Original article

Chemical characterization and evaluation of the neuroprotective potential of \textit{Indigofera sessiliflora} through in-silico studies and behavioral tests in scopolamine-induced memory compromised rats

Muhammad Sajjad Haider\textsuperscript{a}, Waseem Ashraf\textsuperscript{a}, Sana Javaid\textsuperscript{a,b}, Muhammad Fawad Rasool\textsuperscript{c}, Hafiz Muhammad Abdur Rahman\textsuperscript{a}, Hammad Saleem\textsuperscript{d}, Syed Muhammad Muneeb Anjum\textsuperscript{d}, Farhan Siddique\textsuperscript{e,f}, Alejandro Morales-Bayuelo\textsuperscript{g}, Savas Kaya\textsuperscript{h}, Faleh Alqahtani\textsuperscript{i,\textsuperscript{l}}, Fawaz Alasmari\textsuperscript{i}, Imran Imran\textsuperscript{a,\textsuperscript{m}}

\textsuperscript{a} Department of Pharmacology, Faculty of Pharmacy, Bahauddin Zakariya University, Multan 60800, Pakistan
\textsuperscript{b} Department of Pharmacy, The Women University, Multan 60000, Pakistan
\textsuperscript{c} Department of Pharmacy Practice, Faculty of Pharmacy, Bahauddin Zakariya University, Multan 60800, Pakistan
\textsuperscript{d} Institute of Pharmaceutical Sciences, University of Veterinary & Animal Sciences, Lahore, Pakistan
\textsuperscript{e} Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Bahauddin Zakariya University, Multan 60800, Pakistan
\textsuperscript{f} School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China
\textsuperscript{g} Facultad de Ingenierías, Centro de Investigación de Procesos del Tecnologico Conffenalco, (CIPTEC), Programa de Ingeniería Industrial, Fundacion Universitaria Tecnológica, Conffenalco –Cartagena, Bolívar, Colombia
\textsuperscript{h} Sivas Cumhuriyet University Health Services Vocational School, Department of Pharmacy, 8140 Sivas, Turkey
\textsuperscript{i} Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

\textsuperscript{m} Corresponding authors: Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia (F Alqahtani). Department of Pharmacology, Faculty of Pharmacy, Bahauddin Zakariya University, 60800 Multan, Pakistan (I. Imran).

E-mail addresses: afaleh@ksu.edu.sa (F. Alqahtani), imran.ch@bzu.edu.pk (L. Imran), imran.ch@bzu.edu.pk (L. Imran).

Peer review under responsibility of King Saud University.

\textbf{ARTICLE INFO}

Article history:
Received 26 February 2021
Revised 9 April 2021
Accepted 11 April 2021
Available online 21 April 2021

Keywords:
Indigofera sessiliflora
Artocommunol
Anxiety
Learning/memory
Behavioral studies

\textbf{ABSTRACT}

In the current study, we investigated the phytochemical and neuropharmacological potential of \textit{Indigofera sessiliflora}, an indigenous least characterized plant widely distributed in desert areas of Pakistan. The crude extract of the whole plant \textit{Indigofera sessiliflora} (IS.CR) was preliminary tested \textit{in-vitro} for the existence of polyphenol content, antioxidant and anticholinesterase potential followed by detailed chemical characterization through UHPLC-MS. Rats administered with different doses of IS.CR (100–300 mg/kg) for the duration of 4-weeks were behaviorally tested for anxiety and cognition followed by biochemical evaluation of dissected brain. The \textit{in-silico} studies were employed to predict the blood–brain barrier crossing tendencies of secondary metabolites with the elucidation of the target binding site. The \textit{in-vitro} assays revealed ample phenols and flavonoids content in IS.CR with adequate anti-oxidant and anti-cholinesterase potential. The dose-dependent anxiolytic potential of IS.CR was demonstrated in open field (OFT), light/dark (L/D) and elevated plus maze (EPM) tests as animals spent more time in open, illuminated and elevated zones (\textit{P < 0.05}). In the behavioral tests for learning/memory, the IS.CR reversed the scopolamine-induced cognitive deficits, as animals showed better (\textit{P < 0.05}) spontaneous alternation and discrimination index in y-maze and novel object recognition (NOR) tests. Similarly, as compared to amnesic rats, the step-through latencies were increased (\textit{P < 0.05}) and escape latencies were decreased (\textit{P < 0.05}) in passive avoidance (PAT) and Morris water maze (MWM) tests, respectively. Biochemical analysis of rat brains showed significant reduction in malondialdehyde and acetylcholinesterase levels, alongwith preservation of glutathione peroxidase and superoxide dismutase activity. The docking studies further portrayed a possible interaction of detected phytoconstituents with acetylcholinesterase target. The results of the study show valuable therapeutic potential of phytoconstituents present in IS.CR to...
1. Introduction

