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Saraca asoca (Ashoka) flower extract improves scopolamine-impaired task performance in mice; changes in brain cholinesterase activity and oxidative stress markers

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Saraca asoca (Ashoka) flower extract improves scopolamine-impaired task performance in mice; changes in brain cholinesterase activity and oxidative stress markers

Abstract

Cognitive impairment and anxiety often coexist, and cholinergic dysfunction and oxidative stress are implicated in both. The current study aimed to evaluate the ethanolic flower extract of *Saraca asoca* (SAF) against scopolamine-induced impairment of task performance in Swiss albino mice, together with the brain cholinesterase and oxidative changes that accompany it. SAF contained 90.94 ± 0.75 mg gallic acid equivalents/g and 51.54 ± 0.78 mg quercetin equivalents/g, was fingerprinted by LC–DAD–ESI–TOF–MS, and scavenged DPPH radicals with an IC_{50} of 62.7 µg/mL as opposed to 34.8 µg/mL for vitamin E. Mice received SAF (50, 100, and 250 mg/kg, p.o.) for 14 days, with scopolamine (0.4 mg/kg, i.p.) before passive avoidance, novel object recognition, and Y-maze tests. A second cohort received one scopolamine injection on day 14 for brain biochemistry. Scopolamine shortened retention latency about 3.1-fold and lowered the discrimination index about 3.0-fold; it also raised brain cholinesterase activity and malondialdehyde (MDA) and lowered catalase about 2.5-fold. SAF at 100 and 250 mg/kg improved performance in all three tasks and lowered cholinesterase activity and MDA, and all doses partly restored catalase. These effects were confirmed after false discovery rate correction. In a third cohort, SAF increased open-arm time and social interaction, but these anxiety data are exploratory because no locomotor control or usable diazepam comparison was available. SAF thus improved scopolamine-impaired task performance, alongside lower brain cholinesterase activity and oxidative stress.

Keywords *Saraca asoca* · Scopolamine · Cholinesterase · Oxidative stress · Memory · Anxiety-like behavior

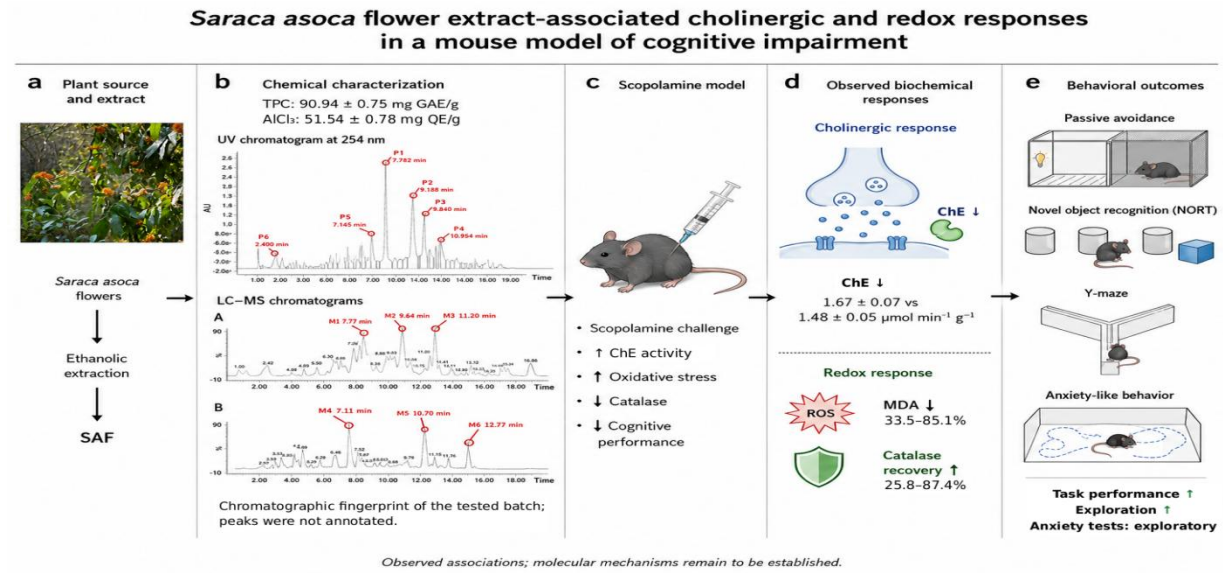


Figure 1. Graphical abstract. Preparation, chemical characterization, and testing of ethanolic *Saraca asoca* flower extract (SAF) in mice. The cognitive, biochemical, and anxiety data come

from separate cohorts; anxiety data are exploratory, and the figure shows associations, not a mechanism.

1. Introduction

Cholinergic dysfunction is closely linked to memory loss in ageing and Alzheimer's disease (AD) [1]. This cholinergic hypothesis is the basis of symptomatic therapy with acetylcholinesterase inhibitors such as donepezil [2] and the scopolamine model, in which muscarinic blockade produces a short-lived central cholinergic deficit [3,4]. Scopolamine also increases brain acetylcholinesterase activity and oxidative stress in rodents [5]. Repeated or high-dose regimens have been linked to neuronal damage and changes in amyloid-β and tau markers [6], but the acute regimen used here does not reproduce AD pathology [7]; it serves as a model of cholinergic dysfunction and oxidative imbalance.

Oxidative stress can worsen cholinergic dysfunction [8], and polyphenol-rich plant extracts have therefore been widely tested in scopolamine-treated animals [9]. *Saraca asoca* W. J. de Wilde (Ashoka), a tree used in traditional Indian medicine, has antibacterial and antioxidant activities [10]. Its flowers contain tannins, flavonoids, and phenolic acids [11]; gallic acid, (+)-catechin, procyanidin-B2, (−)-epicatechin, and epicatechin gallate have been quantified in the species [12], and myricetin, rhamnazin, and quercetin have been reported in a fraction of the flower extract [13]. The ethanolic flower extract improved scopolamine-impaired performance in the elevated plus maze transfer-latency test and the Morris water maze [14] and reduced immobility in restrained mice [13], and a bark extract showed antidepressant-like activity in chronic unpredictable mild stress [15].

Anxiety and cognitive impairment often occur together; anxiety is more common in older adults with cognitive impairment and is linked to later decline [16,17]. Plant-derived preparations have been studied for both conditions [18,19]; *for example, Grewia asiatica berry juice* reduced anxiety- and depression-like behavior and scopolamine-induced memory impairment [20].

Therefore, this study aimed to address three questions not previously addressed for *S. asoca* flower extract: whether it improves scopolamine-impaired passive avoidance, novel object recognition, and Y-maze performance; whether these changes are accompanied by lower brain cholinesterase activity and MDA and restored catalase; and whether it alters behavior in the elevated plus maze, light–dark box, and social interaction test. The tested batch was characterized by Folin–Ciocalteu and aluminum chloride assays, LC–DAD–ESI–TOF–MS fingerprint, and *in vitro* antioxidant and enzyme-inhibitory assays. Behavior and biochemistry were measured in separate cohorts.

2. Materials and methods

2.1. Drugs and chemicals

Acetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), aluminum chloride, ascorbic acid, chloroform, ethanol, Folin–Ciocalteu reagent, gallic acid, methanol, n-butanol, potassium

acetate, potassium ferricyanide, sodium carbonate, trichloroacetic acid, ferric chloride, ferrous sulfate, hydrogen peroxide, egg albumin, egg lecithin, xanthine oxidase, oxypurinol, diclofenac sodium, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, α-tocopherol and buffer salts were of analytical grade. Scopolamine hydrobromide, piracetam and donepezil were gifts. Solutions were prepared fresh on the day of use.

2.2. Plant material, authentication and extraction

Flowers of *S. asoca* were collected in West Bengal, India, during April–May 2016; the exact collection site and coordinates were not recorded. The material was authenticated at the Department of Pharmacognosy, JSS College of Pharmacy, Mysuru, India, and a voucher specimen (No. SA-10601/Pharma) was deposited in the departmental herbarium. The flowers were shade-dried at room temperature for 6–8 d and coarsely powdered, and 800 g of powder was extracted with 2.0 L of 95% (v/v) ethanol (2.5:1, v/w) in a Soxhlet apparatus for 24 h. The extract was filtered through Whatman No. 1 paper, concentrated under reduced pressure at no more than 40 °C, and dried to constant mass, yielding 72 g (9.0% w/w yield). No marker-based standardization was performed; therefore, the extract was characterized but not standardized. The dried extract (SAF) was stored in an airtight amber container at −20 °C and suspended in normal saline just before oral dosing.

