Drosophila*, makes it an indispensable asset in preclinical research (46,47). Thus, the present investigation sought to determine the neuroprotective effects of *R. damascena* and *C. wightii* extracts and their major bioactive compounds, geraniol and guggulsterone, in $AlCl_3$-induced neurotoxicity in *Drosophila*.

Metal-induced oxidative trauma has long been recognized to promote several processes. Aluminum acts as a prooxidant that augments free radicals in the brain, kidney, and liver via amplifying lipid peroxidation, followed by a reduction in the level of several antioxidative enzymes. Furthermore, it also augments the formation of 4-hydroxynonenal (4-HNE), a key marker of oxidative damage to cellular membranes (48). The amplified levels of MDA and nitrite content in the $AlCl_3$-treated group highlight the oxidative stress-induced damage associated with aluminum exposure. The significant reduction in these markers after treatment with *R. damascena* and *C. wightii* suggests the potential of natural products in mitigating oxidative stress and protecting against neurodegeneration.

Our study also demonstrated that flies exhibited decreased enzymatic activity of several endogenous antioxidant enzymes, specifically SOD and catalase, compared to healthy control flies. SOD is indeed a critical enzyme that breaks down harmful superoxide radicals into less damaging oxygen or hydrogen peroxide (49). Moreover, the enzymatic dismutation of superoxide anions to hydrogen peroxide by SOD, followed by the catalytic conversion of hydrogen peroxide to water by catalase, is a perilous mechanism for survival in *Drosophila* (50). Interestingly, aluminum exposure also augments hydrogen peroxide to interact with free iron via the Fenton reaction, producing more extensive ROS that further drives oxidative cellular damage (51). Administration of *R. damascena* and *C. wightii* extracts within food significantly increased the activity of these enzymes in flies, subsequently augmenting their survival and life span. In addition, the increased catalase activity is likely to contribute to a reduction in hydroxyl radicals and the generation of harmful ROS following inhibition of the Fenton reaction. Furthermore, the observed reduction in GSH activity in untreated flies is also indicative of tissue and/or cellular damage resulting from oxidative stress induced by increased ROS production, which was altered after the treatment with *R. damascena* and *C. wightii*. The present findings corroborate prior research indicating that dietary plant matter enhances antioxidant activity in fruit flies (52).

Nevertheless, the outcomes furthermore demonstrated that the combined administration of *R. damascena* and *C. wightii* extracts mitigates oxidative stress more profoundly than the administration of either extract alone. This enhanced effect may be attributed to the presence of major phytoconstituents within these extracts. However, the combination of geraniol and guggulsterone also demonstrated a more pronounced efficacy in mitigating oxidative stress, as reflected by significant improvements in SOD, catalase, and GSH levels compared to their individual administration. Geraniol, a plant compound found in *R. damascena*, possesses distinguished antioxidant properties. It acts by neutralizing free radicals, stimulating antioxidant enzyme activity, and chelating metals that catalyze free radical formation. Furthermore, geraniol effectively reduces inflammatory responses, contributing to its overall protective effect (53). Similarly, guggulsterone, extracted from the *C. wightii* (guggul) tree, demonstrates a compelling combination of antioxidant and lipid-regulating capabilities by combating oxidative stress by directly scavenging free radicals and augmenting the activity of antioxidant enzymes (54).

Aluminum-induced oxidative stress also exacerbates cholinergic dysfunction, leading to an increase in choline synaptic uptake (55). Furthermore, chronic aluminum exposure downregulates cerebral AChE activity, which subsequently augments cognitive and memory impairment (56). Inhibition of AChE has been established as a beneficial approach in AD and other forms of dementia (57). Consequently, elevated AChE activity, as observed in Drosophila after exposure to $AlCl_3$, serves as a clear indicator of underlying neurodegeneration, reflecting the disruption of normal cholinergic function within the brain. However, treatment with *R. damascena* and *C. wightii* inclusive diet led to a momentous decrease in AChE activity, suggesting an increase in ACh levels in the synaptic cleft and improved cholinergic function. These outcomes are consistent with previous reports highlighting the anti-neurodegenerative potential of phytoconstituents in flies (47,58,59).

Notably, aluminum-induced neurodegeneration is also significantly influenced by glutamate. Elevated glutamate levels can directly trigger excitotoxicity, causing neuronal damage through excessive stimulation. Furthermore, glutamate metabolism can generate neurotoxic byproducts, further contributing to cellular dysfunction (60). The reduction in the glutamate levels after drug

intervention also suggests that both *R. damascena* and *C. wightii* may exert their effects through modulation of the NMDA receptor, which later on diminishes excitotoxicity and improves antioxidant enzymatic activity.