Neurological disorders (NDs) are ailments involving the central nervous system and are usually characterized by behavioral changes and cognitive deficit (Sharifi-Rad et al., 2020). These disorders are contributing to one of the leading reasons for life-long disabilities and socioeconomic burden at the global level (Collaborators, 2019). The increasing life expectancy and aging population are additional reasons to look for new therapeutic options capable to alleviate the age-related biological dysfunctions causing dementia, the principal cause of which is Alzheimer’s disease (AD) (Agatonovic-Kustrin et al., 2018). The available therapeutic options to target the majority of NDs primarily focus on symptomatic relief only. Therefore, it is of great interest to look for novel neuroprotective agents capable to decelerate the progression of NDs.

Humans have been relying on plants as a therapeutic source for centuries due to their easy accessibility, cost-efficiency and lesser unwanted effects (Ekor, 2014). The remedial characteristics of plants are often ascribed to their secondary metabolites (SMs) such as flavonoids, terpenoids and fatty acids, the compounds that do not take part in their growth but aid them for survival (Mushtaq et al., 2018). The plants are enriched with a variety of such bioactive compounds that when used alone or as a mixture, provide effectiveness and safety even when synthetic drugs fail (Seca and Pinto 2019). The beneficial role of plant’s secondary metabolites in treating neurological disorders has been reported by various researchers worldwide (Sharifi-Rad et al., 2020). These secondary metabolites have the capability to work simultaneously through different targets and treat diseases with multifaceted pathophysiology. They can prevent the protein misfolding that is the key pathophysiological factor behind various NDs including Alzheimer’s disease (AD) (Soto and Pritzkow, 2018). The antioxidant capacity of these compounds is of considerable importance in limiting the pathogenesis and progression of NDs as neuronal tissues are more vulnerable to oxidative stress because of higher oxygen requirements, increased metabolic activity and higher content of polyunsaturated fatty acids (Uttara et al. 2009). Flavonoids are well-known examples of plant secondary metabolites which can cross the blood–brain barrier and comprise of polyphenolic structures (De Andrade Teles et al., 2018). They are efficient antioxidant and metal chelators. Their neuroprotective (Gomes et al., 2015; Prakash and Sudhandiran, 2015), anticholinesterase (Abedayo et al., 2015), and anti-inflammatory (Islam et al., 2013) abilities have been established. Many researchers have reported the memory and learning improving potential of flavonoids (administered for 2–6 weeks) through behavioral models in rodents (Hoffman et al., 2004; Rendeiro et al., 2013; Shif et al., 2006; Williams et al., 2004).

Fabaceae is the 3rd largest family of land plants and is ranked as the 2nd largest family regarding medicinal plants as it includes around 490 species that have traditional utilization as medicines in different countries (Dzoyem et al., 2014; Christenhusz and Byng, 2016). Glycyrrhiza glabra, Physostigma venenosum, and Clitoria ternatea are a few examples of the eminent therapeutic properties of this family. A study revealed that Glycyrrhiza glabra reversed the scopolamine-induced amnesia in mice (Dhingra et al., 2004) and its marvelous neuropharmacological potential is linked to the presence of triterpenes and flavonoids (Ravanfar et al., 2018). Physostigmine, a secondary metabolite owned by Physostigma venenosum, is an acetylcholinesterase inhibitor that enhances cholinergic neurotransmission and has been shown to increase long-term memory (Davis et al., 1978). Furthermore, a study has reported that the root extracts of Clitoria ternatea have the potential to prevent cognitive deterioration occurring in AD (Damodaran et al., 2018).