2.3. Preliminary phytochemical screening

Qualitative screening used standard tests [21]: Hager's reagent for alkaloids (yellow precipitate), the Shinoda test for flavonoids (magnesium and concentrated HCl; pink to red color), the biuret reaction for proteins (violet color), 10% ferric chloride for tannins (blue-black or green color), the Salkowski test for steroids (chloroform and concentrated H_2SO_4; reddish-brown interface), Barfoed's reagent for reducing sugars (red precipitate), Borntrager's test for anthraquinone glycosides (pink ammoniacal layer), and the foam test for saponins (froth lasting at least 10 min). Each test was run in triplicate against a reagent blank. Reference compounds were tested alongside six of the tests: quercetin (Shinoda), bovine serum albumin (biuret), tannic acid (ferric chloride), cholesterol (Salkowski), glucose (Barfoed's), and saponin (foam test). No reference compound was used for the Hager and Borntrager tests.

2.4. Total phenolic content

Total phenolic content was measured using the Folin–Ciocalteu method. SAF (4 µL, 1 mg/mL) was mixed with 98 µL of 10-fold diluted Folin–Ciocalteu reagent; after 5 min, 98 µL of 6% (w/v) sodium carbonate was added, and the mixture was kept at 25 °C for 90 min in the dark and read at 725 nm against a reagent blank. Gallic acid (20–100 µg/mL) was the standard, and the results are expressed as mg gallic acid equivalents per g of dry extract (mg GAE/g). The reagent also reacts with non-phenolic reducing substances [22], so the value is a measure of Folin–Ciocalteu reducing capacity expressed as GAE, not a phenolic-specific measure. The assay was performed in triplicate in three independent experiments.

2.5. Total flavonoid content

Flavonoid-type response was measured using a modification of the aluminum chloride method described by Ebrahimzadeh et al. [23]. SAF (0.5 mL, 1 mg/mL) was mixed with 0.1 mL of 10% aluminum chloride and 0.1 mL of 1 M potassium acetate and brought to a volume of 5 mL with 80% methanol, in place of the methanol and water volumes of the original method; quercetin standards (12.5–100 µg/mL) were treated in the same way. After 30 min at room temperature, absorbance was read at 415 nm, and the result was expressed as mg quercetin equivalents per g of dry extract (mg QE/g). At 410–430 nm, the aluminum complex forms mainly with flavonols and flavones, and its absorbance depends on the structure of each flavonoid; therefore, the value is a quercetin-equivalent assay response and not a structurally specific measure of total flavonoid content [24]. Each sample was measured in triplicate within a single run; therefore, the SEM reflects the within-run variation only.

2.6. *In vitro* antioxidant assays

Concentrations. The final concentrations in each reaction were the SAF and reference concentrations (10–160 µg/mL), and IC_{50} values follow the same convention. In every assay, an extract blank (SAF at each concentration without the color-forming reagent or substrate) was read and subtracted. **DPPH scavenging.** DPPH (195 µL; 0.1 mM in methanol) was mixed with 5 µL of SAF or vitamin E stock (0.4–6.4 mg/mL) in a 96-well plate, kept in the dark at 37 °C for 30 min, and read at 515 nm. Scavenging (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$, where the control contained methanol instead of the sample and the extract blank contained methanol instead of DPPH. **FRAP.** FRAP reagent was prepared fresh from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ (10:1:1, v/v/v) [25]. Reagent (3.0 mL, 37 °C) was mixed with 100 µL of SAF, held at 37 °C for 10 min, and read at 593 nm. The extract blank contained acetate buffer instead of FRAP reagent. $FeSO_4 \cdot 7H_2O$ was run at the same concentrations, and the results are given as absorbance at 593 nm. **Lipid peroxidation.** Egg lecithin liposomes were peroxidized with $FeCl_3$ (400 µM) and ascorbic acid (200 µM) at 37 °C for 60 min. The reaction mixture (0.9 mL) received 0.1 mL of SAF, giving a final volume of 1.0 mL and final SAF concentrations of 10–160 µg/mL. The reaction was stopped with 1.0 mL of 0.25 M HCl containing 15% (w/v) trichloroacetic acid; 1.0 mL of 0.67% (w/v) thiobarbituric acid was added, and the tubes were heated in a boiling water bath for 15 min, cooled, centrifuged, and read at 532 nm. The control lacked SAF, and the extract blank lacked liposomes. IC_{50} values were obtained by nonlinear regression, with vitamin E as the positive control. Each concentration was assayed in triplicate in a single run. The SEM therefore reflects within-run variation only, not reproducibility across runs or extract batches.

2.7. LC–DAD–ESI–TOF–MS fingerprinting

LC–high-resolution mass spectrometry was used to fingerprint SAF, not to identify compounds. Analysis was performed at the CSIR–Central Food Technological Research Institute, Mysuru, India, on an ultra-high-performance liquid chromatograph coupled to an AB Sciex TripleTOF 5600 mass spectrometer with an electrospray source and a diode-array detector. A Waters ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 µm; 40 °C) was eluted with water (A) and acetonitrile (B), each with 0.1% formic acid, using the linear gradient in Table 1 at 0.3 mL/min (5-µL injection; DAD at 254 nm). Spectra were acquired in positive and negative ESI modes over m/z 100–1500. Sample preparation, source, resolution, calibration, and processing settings

followed the facility's standard method; however, the method and raw files could not be recovered, so the accurate-mass values were not used for formula assignment or annotation. Representative ion features (Table 5) were selected manually as the most intense apices of the base-peak-intensity (BPI) chromatogram in each mode, and their m/z values were read from the full-scan spectra. Adducts and isotopes were not grouped; therefore, one constituent may give several ion features. The MS ion features are at a confidence level of 5 (exact mass of interest) of Schymanski et al. [26] and are reported without annotation [27]; the UV peaks were not annotated or matched to them.

Table 1. Gradient elution program for LC–DAD–ESI–TOF–MS analysis of SAF

Time (min)	Mobile Phase B (%)
0.0	5
2.0	5
20.0	95
25.0	95
27.0	5
30.0	5

Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The changes in %B between the time points were linear. Column: Waters ACQUITY BEH C18 (2.1 × 100 mm, 1.7 µm); 40 °C; 0.3 mL/min; injection 5 µL; DAD at 254 nm.

2.8. Xanthine oxidase inhibition and albumin denaturation

For xanthine oxidase inhibition, SAF was incubated with xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.5) at 37 °C for 15 min, xanthine (0.15 mM, final) was added, and after 30 min at 37 °C, the reaction was stopped with 0.5 M HCl and read at 295 nm. Polyphenols absorb in the UV range; therefore, an extract blank without the enzyme was subtracted at each concentration. Inhibition (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$, with oxypurinol as the positive control. Xanthine oxidase produces superoxide and hydrogen peroxide during purine oxidation [28], and phenolic compounds can inhibit it [29]. For albumin denaturation, SAF was mixed with egg albumin in phosphate-buffered saline, held at 37 °C for 15 min, heated at 70 °C for 5 min, cooled, and read at 660 nm; an extract blank without albumin was subtracted to correct for color and turbidity, and diclofenac sodium was the positive control. Both assays used final SAF concentrations of 10–160 µg/mL and technical triplicates in a single run. Inhibition of albumin denaturation *in vitro* does not by itself indicate anti-inflammatory activity *in vivo*.

2.9. Animals and ethics

Healthy adult Swiss albino mice (*Mus musculus*; 8–10 weeks, 25–30 g) were obtained from the animal facility of JSS Medical College (Mysuru, India). They were housed six per cage at 22 ± 2 °C and 55 ± 10% relative humidity on a 12-h light/dark cycle (lights on 07:00) with a pelleted diet and water *ad libitum*, and were acclimatized for 7 days. Behavioral tests were performed in the light phase in a quiet, temperature-controlled room, and health, food and water intake, and signs of distress were checked daily. The cognitive and biochemical cohorts were all female. The sex of the anxiolytic cohort was not recorded, and whether social interaction pairs were sex-matched is unknown; since mouse social behavior differs with sex and pairing, this adds unexplained variation to the data [30].

All procedures followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and were approved by the Institutional Animal Ethics Committee [name and approval number withheld for blind review]. The work predates ARRIVE 2.0 [31]; the manuscript was checked against that checklist afterwards and does not meet all Essential 10 items (Supplementary Table S1). No sample size was determined in advance, and this is recorded as an unmet item. The use of six animals per group followed earlier studies of this extract from our laboratory [13,14]; this is precedent, not a statistical justification. The smallest effect that this design could detect is given in Section 2.14. Animals were allocated using a computer-generated random sequence, and testing and scoring were conducted blind to treatment.