Aluminum notably impedes complex I, complex II, and complex IV in the cortex, midbrain, and cerebellum, leading to a reduction in the activity of the mitochondrial electron transport chain (61). A significant decrease in cytochrome oxidase activity following aluminum exposure also resulted in noticeable alterations in the Arrhenius plot of the enzyme (62). The reduction in the level of mitochondrial complexes (Complex I, II, IV, and V) was also observed in the $AlCl_3$-treated group, underscoring the detrimental impact of aluminum on mitochondrial function, contributing to the overall neurodegenerative process. However, the significant improvement in these parameters by the extracts suggests their ability to preserve mitochondrial integrity and function. Both *R. damascena* and *C. wightii* exert beneficial impacts on mitochondrial function, either by enhancing the efficiency of cellular respiration through increased oxygen consumption or by mitigating oxidative stress through a reduction in the generation of mitochondrial ROS. This diminished endogenous ROS production and/or elevated oxygen consumption promotes defensive mechanisms against exogenous oxidative damage (63). Notably, co-exposure to geraniol and guggulsterone also resulted in augmenting NADH dehydrogenase activity. This enhancement may be attributed to alterations in phospholipid content, which could influence electron transfer from NADH to ubiquinone. These findings are, however, consistent with previous reports highlighting the neuroprotective effects of rutin on mitochondrial function (64). Similar effects were observed in Complex II, whose activity was enhanced by co-administration of geraniol and guggulsterone, and slightly more by the combination of *R. damascena and C. wightii* extracts. Further analysis revealed that $AlCl_3$ exposure led to significant alterations in mitochondrial function, including decreased cytochrome c levels and ATP hydrolysis. When used in conjunction, *R. damascena* and *C. wightii* extracts demonstrated a more notable improvement in preserving mitochondrial integrity, suggesting that their combined therapeutic action surpassed the efficacy of individual treatments. Furthermore, the combination of specific phytoconstituents from *R. damascena* and *C. wightii* also showed a more profound mitochondrial protective effect compared to the crude extracts.

Behavioural assays, like negative geotaxis and aversive olfactory assay, are used to recapitulate behavioural phenotypes associated with human neurodegenerative diseases in fruit flies. These assays provide a powerful platform for studying the underlying mechanisms of neurodegeneration and for identifying potential therapeutic targets (65). The negative geotaxis assay, which measures a fly's ability to climb against gravity, was used to assess locomotor function in AD flies (47,66,67). Untreated flies displayed a significant deficit in short-term memory compared to control flies, and this impairment in memory function may be attributed to reduced ACh levels in the synaptic cleft (68). Similarly, our findings demonstrate a significant decline in climbing ability in flies with AD compared to healthy controls, suggesting impaired locomotion after $AlCl_3$ treatment. However, the administration of *R. damascena* and *C. wightii* individually improved their climbing capacity. Notably, their combination exhibited the most significant enhancement in climbing ability, indicating improved locomotion. Furthermore, combining geraniol or guggulsterone also yields effective results in improving learning and memory than using either geraniol or guggulsterone alone. This enhanced efficacy may be attributed to their potent antioxidant properties. Furthermore, this result aligns with prior studies showing that polyphenols improve memory and cognitive function in flies (59,69).

Similarly, in the aversive olfactory assay, $AlCl_3$-induced flies typically exhibit impaired olfactory learning and memory compared to control flies, affirming that they face difficulty learning avoidance of an odour that is associated with an aversive stimulus. However, a significant reversal of these cognitive deficits was observed in flies treated with *R. damascena* and *C. wightii*, displaying a significantly higher likelihood of avoiding the odour, and protection against $AlCl_3$-induced olfactory learning and memory impairments. Interestingly, the combination of geraniol or guggulsterone also demonstrated a considerable improvement in their olfactory avoidance behaviour compared to those treated with either drug alone. This compelling result underscores the potential of the combined effect of both geraniol and guggulsterone in ameliorating olfactory learning and memory impairments.

Conclusion

In conclusion, it can be affirmed that dietary supplementation with *R. damascena* and *C. wightii* extracts and their respective phytoconstituents exerts noteworthy improved therapeutic effects in flies, providing a more comprehensive approach to neuroprotection by targeting multiple pathways implicated in neurodegeneration, as evidenced by improved climbing ability, restored activity of antioxidant enzymes, modulated neurotransmitter signaling pathways and elevated mitochondrial function. Moreover, their phytoconstituents combination was also found to be effective in managing these neurological conditions. While this study provides valuable insights, further research is necessary to elucidate the underlying mechanisms of action and to evaluate their therapeutic potential in clinical settings.

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Authors' contribution

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Supervision: Govind Singh.
Validation: Govind Singh.
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Writing–review and editing: Payal Chauhan, Karan Wadhwa, Himanshu Sachdeva.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical considerations

According to the Committee for Control and Supervision of Experiments on Animals (CPCSEA) guidelines, which regulates animal research in India, no CCSEA and institutional animal ethics committee (IAEC) approval is required to conduct experiments on invertebrates such as fruit flies (*Drosophila melanogaster*) and worms or nematodes (*Caenorhabditis elegans*). However, the whole experiment and procedure were conducted with ethical and humane care under the supervision of the IAEC.

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Supplementary files

Supplementary file 1 contains Figure S1.

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