2.10. Experimental design

Model and reference drugs. Scopolamine (0.4 mg/kg, i.p., 45 min before the session) was used to produce an acute cholinergic deficit. The dose, route, and interval followed earlier mouse studies of plant preparations in the passive avoidance task [32] and were not re-established by dose-finding in this laboratory; the scopolamine-versus-normal differences in all three tasks (Section 3.5) show that the regimen was effective here. Scopolamine also impairs object recognition in mice [3], and its cognitive and locomotor effects are not readily dissociated even at low doses [33]. Piracetam (100 mg/kg, p.o.) was the behavioral reference. This dose came from the earlier study of this extract in the same laboratory [14] and is lower than the 200 mg/kg, i.p. used in other mouse studies [32], so piracetam served to confirm that the deficit could be reversed rather than as a benchmark of potency. Donepezil [2] was the reference in the biochemical cohort and diazepam (1 mg/kg) in the anxiolytic cohort, although diazepam at 0.5–2 mg/kg had no anxiolytic effect in the elevated plus maze in C57BL/6J mice and impaired locomotion at the highest dose [34]. Novel object recognition [35] and the Y-maze [36] assess recognition and spatial working memory, and step-through passive avoidance is an inhibitory avoidance task. The elevated plus maze, light–dark box, and social interaction test are unconditioned tests of anxiety-like behavior [37] whose sensitivity differs by endpoint [38].

Dose selection. Acute toxicity was not re-examined. In an earlier OECD 423 study of the same extract in female Swiss albino mice, no mortality occurred up to 2000 mg/kg, p.o., and one death with pathological changes was reported at 2500 mg/kg [13,39]. The doses used here (50, 100,

and 250 mg/kg) are one-eighth or less of 2000 mg/kg and match those of that study. This single-dose result supports dose selection only; repeated-dose safety over 14 days was not assessed beyond daily health checks.

Cohort structure. Cognitive, biochemical, and anxiolytic assessments were performed on three independent cohorts of 36 mice (six groups of six); no animal was used in more than one cohort.

Cognitive cohort. Treatments were administered orally once daily for 14 days, and the three tasks were run in sequence in the same animals (n = 6 per group). The groups were normal control (saline 10 mL/kg, p.o.), scopolamine control, SAF (50, 100, or 250 mg/kg, p.o.) + scopolamine, and piracetam (100 mg/kg, p.o.) + scopolamine. Scopolamine (0.4 mg/kg, i.p.) was administered 45 min before passive avoidance acquisition (day 8), the novel object recognition sample trial (day 12), and the Y-maze test (day 14); the passive avoidance retention test (day 9) and the object recognition test (day 13) were drug-free. Each mouse therefore received three scopolamine challenges, and tolerance or carry-over between sessions may have affected the later tasks [27]. No locomotor, sensory, or attentional measurements were collected.

Biochemical cohort. Six groups (n = 6) received oral saline (normal control and scopolamine control), donepezil (5 mg/kg, p.o.), or SAF (50, 100, or 250 mg/kg, p.o.) once daily for 14 days. No scopolamine was administered during the 14 days of oral dosing. On day 14, every group except the normal control received a single injection of scopolamine (0.4 mg/kg, i.p.) 60 min after the last oral dose, and brains were collected 60 min later for cholinesterase, MDA, and catalase assays.

Anxiolytic cohort. Six groups (n = 6; three pairs per group for the social interaction test) received vehicle (saline 10 mL/kg, p.o.), diazepam (1 mg/kg, p.o.), SAF (50, 100, or 250 mg/kg, p.o.), or SAF 50 mg/kg + diazepam 1 mg/kg once daily for 7 days, without scopolamine. Testing began approximately 30 min after the last dose; the basis of this interval was not documented, and no locomotor, open-field, or rotarod tests were performed.

Table 2. Treatment groups used for cognitive behavioral assessment

Group	Treatment	Dose and route of administration	Schedule
Normal control	Saline p.o. + saline i.p.	10 mL/kg, p.o.; saline, i.p.	Saline p.o. daily for 14 days; saline i.p. at the times of scopolamine administration
Scopolamine control	Saline p.o. + scopolamine	10 mL/kg, p.o.; 0.4 mg/kg, i.p.	Saline p.o. daily for 14 days; scopolamine on days 8, 12 and 14

Low-dose ethanolic flower extract of *Saraca asoca* (SAF)	SAF + scopolamine	SAF 50 mg/kg, p.o.; scopolamine 0.4 mg/kg, i.p.	SAF daily for 14 days; scopolamine on days 8, 12 and 14
Medium-dose SAF	SAF + scopolamine	SAF 100 mg/kg, p.o.; scopolamine 0.4 mg/kg, i.p.	SAF daily for 14 days; scopolamine on days 8, 12 and 14
High-dose SAF	SAF + scopolamine	SAF 250 mg/kg, p.o.; scopolamine 0.4 mg/kg, i.p.	SAF daily for 14 days; scopolamine on days 8, 12 and 14
Positive control	Piracetam + scopolamine	Piracetam 100 mg/kg, p.o.; scopolamine 0.4 mg/kg, i.p.	Piracetam daily for 14 days; scopolamine on days 8, 12 and 14

Note: SAF, ethanolic flower extract of *Saraca asoca*; p.o., oral; i.p., intraperitoneal; n = 6 per group. The normal control received oral saline and i.p. saline in place of scopolamine, and the scopolamine control received oral saline in place of SAF; therefore, oral and i.p. dosing were matched across groups. The timing for all cohorts is shown in Figure 2.

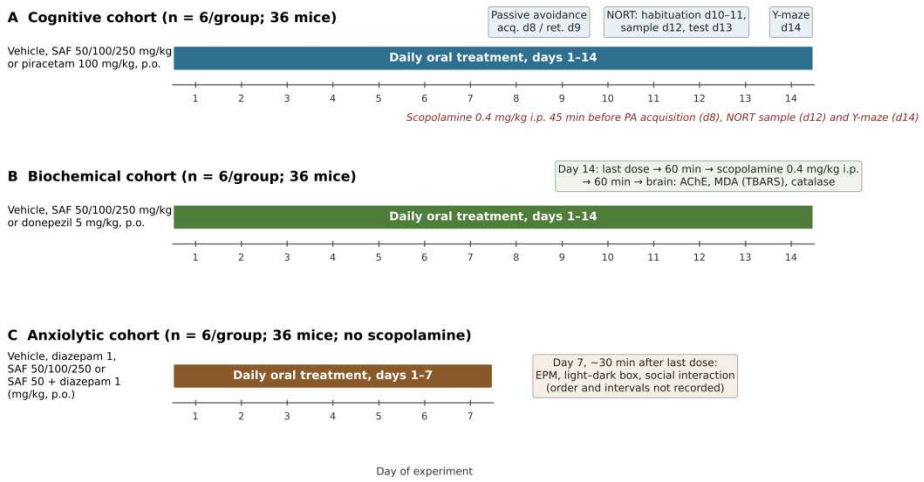


Figure 2. Experimental design. (A) Cognitive cohort: oral treatment for 14 days; scopolamine (0.4 mg/kg, i.p.) 45 min before passive avoidance acquisition (day 8), the novel object recognition sample trial (day 12), and the Y-maze test (day 14); retention (day 9) and recognition testing (day 13) were drug-free. (B) Biochemical cohort: oral treatment for 14 days without scopolamine; on day 14, one scopolamine injection 60 min after the last dose and brain collection 60 min later. (C) Anxiolytic cohort: oral treatment for 7 days without scopolamine, followed by the elevated plus maze, light–dark box, and social interaction test about 30 min after the last dose (order and spacing not recorded). Each cohort had six groups of six mice; in the

social interaction test, the six mice of a group formed three pairs, and the pair was the unit of analysis.

2.11. Cognitive tasks

The tests were performed in a quiet, temperature-controlled room during the light phase. The researchers acclimatized the mice for at least 30 min before each session, cleaned the apparatus with 70% ethanol between animals, and conducted testing and scoring blind to treatment.

Step-through passive avoidance. The apparatus had an illuminated chamber (approximately 400 lx) and a dark chamber (each 20 × 20 × 20 cm) joined by a 5 × 5 cm guillotine door with a grid floor in the dark chamber. At acquisition, each mouse was placed in the illuminated chamber facing away from the door; after 60 s, the door was opened, the latency to enter the dark chamber with all four paws was recorded, and an inescapable foot shock (0.2 mA, 2 s) was administered. The maximum acquisition time and handling of any mouse that did not enter were not recorded; group mean acquisition latencies were 21.8–24.1 s. Individual acquisition records were not retained; therefore, it cannot be verified that every mouse entered the dark chamber and received the shock, or that shock exposure was equal across animals. All six mice in each group were included in the retention analysis. Retention latency was measured 24 h later without shock, with a 300-s cut-off.

Novel object recognition test. The test uses the preference of mice for exploring a novel object as an index of recognition memory [35]. After two 5-min habituation sessions in a 40 × 40 × 40 cm arena, mice explored two identical objects (A1, A2) for 5 min, 45 min after scopolamine administration. Twenty-four hours later, one object was replaced by a novel object (B), and exploration of each object (nose within 2 cm of it) was timed for 5 min. It was not recorded whether object identity and the side of the novel object were counterbalanced across mice; therefore, a preference for one object or one side that is unrelated to memory cannot be excluded. Discrimination index (DI) = (Tnovel − Tfamiliar)/(Tnovel + Tfamiliar), and novel-object preference (%) = Tnovel/(Tnovel + Tfamiliar) × 100. Both are calculated from the same exploration times; therefore, preference (%) = 50 × (1 + DI) for every animal.

Y-maze spontaneous alternation test. The maze had three opaque arms (35 × 5 × 20 cm) at 120° to each other. Spontaneous alternation is used as an index of spatial working memory in mice [40]. Scopolamine (0.4 mg/kg, i.p.) was administered 45 min before testing, and each mouse was placed in the center and allowed to explore for 8 min. An arm entry was scored when all four paws were inside an arm [41], and every entry was counted, including re-entry into the arm immediately adjacent to the arm entered. An alternation was three consecutive entries into different arms, and spontaneous alternation (%) = [alternations/(total arm entries − 2)] × 100. With random choice among the three arms, three consecutive entries fell in different arms with probability 1 × (2/3) × (1/3) = 2/9; therefore, chance alternation was 22.2%. Total arm entries were recorded as a measure of activity within this task only [41].

2.12. Anxiety-like behavior tests

Anxiety-like behavior was tested approximately 30 min after the last dose, during the light phase, in a quiet, temperature-controlled room.

Elevated plus maze (EPM). The EPM is based on the conflict between exploration and the aversion of mice to open, elevated spaces [42]. The maze had two open and two closed arms (50 cm long; closed arms with 40-cm walls) 50 cm above the floor. Each mouse was placed in the center facing an open arm, and its behavior was recorded for 5 min, and open-arm entries (all four paws) and open-arm time were analyzed separately. Both measures also change with general activity [43].

Light–dark box test (LDBT). The LDBT is based on the conflict between exploration and the aversion of mice to brightly lit areas [44,45]. The box (45 × 27 × 27 cm) had light and dark compartments joined by a central 7.5 × 7.5 cm opening. Following Bourin and Hascoët [7], the light compartment was lit at approximately 400 lx, and each mouse was placed in its center facing away from the opening at the start of the 5-min test. Time spent in the light compartment was used as an anxiety-related measure. Shuttle crossings were recorded but not retained.

Social interaction test (SIT). The SIT measures the amount of investigation of an unfamiliar conspecific in a novel setting [46]. Each mouse was isolated for 1 h and paired, in a covered box (22 × 15 × 12 cm), with an unfamiliar mouse from the same treatment group, and the total time spent in active social behavior (sniffing, genital investigation, following, grooming, wrestling, climbing, and boxing) was recorded over 5 min. The pair was the experimental unit (n = 3 pairs per group).

The EPM, LDBT, and SIT were run in the same cohort. Their order and the intervals between them were not recorded, and the order was not counterbalanced; therefore, carry-over between tests cannot be excluded.

2.13. Biochemical assays

On day 14, 60 min after scopolamine, mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. and killed by cervical dislocation. Brains were removed on ice with the cerebellum excluded, homogenized (10% w/v) in ice-cold 0.1 M phosphate buffer (pH 7.4) and centrifuged at 10,000 × g for 15 min at 4 °C. Protein was measured using the method described by Lowry et al. [47]. **Cholinesterase.** Activity was measured by Ellman's method [48]. Supernatant (0.4 mL) was mixed with 2.6 mL of 0.1 M phosphate buffer (pH 8.0) and 100 µL of DTNB; 20 µL of acetylthiocholine iodide was added, and absorbance at 412 nm was read every 2 min for 10 min. Activity was calculated from the linear rate as $R = 5.74 \times 10^{-4} \times \Delta A/C_0$ [48], where R is mol acetylthiocholine hydrolyzed min^{-1} g^{-1} tissue, ΔA is the change in absorbance per min, and C_0 is the tissue concentration of the supernatant (100 mg/mL for a 10% w/v homogenate). The constant follows from the molar absorption coefficient of 5-thio-2-nitrobenzoate (1.36×10^4 M^{-1} cm^{-1}; 1-cm path), the 3.12-mL reaction volume, and the 0.4-mL sample: $5.74 \times 10^{-4} = (3.12 \times 10^{-3}\ L \times 10^3\ mg\ g^{-1})/(1.36 \times 10^4\ M^{-1} \times 0.4\ mL)$. R was multiplied by 10^6 to give µmol min^{-1} g^{-1} tissue. Acetylthiocholine is also hydrolyzed by butyrylcholinesterase (BChE), and no selective BChE inhibitor was used; therefore, the values are total acetylthiocholine-hydrolyzing cholinesterase (ChE) activity and are not specific to

AChE [49]. They describe tissue activity after treatment, not direct enzyme inhibition. **Lipid peroxidation.** Thiobarbituric acid-reactive substances (TBARS) were measured using the method described by Ohkawa et al. [50]: homogenate (100 µL) was mixed with 1.5 mL of 0.8% thiobarbituric acid, 1.5 mL of 20% acetic acid, and 200 µL of 8% sodium dodecyl sulfate, heated at 95 °C for 1 h, cooled, extracted with n-butanol, and read at 532 nm. Because other aldehydes also react, the results are expressed as MDA equivalents (nmol/mg protein) from a 1,1,3,3-tetramethoxypropane standard curve. **Catalase.** Activity was measured using the dichromate–acetic acid method [51,52]. Homogenate (0.1 mL) was incubated with 1.0 mL of 0.01 M phosphate buffer (pH 7.0) and 0.4 mL of 0.2 M H_2O_2 at 37 °C for 1 min; the reaction was stopped with dichromate–acetic acid reagent, heated in a boiling water bath for 15 min, and read after cooling. The H_2O_2 remaining was read from an H_2O_2 standard curve treated in the same way, and the activity was calculated as µmol H_2O_2 decomposed min^{-1} mg^{-1} protein. The original method reads chromic acetate at 570 nm. This laboratory read standards and samples at 620 nm, and the linearity and sensitivity of the assay at 620 nm were not checked against 570 nm. Therefore, the catalase values are used only to compare groups within this study and should not be compared directly with values obtained at 570 nm.

2.14. Statistical analysis

Data are presented as mean ± SEM. Individual-animal data were not retained; therefore, normality, equality of variance, and outliers could not be examined. For the cognitive and biochemical endpoints, one-way ANOVA, Dunnett's test of the four treated groups against the scopolamine control, and a planned two-sided t-test of scopolamine versus normal control were computed from the reported group means, SEMs, and n, using the pooled error term of all six groups (df = 30). The recomputed F values matched the original values. These calculations reproduce the test statistics implied by the summaries but cannot confirm that the raw data met the model assumptions; the inputs, results, and 95% confidence intervals of each difference from the scopolamine control (pooled SE, df = 30, unadjusted) are listed in Supplementary Table S2 so that they can be checked. Because six endpoints were tested in these two cohorts, the 18 Dunnett-adjusted p-values for SAF (three doses × six endpoints) were also adjusted by the Benjamini–Hochberg false discovery rate (FDR) procedure, and the q-values are given in Table S2. For the anxiety endpoints, the original ANOVA and Dunnett results are reported where they were retained; they were not corrected across endpoints and are exploratory. Degrees of freedom were 5 and 30 for n = 6 per group, and 5 and 12 for the SIT (pairs). Effect sizes are partial eta-squared ($\eta p^2 = F \cdot df_1/(F \cdot df_1 + df_2)$). Y-maze alternation was compared with the 22.2% chance level by one-sample t-tests with Bonferroni correction for six tests. With six animals per group, a two-sided two-group comparison at $\alpha = 0.05$ has 80% power only for standardized differences of about 1.8 or more (sensitivity analysis, not post hoc power), so smaller effects could be missed. Reversal (%) = (scopolamine − treated)/(scopolamine − normal) × 100 for endpoints raised by scopolamine (ChE, MDA) and (treated − scopolamine)/(normal − scopolamine) × 100 for endpoints lowered by scopolamine (retention latency, discrimination index, catalase). These percentages were calculated from the rounded group means shown in the tables and were not tested. Significance was set at $p < 0.05$. *In vitro* data are descriptive.

3. Results

3.1. Phytochemical screening

SAF gave positive tests for flavonoids (++), proteins, tannins, carbohydrates, glycosides, and saponins, and negative tests for alkaloids and steroids (Table 3).

Table 3. Preliminary phytochemical screening of SAF

Phytoconstituent	Test or reagent	Result
Alkaloids	Hager's test	−
Flavonoids	Shinoda test	++
Proteins	Biuret test	+
Tannins	Ferric chloride test	+
Steroids	Salkowski test	−
Carbohydrates	Barfoed's test	+
Glycosides	Borntrager's test	+
Saponins	Foam test	+

+, clear positive reaction; ++, visibly stronger color or precipitate than +, judged by the same observer across triplicate tests (qualitative grading); −, no reaction.

3.2. Folin–Ciocalteu and aluminum chloride assay values

SAF gave 90.94 ± 0.75 mg GAE/g and 51.54 ± 0.78 mg QE/g, respectively. The extraction yield was 9.0% (w/w).

3.3. LC–DAD–ESI–TOF–MS fingerprint

The LC–DAD chromatogram at 254 nm resolved 51 integrated UV peaks between 0.972 and 18.180 min (Figure 3 and Table 4). The six largest peaks, labeled P1–P6 in order of integrated area rather than elution, accounted for 41.815% of the total area: P1 (7.782 min, 12.414%), P2 (9.180 min, 11.498%), P3 (9.840 min, 5.757%), P4 (10.954 min, 4.815%), P5 (7.050 min, 3.729%), and P6 (2.390 min, 3.602%). No single peak dominated the profiles. ESI-TOF-MS in positive and negative modes (m/z 100–1500) provided base-peak-intensity profiles (Figure 4; Table 5), with representative ion features at 7.77, 9.64, and 11.20 min in positive mode (m/z 131.0949, 429.2911, and 307.1996) and at 7.11, 10.70, and 12.77 min in negative mode (m/z

327.2426, 293.2346, and 277.2403). No correspondence between the UV peaks and MS ion features was established, and none of the signals were annotated.

Table 4. Six major LC–DAD peaks of SAF at 254 nm, labeled in order of integrated UV area

Feature	Retention time (min)	Peak height	Integrated area	Relative area (%)
P1	7.782	2,983,029	654,297.688	12.414
P2	9.180	2,055,753	605,984.313	11.498
P3	9.840	1,544,996	303,402.406	5.757
P4	10.954	839,115	253,750.953	4.815
P5	7.050	1,091,503	196,528.063	3.729
P6	2.390	492,880	189,861.828	3.602
Subtotal for P1–P6	–	–	–	41.815

Fifty-one UV peaks were integrated (0.972–18.180 min) in the chromatogram. P1–P6 were ranked by area, not retention time. Peak height and integrated area are in instrument counts (arbitrary units), and relative area (%) = peak area/summed area of all 51 integrated peaks × 100. UV peaks are chromatographic signals, not identified as compounds.

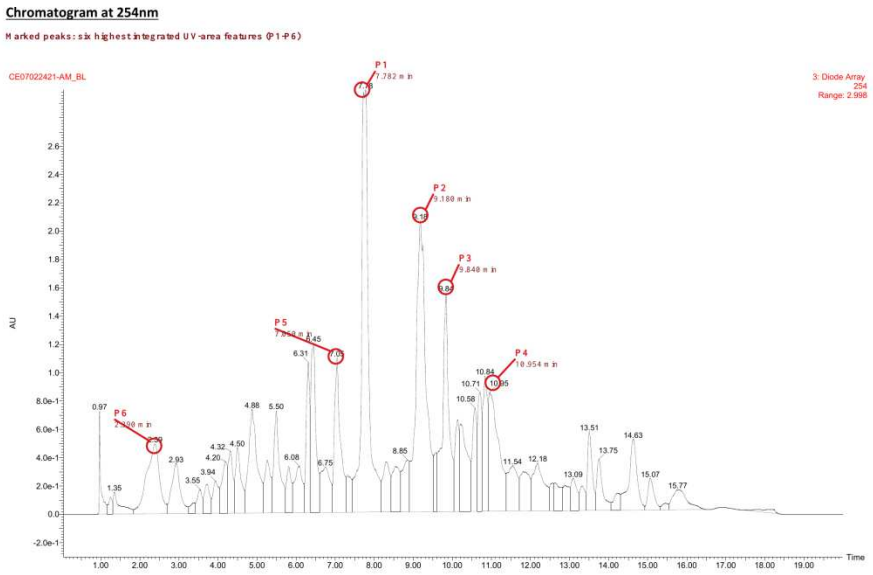


Figure 3. LC–DAD chromatogram of SAF at 254 nm. Fifty-one UV peaks were integrated between 0.972 and 18.180 min; the six largest (P1–P6, ranked by area) accounted for 41.815% of the integrated area. No peak was annotated.

Table 5. Representative ion features of SAF from LC–ESI–TOF–MS

Feature	ESI Mode	RT (min)	m/z
M1	ESI+	7.77	131.0949
M2	ESI+	9.64	429.2911
M3	ESI+	11.20	307.1996
M4	ESI−	7.11	327.2426
M5	ESI−	10.70	293.2346
M6	ESI−	12.77	277.2403

RT is the apex of the base-peak-intensity chromatogram (Figure 4). The m/z values were read from full-scan spectra at 7.719 (M1), 9.587 (M2), 11.204 (M3), 7.113 (M4), 10.598 (M5), and 12.769 min (M6), close to each apex; the M5 scan lies 0.10 min before the apex. Adducts and isotopes were not grouped, and no standards or MS/MS spectra were used; therefore, these are ion features, not compounds.

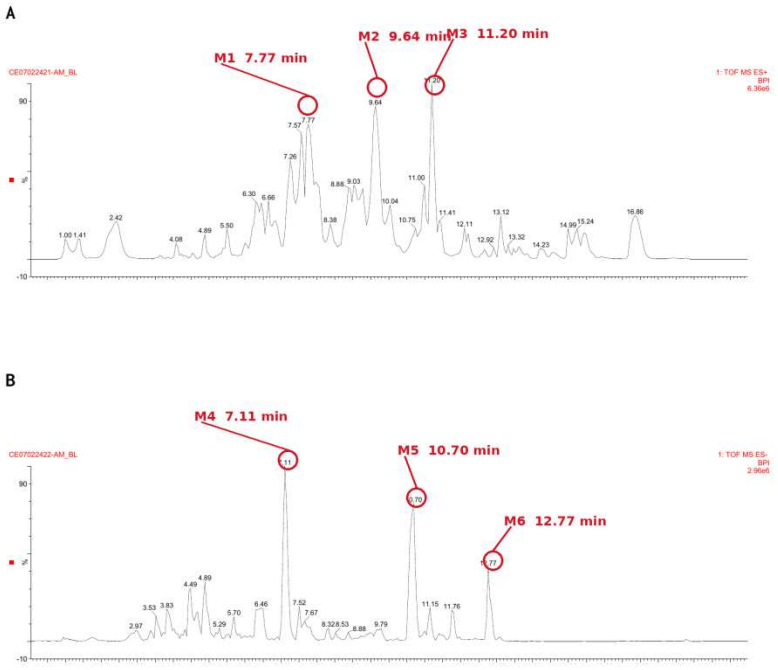


Figure 4. Base-peak-intensity chromatograms of SAF from LC–ESI–TOF–MS in (A) positive-ion mode, with representative ion features M1 (7.77 min), M2 (9.64 min), and M3 (11.20 min), and (B) negative-ion mode, with M4 (7.11 min), M5 (10.70 min), and M6 (12.77 min). m/z values are listed in Table 5. No feature was annotated.

3.4. Antioxidant and enzyme-inhibitory properties of SAF

SAF possessed concentration-dependent activity in all five *in vitro* assays (Figure 5; Table 6). It scavenged DPPH radicals with an IC_{50} of 62.7 µg/mL compared with vitamin E (IC_{50} 34.8 µg/mL), reaching 66.0 ± 3.5% at 100 µg/mL (vitamin E 83.0 ± 2.2%). FRAP absorbance increased from 0.45 ± 0.05 to 1.12 ± 0.06 over 20–100 µg/mL, about three-quarters of the $FeSO_4 \cdot 7H_2O$ response at each concentration (0.60 ± 0.04 to 1.48 ± 0.05). SAF inhibited lipid peroxidation (58.0 ± 3.5% at 100 µg/mL; IC_{50} 58.4 µg/mL; vitamin E 32.6 µg/mL), xanthine oxidase (60.0 ± 4.0% at 160 µg/mL; IC_{50} 74.5 µg/mL; oxypurinol 18.9 µg/mL), and albumin denaturation (65.0 ± 3.5% at 160 µg/mL; IC_{50} 69.3 µg/mL; diclofenac 27.6 µg/mL), so the reference standards were ~1.8- to 3.9-fold more potent. Each assay was run once in technical triplicate, and the activities belong to the whole extract.

Table 6. *In vitro* antioxidant, xanthine oxidase-inhibitory and anti-denaturation activity of SAF (mean ± SEM; technical triplicates from a single run)

Assay	Test Sample	Activity at the indicated concentration	Half-maximal inhibitory

			concentration, IC_{50} (µg/mL)
2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging	SAF	66.0 ± 3.5% at 100 µg/mL	62.7
	Vitamin E	83.0 ± 2.2% at 100 µg/mL	34.8
Ferric-reducing antioxidant power	SAF	1.12 ± 0.06 AU at 593 nm and 100 µg/mL	—
	$FeSO_4 \cdot 7H_2O$ standard	1.48 ± 0.05 AU at 593 nm and 100 µg/mL	—
Lipid peroxidation inhibition	SAF	58.0 ± 3.5% at 100 µg/mL	58.4
	Vitamin E	84.0 ± 2.6% at 100 µg/mL	32.6
Xanthine oxidase inhibition	SAF	60.0 ± 4.0% at 160 µg/mL	74.5
	Oxypurinol	83.0 ± 3.0% at 160 µg/mL	18.9
Albumin denaturation inhibition	SAF	65.0 ± 3.5% at 160 µg/mL	69.3
	Diclofenac sodium	86.0 ± 2.8% at 160 µg/mL	27.6

IC50 is the concentration that inhibits 50% of the reaction, estimated from the nonlinear regression of the mean concentration–response data (final concentrations). IC50 values are point estimates: with technical triplicates from one run, a confidence interval would not reflect between-run variability, so none is given. DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric-reducing antioxidant power; AU, absorbance units.

3.5. Effect of SAF on scopolamine-impaired task performance

All 36 mice in the cognitive cohort completed the three tasks successfully.

Step-through passive avoidance. Scopolamine shortened retention latency by approximately 3.1-fold, from 268.5 ± 18.6 s to 86.7 ± 9.4 s ($t = 8.41$, $p < 0.001$; $F = 24.49$, $p < 0.0001$, $\eta p^2 = 0.80$; Figure 6; Table 7). SAF at 100 and 250 mg/kg prolonged retention latency by approximately 1.9-fold (165.8 ± 14.7 s; Dunnett $p = 0.003$; 43.5% reversal) and 2.6-fold (223.4 ± 16.3 s; $p < 0.001$; 75.2%), whereas 50 mg/kg had no significant effect (112.6 ± 12.8 s; $p = 0.58$). Piracetam gave the largest reversal (254.7 ± 17.9 s; 92.4%; $p < 0.001$). No difference in acquisition latency was detected (21.8–24.1 s; $F = 0.16$, $p = 0.97$). Because shock exposure at acquisition could not be verified, longer retention latencies may also reflect differences in conditioning or shock sensitivity.

Table 7. Effect of SAF on step-through passive avoidance performance

Treatment Group	Acquisition Latency (s)	Retention Latency (s)	Reversal Rate (%)	Statistical Significance
Saline control	22.4 ± 2.1	268.5 ± 18.6	—	—
Scopolamine (0.4 mg/kg)	21.8 ± 1.9	86.7 ± 9.4	0	### $p < 0.001$
SAF 50 mg/kg + scopolamine	23.1 ± 2.0	112.6 ± 12.8	14.2	NS ($p = 0.58$)
SAF 100 mg/kg + scopolamine	22.6 ± 2.3	165.8 ± 14.7	43.5	** $p = 0.003$
SAF 250 mg/kg + scopolamine	23.8 ± 2.5	223.4 ± 16.3	75.2	*** $p < 0.001$
Piracetam 100 mg/kg + scopolamine	24.1 ± 2.2	254.7 ± 17.9	92.4	*** $p < 0.001$

Mean ± SEM; n = 6 per group; 300-s cut-off. The Significance column refers to retention latency: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p-values for each treatment versus the scopolamine control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; NS, not significant). One-way ANOVA: retention latency $F = 24.49$, $p < 0.0001$, $\eta p^2 = 0.80$; acquisition latency $F = 0.16$, $p = 0.97$. Reversal (%) is defined in Section 2.14.

Novel object recognition. Scopolamine lowered the discrimination index approximately 3.0-fold, from 0.62 ± 0.04 to 0.21 ± 0.03 ($t = 7.44$, $p < 0.001$; $F = 19.90$, $p < 0.0001$, $\eta p^2 = 0.77$;

Figure 7; Table 8). SAF at 100 and 250 mg/kg raised the index approximately 2.0-fold (0.42 ± 0.03; $p = 0.002$; 51.2% reversal) and 2.7-fold (0.56 ± 0.04; $p < 0.001$; 85.4%), and 50 mg/kg had no significant effect (0.28 ± 0.04; $p = 0.53$). Piracetam restored the index to 0.60 ± 0.05 (95.1%; $p < 0.001$). The total object exploration time was not retained.

Table 8. Effect of SAF on novel object recognition

Treatment Group	Discrimination Index (DI)	Novel-Object Preference (%) [= 50 × (1 + DI)]	Reversal Rate (%)	Significance
Control (saline)	0.62 ± 0.04	81.0	—	—
Scopolamine (0.4 mg/kg)	0.21 ± 0.03	60.5	0	*** $p < 0.001$
SAF 50 mg/kg + scopolamine	0.28 ± 0.04	64.0	17.1	NS ($p = 0.53$)
SAF 100 mg/kg + scopolamine	0.42 ± 0.03	71.0	51.2	** $p = 0.002$
SAF 250 mg/kg + scopolamine	0.56 ± 0.04	78.0	85.4	*** $p < 0.001$
Piracetam 100 mg/kg + scopolamine	0.60 ± 0.05	80.0	95.1	*** $p < 0.001$

Note: Mean ± SEM; $n = 6$ per group. $DI = (T_{novel} - T_{familiar})/(T_{novel} + T_{familiar})$ [53]; preference (%) = 50 × (1 + DI); descriptive only. One-way ANOVA: $F = 19.90$, $p < 0.0001$, $\eta p^2 = 0.77$. The Significance column refers to the DI: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p-values versus the scopolamine control (**$p < 0.01$, ***$p < 0.001$; NS, not significant).

Y-maze spontaneous alternation. Scopolamine reduced alternation from 71.4 ± 3.5% to 42.6 ± 2.9% ($t = 5.97$, $p < 0.001$; $F = 12.18$, $p < 0.0001$, $\eta p^2 = 0.67$; Figure 8; Table 9). SAF at 100 mg/kg (58.7 ± 3.4%; $p = 0.008$) and 250 mg/kg (66.9 ± 3.6%; $p < 0.001$) raised alternation; 50 mg/kg did not (48.9 ± 3.2%; $p = 0.51$), and piracetam brought it close to control (70.2 ± 3.8%; $p < 0.001$). All groups alternated above the 22.2% chance level ($t = 7.03$–14.05; Bonferroni-corrected $p < 0.01$). Total arm entries did not differ (21.9–24.0; $F = 0.22$, $p = 0.95$); therefore, the alternation differences were not due to fewer entries in this session; arm entries are not a separate locomotor test.

Table 9. Effect of SAF on Y-maze spontaneous alternation

Treatment Group	Spontaneous Alternation (%)	Total Arm Entries	Significance
Control (saline)	71.4 ± 3.5	22.8 ± 1.6	—
Scopolamine (0.4 mg/kg)	42.6 ± 2.9	21.9 ± 1.4	### $p < 0.001$
SAF 50 mg/kg + scopolamine	48.9 ± 3.2	22.3 ± 1.5	NS ($p = 0.51$)
SAF 100 mg/kg + scopolamine	58.7 ± 3.4	23.1 ± 1.7	** $p = 0.008$
SAF 250 mg/kg + scopolamine	66.9 ± 3.6	23.5 ± 1.8	*** $p < 0.001$
Piracetam 100 mg/kg + scopolamine	70.2 ± 3.8	24.0 ± 1.9	*** $p < 0.001$

Note: Mean ± SEM; n = 6 per group. Alternation (%) = [alternations/(total entries − 2)] × 100; chance level: 22.2%. One-way ANOVA: alternation $F = 12.18$, $p < 0.0001$, $\eta p^2 = 0.67$; arm entries $F = 0.22$, $p = 0.95$. The Significance column refers to alternation: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p values versus the scopolamine control.

3.6. Effect of SAF on brain cholinesterase activity

Scopolamine raised brain cholinesterase activity approximately 1.5-fold, from 1.56 ± 0.06 to 2.41 ± 0.11 μmol min^{-1} g^{-1} tissue ($t = 7.41$, $p < 0.001$; $F = 19.44$, $p < 0.0001$, $\eta p^2 = 0.76$; Figure 9; Table 10), in line with earlier reports for this model [54]. SAF lowered activity in a dose-dependent manner to 2.12 ± 0.10 (34.1% reversal; $p = 0.056$), 1.86 ± 0.08 (64.7%; $p < 0.001$) and 1.67 ± 0.07 μmol min^{-1} g^{-1} (87.1%; $p < 0.001$) at 50, 100 and 250 mg/kg. The 250 mg/kg value approached that of donepezil (1.48 ± 0.05; $p < 0.001$), although the two were not compared statistically.

Table 10. Effect of treatment on brain total cholinesterase (acetylthiocholine-hydrolyzing) activity

Group	Total cholinesterase (ChE; μmol min^{-1} g^{-1} tissue)	Reversal (%)	Significance
Normal control	1.56 ± 0.06	—	—
Scopolamine control	2.41 ± 0.11	0	### $p < 0.001$

Donepezil (5 mg/kg)	1.48 ± 0.05	109.4 †	*** $p < 0.001$
SAF (50 mg/kg)	2.12 ± 0.10	34.1	NS ($p = 0.056$)
SAF (100 mg/kg)	1.86 ± 0.08	64.7	*** $p < 0.001$
SAF (250 mg/kg)	1.67 ± 0.07	87.1	*** $p < 0.001$

Note: Mean ± SEM; n = 6 per group. Reversal (%) = (scopolamine-treated)/(scopolamine-normal) × 100. † The donepezil mean was below the normal-control mean, so its reversal exceeds 100%; this compares means only and was not tested. One-way ANOVA: $F = 19.44$, $p < 0.0001$, $\eta p^2 = 0.76$. Significance column: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p-values versus the scopolamine control.

3.7. Effect of SAF on brain MDA and catalase

Scopolamine increased brain MDA approximately 1.7-fold (62.4 ± 3.2 to 104.8 ± 5.1 nmol/mg protein; $t = 7.60$, $p < 0.001$) and lowered catalase activity approximately 2.5-fold (286.4 ± 13.7 to 114.6 ± 5.8 μmol H_2O_2 min^{-1} mg^{-1} protein; $t = 11.37$, $p < 0.001$) (Figures 10 and 11; Tables 11 and 12; MDA $F = 17.41$, $\eta p^2 = 0.74$; catalase $F = 41.99$, $\eta p^2 = 0.87$; both $p < 0.0001$). SAF lowered MDA to 90.6 ± 4.5, 77.9 ± 3.8 and 68.7 ± 3.3 nmol/mg protein at 50, 100 and 250 mg/kg ($p = 0.054$, $p < 0.001$ and $p < 0.001$; 33.5%, 63.4% and 85.1% reversal). Catalase activity was partly restored at all three doses, to 158.9 ± 7.6, 232.6 ± 10.4, and 264.7 ± 11.8 (25.8%, 68.7%, and 87.4% recovery; $p = 0.022$, $p < 0.001$, and $p < 0.001$), and remained below the normal control. Donepezil lowered MDA to 65.8 ± 3.4 (92.0% reversal) and restored catalase activity to 270.8 ± 12.6 (90.9%; both $p < 0.001$). After FDR correction across the six endpoints of the cognitive and biochemical cohorts, all effects of SAF at 100 and 250 mg/kg and the catalase effect at 50 mg/kg remained significant ($q \leq 0.031$; Supplementary Table S2).

Table 11. Effect of SAF on brain malondialdehyde (MDA equivalents)

Experimental Group	Brain malondialdehyde (MDA) concentration (nmol MDA equivalents/mg protein)	Reversal (%)	Statistical significance
Normal control	62.4 ± 3.2	—	—
Scopolamine control (0.4 mg/kg)	104.8 ± 5.1	0	### $p < 0.001$
Donepezil (5 mg/kg)	65.8 ± 3.4	92.0	*** $p < 0.001$
SAF (50 mg/kg)	90.6 ± 4.5	33.5	NS ($p = 0.054$)

SAF (100 mg/kg)	77.9 ± 3.8	63.4	*** $p < 0.001$
SAF (250 mg/kg)	68.7 ± 3.3	85.1	*** $p < 0.001$

Note: Mean ± SEM; n = 6 per group. Reversal (%) = (scopolamine-treated)/(scopolamine-normal) × 100. One-way ANOVA: $F = 17.41$, $p < 0.0001$, $\eta p^2 = 0.74$. Significance column: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p-values versus the scopolamine control.

Table 12. Effect of SAF on brain malondialdehyde (MDA) concentration

Experimental Group	Catalase (μmol H_2O_2 min^{-1} mg^{-1} protein)	Recovery (%)	Significance
Normal control	286.4 ± 13.7	—	—
Scopolamine control (0.4 mg/kg)	114.6 ± 5.8	0	### $p < 0.001$
Donepezil (5 mg/kg)	270.8 ± 12.6	90.9	*** $p < 0.001$
SAF (50 mg/kg)	158.9 ± 7.6	25.8	* $p = 0.022$
SAF (100 mg/kg)	232.6 ± 10.4	68.7	*** $p < 0.001$
SAF (250 mg/kg)	264.7 ± 11.8	87.4	*** $p < 0.001$

Note: Mean ± SEM; n = 6 per group. Recovery (%) = (treated − scopolamine)/(normal − scopolamine) × 100; all treated groups remained below the normal control level. One-way ANOVA: $F = 41.99$, $p < 0.0001$, $\eta p^2 = 0.87$. Significance column: ### $p < 0.001$, scopolamine versus normal control (planned t-test); all other entries are Dunnett-adjusted p-values versus scopolamine control.

3.8. Effect of SAF on anxiety-related behavior

This cohort (n = 6 per group; sex not recorded) received vehicle, diazepam (1 mg/kg), SAF (50, 100, or 250 mg/kg), or SAF 50 mg/kg + diazepam 1 mg/kg for 7 days without scopolamine (Figure 12). All three tests depend on exploration, and no activity control was included; therefore, the results are exploratory [42,43,46,55]. Diazepam means were higher than those for vehicle on all four measures (Figure 12), but the diazepam-versus-vehicle comparison was not retained.

Treatment affected EPM open-arm time ($F = 11.51$, $p < 0.0001$, $\eta p^2 = 0.66$; Figure 12B), which was longer with SAF 250 mg/kg and the combination than that in the vehicle group (a single adjusted $p = 0.0014$ was retained for these two contrasts). SAF (50 mg/kg) had no significant

effect. The mean open-arm entries were higher in the SAF 250 mg/kg and combination groups than in the vehicle group (Figure 12A). As the test statistics for this endpoint were not retained, it was reported descriptively.

Treatment affected LDBT light-compartment time ($F = 8.10$, $p < 0.0001$, $\eta p^2 = 0.57$; Figure 12C), which was longer with SAF 250 mg/kg ($p = 0.0317$) and the combination ($p = 0.0037$) than in the vehicle group. Light-compartment time is informative only when the test detects a reference anxiolytic in the same study [38], so this result cannot be interpreted as an anxiolytic-like effect.

Social interaction time, analyzed by pair, was affected by treatment ($F = 9.989$, $p = 0.00059$, $\eta p^2 = 0.81$; Figure 12D) and was longer with 100 mg/kg, 250 mg/kg, and the combination ($p = 0.0012$) than in the vehicle group, but not with 50 mg/kg. These comparisons are based on three pairs per group.

No trend test was performed, and the combination was not compared with diazepam alone.

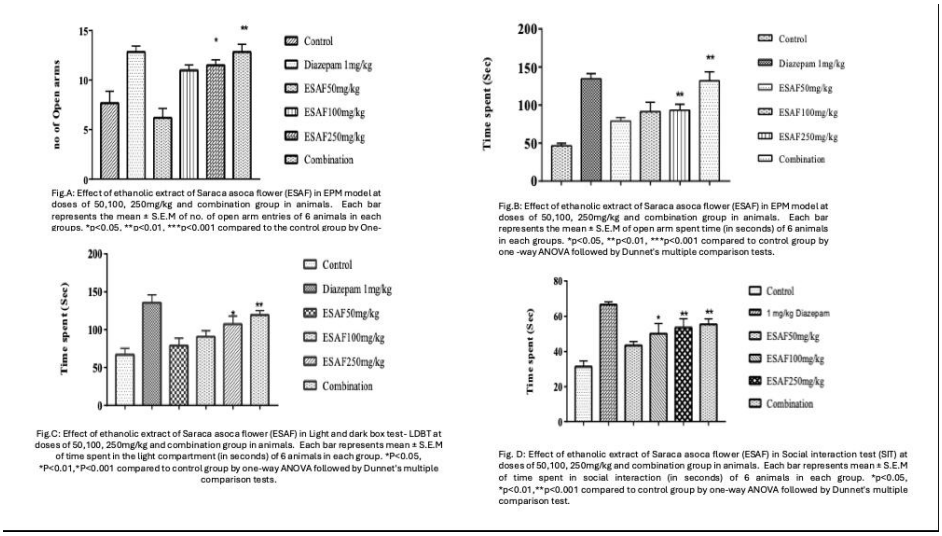


Fig.A: Effect of ethanolic extract of Saraca asoca flower (ESAF) in EPM model at doses of 50,100, 250mg/kg and combination group in animals. Each bar represents the mean ± S.E.M of no. of open arm entries of 6 animals in each groups. *p<0.05, **p<0.01, ***p<0.001 compared to the control group by One-

Fig.B: Effect of ethanolic extract of Saraca asoca flower (ESAF) in EPM model at doses of 50,100, 250mg/kg and combination group in animals. Each bar represents the mean ± S.E.M of open arm spent time (in seconds) of 6 animals in each groups. *p<0.05, **p<0.01, ***p<0.001 compared to control group by one -way ANOVA followed by Dunnet's multiple comparison tests.

Fig.C: Effect of ethanolic extract of Saraca asoca flower (ESAF) in Light and dark box test- LDBT at doses of 50,100, 250mg/kg and combination group in animals. Each bar represents mean ± S.E.M of time spent in the light compartment (in seconds) of 6 animals in each group. *P<0.05, *P<0.01,*P<0.001 compared to control group by one-way ANOVA followed by Dunnet's multiple comparison tests.

Fig. D: Effect of ethanolic extract of Saraca asoca flower (ESAF) in Social interaction test (SIT) at doses of 50,100, 250mg/kg and combination group in animals. Each bar represents mean ± S.E.M of time spent in social interaction (in seconds) of 6 animals in each group. *p<0.05, *p<0.01,**p<0.001 compared to control group by one-way ANOVA followed by Dunnet's multiple comparison test.

Figure 12. Anxiety-related measures after 7 days of treatment. (A) EPM open-arm entries (descriptive; statistics not retained). (B) EPM open-arm time. (C) LDBT light-compartment time. (D) SIT social interaction time. Mean ± SEM; n = 6 mice per group in A–C and n = 3 pairs per group in D. Groups: vehicle, diazepam (1 mg/kg), SAF (50, 100, 250 mg/kg), and SAF 50 mg/kg + diazepam 1 mg/kg. One-way ANOVA with Dunnett's test against vehicle (B, C, D); *$p < 0.05$, **$p < 0.01$ versus vehicle.

4. Discussion

The current study showed that oral SAF at 100 and 250 mg/kg improved scopolamine-impaired passive avoidance, novel object recognition, and Y-maze performance, and in a separate cohort lowered brain cholinesterase activity and MDA, while all three doses partly restored catalase. At 250 mg/kg, SAF reversed 75–85% of the scopolamine effect on the three tasks and 85–87% of the effect on the three brain markers, close to piracetam and donepezil, though neither

comparison was tested. Behavior and biochemistry moved in the same direction but were measured in different animals; therefore, the link between them is an association.

The three tasks draw on different memory processes [56] and partly different temporal lobe structures [57], so an effect of SAF on all three is not explained by one task alone. We still describe the changes as improved task performance rather than memory rescue, because scopolamine also affects attention, sensory processing, and psychomotor activity [4,58], and its cognitive and locomotor effects are hard to separate [33,59]. Equal Y-maze arm entries rule out fewer entries as the cause of lower alternation in that session, but they do not control for activity in the other tasks. Of the three tasks, the passive avoidance result was the least secure, because shock exposure at acquisition could not be verified. Piracetam reversed the deficit even at a low dose, which shows that the impairment in this cohort was reversible.

In the anxiety cohort, SAF 250 mg/kg increased EPM open-arm time and LDBT light time, and SAF 100 and 250 mg/kg increased social interaction; the combination increased all three. These tests read anxiety-like behavior as a balance between exploration and avoidance [46,60,61]; therefore, higher values could reflect higher activity [43]. In a meta-analysis of 17 common measures, only EPM open-arm time and light-compartment time reliably detected anxiolytic compounds, and social interaction time was not sensitive [38]; even these two depend on the test detecting a reference drug in the same study. Without the diazepam comparison, the light–dark box results cannot be interpreted here. The anxiolytic-like effect of SAF remains to be demonstrated with activity controls and a validated reference drug.

These findings extend earlier work on the flower extract in the EPM transfer-latency test and Morris water maze [14] and in restrained mice [13], and on the bark extract in chronic mild stress [15]. Other polyphenol- and flavonoid-rich preparations have improved performance in scopolamine models, including quercetin and rutin [62], protocatechuic acid [63], safflower seed [64], soursop leaf [65], *Achras sapota* [66], and *Grewia asiatica* [20]. The comparison concerns outcomes only; the constituents of SAF were not identified here.

The two cohorts were also dosed differently: the cognitive cohort received three scopolamine challenges, while the biochemical cohort received one. Biochemical values describe the response to a single challenge.

Brain cholinesterase activity is a relevant cholinergic correlate in this model, although scopolamine acts by muscarinic blockade, and cholinesterase activity is not a direct readout of that blockade [6]. SAF lowered the scopolamine-induced rise by 64.7% and 87.1% at 100 and 250 mg/kg. Because the assay measured total acetylthiocholine-hydrolyzing activity, the result showed lower tissue cholinesterase activity and did not show AChE inhibition. MDA and catalase followed the same dose order, which indicates reduced oxidative stress in the brain. *In vitro*, SAF scavenged DPPH, reduced ferric iron, and inhibited lipid peroxidation and xanthine oxidase, an enzyme that produces superoxide and hydrogen peroxide [28]. These cell-free results fit the *in vivo* changes, but the brain exposure of the active constituents is unknown.

The chemical data describe the tested batch, not its active constituents. The Folin–Ciocalteu value is a reducing-capacity estimate, and the aluminum chloride value is a quercetin-equivalent

response that depends on flavonoid structure [24]. Rhamnazin, myricetin and quercetin were reported by HPLC in an acidified fraction of this extract [13] but were not confirmed here; that would need library-matched MS/MS spectra and authentic standards, and linking them to activity would require fractionation or tests of isolated compounds. The doses used were well below the highest non-lethal dose (2000 mg/kg) in the earlier acute toxicity study [13].

This study had several limitations. The acute scopolamine regimen models cholinergic dysfunction, not AD pathology [6,67]. No cohort had locomotor, sensory, or attentional controls, and the cognitive cohort received three scopolamine challenges in a fixed task order. Shock exposure at passive avoidance acquisition, total object exploration, and object counterbalancing could not be verified. In the anxiety cohort, the diazepam comparison, open-arm entry statistics, shuttle-crossing data, sex of the animals, and test order were not retained. Group sizes were not set by a power calculation, and with six mice per group (three pairs in the SIT) only large effects could be detected reliably; individual data were not retained, so model assumptions could not be checked. The cholinesterase assay did not separate AChE from BChE, the catalase assay was read at 620 nm without validation, the LC–MS settings were not retained, and repeated-dose toxicity, pharmacokinetics, and compound identification were not studied.

5. Conclusion

Oral SAF at 100 and 250 mg/kg improved scopolamine-impaired passive avoidance, novel object recognition, and Y-maze performance, and, in a separate cohort, lowered brain cholinesterase activity and MDA, while all three doses partly restored catalase. Thus, oral SAF improved task performance in association with lower cholinesterase activity and oxidative stress in the brain. Without locomotor or sensory controls, these effects cannot be attributed to memory alone, and the anxiety data remain exploratory. Identifying the active constituents of this LC–MS-fingerprinted extract and testing them with activity controls and a validated reference anxiolytic, is the next step.

Declarations

Competing interests The authors declare no competing interests. M/s Protein Design, Bengaluru, India, supplied chemicals, solvents, and technical assistance with the *in vitro* and biochemical assays under a standard commercial arrangement and had no role in study design, analysis, interpretation, or writing.

Consent to participate: Not applicable. **Consent for publication:** Not applicable.

Data availability: All data supporting the findings are presented in the article as group summaries (Tables 3–12, Figure 12, and Supplementary Table S2). Individual-animal data were not retained and cannot be shared.

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; ANOVA, analysis of variance; BChE, butyrylcholinesterase; BPI, base-peak intensity; ChE, cholinesterase; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; DAD, diode-array detection; DI, discrimination index; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EPM, elevated plus maze; ESI, electrospray ionization; FDR, false discovery rate; FRAP, ferric-reducing antioxidant power; GAE, gallic acid equivalents; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; LDBT, light–dark box test; MDA, malondialdehyde; MS, mass spectrometry; NS, not significant; QE, quercetin equivalents; RT, retention time; SAF, ethanolic flower extract of *Saraca asoca*; SEM, standard error of the mean; SIT, social interaction test; TBARS, thiobarbituric acid-reactive substances; TOF, time-of-flight; TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine.

Supplementary Information The online version contains the following supplementary material: Supplementary Table S1 (ARRIVE 2.0 Essential 10, assessed retrospectively) and Supplementary Table S2 (summary statistics, recomputed tests, 95% confidence intervals, and FDR q-values for the cognitive and biochemical endpoints).

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