Indigofera sessiliflora, commonly known as “vekar” is an indigenous desert plant of Pakistan that belongs to the family Fabaceae. It is also commonly found in various countries such as Algeria, Australia, Sudan, Nigeria, Senegal and India where this plant is generally used as animal fodder. The people residing in desert areas of South Punjab, Pakistan have been traditionally using it as an alternative remedial source for infectious and inflammatory diseases. It is also used for neuroprotective and memory-enhancing purposes in some native areas; however, there is no scientific evidence to support these folkloric claims. The chemical composition of the plant has never been explored previously. Therefore, in this study, we attempted to investigate the chemical and neuropharmacological characteristics of this plant. The aqueous-methanol extract of the whole plant of Indigofera sessiliflora (IS.CR) was studied through various in-vitro experiments including UHPLC-MS to identify its secondary metabolites (SMs). Furthermore, the study involves a set of behavioral experiments to explore the possible neuropharmacological potential of IS.CR. Additionally, the in-silico studies are also incorporated to predict the interaction of SMs with the target binding site, acetylcholinesterase, for further validation of their neuromodulating potential in observed neurological disorders.

2. Materials and methods

2.1. Chemicals

Most of the chemicals used in the study including scopolamine and piracetam were procured from Sigma-Aldrich, USA. The diazepam was purchased from Roche Pharma (Switzerland) and isoflurane (Forane® Abbots Laboratories, USA), used for anesthesia, was generously provided by Ahsan Medicine Agency, Multan. All employed chemicals and drugs in this study were of research-grade with high purity.

2.2. Preparation of plant extract

The whole plant of Indigofera sessiliflora was picked fresh from the desert area near Bahawalpur, Pakistan. The plant was authenticated by a taxonomist at the Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan. The voucher number (www.theplantlist.org/tpl1.1/record/tid-41778) was assigned and the herbarium was deposited there. After the removal of extraneous matters, the gathered plant was shade-dried for 2 weeks. Later, the completely dried plant was subjected to coarse grinding. This coarsely powdered plant was soaked in aqueous-methanolic combination (80% v/v) in a light-resistant and airtight container at 25°C for seven days with intermittent vigorous minling. The whole mixture was filtered and this whole procedure was repeated twice (Janbaz et al., 2015). The obtained filtrate was evaporated in a Rota evaporator using low pressure.
unless a concentrated crude extract (IS.CR) was obtained with a 19.9% yield.

2.3. In-vitro analysis

2.3.1. Total phenolic and flavonoid content estimation

The IS.CR was examined for phenolic and flavonoid content by following the previously reported methods (Slinkard and Singleton 1977, El Far and Taie 2009).

2.3.2. Antioxidant activity by ABTS and DPPH radical cation scavenging methods

The antioxidant potential of IS.CR was examined by ABTS and DPPH methods (Li et al., 2009, Cuvelier and Berzet 1995), using ascorbic acid as positive control. The antioxidant capacity of the extract (IS.CR) was determined using the following formula:

\[
\% \text{ of radical scavenging activity} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100
\]

2.3.3. Acetylcholinesterase (AChE) inhibitory activity

The anticholinesterase capacity of IS.CR was evaluated according to the method described (Ellman et al., 1961), using galantamine as positive control.

2.4. Analysis of secondary metabolites through UHPLC-MS

The crude extract of Indigofera sessiliflora (IS.CR) was analyzed through RP-UHPLC-MS for the presence of secondary metabolites according to the previously reported method (Saleem et al., 2019).

2.5. In-vivo behavioral studies

2.5.1. Animals

Sprague Dawley male rats (200–300 g) were used in this study. The animals were purchased from the National Institute of Health Islamabad and kept in the animal’s house at the Faculty of Pharmacy, BZU, Multan under standard conditions i.e. 25 °C, 50% humidity and 12 h L/D cycle (8:00 am to 8:00 pm, lights on). The animals were given free access to standard rodent food and water ad libitum. Before each behavioral test, the animals were acclimatized to the researcher’s handling and atmosphere of the experimental room for a few hours. All behavioral experiments were carried out in the time window from 8:00 am to 6:00 pm. To avoid any confounding effects of one behavioral test on the next, the animals were provided with adequate washout periods and the intensity of aversive stimuli was kept minimum. The protocols of animal study were under the rules described by the Institute of Laboratory Animal Resources (ILAR), National Research Council (NRC, 1996), and permitted by the Ethical Committee of B.Z.U, Multan, Punjab, Pakistan vide # 09/PEC/2015.

2.5.2. Animal grouping with drug treatments

The randomly selected rats were divided into 7 different groups (n = 10). Animals of group A (control) were administered once daily with normal saline (5 ml/kg). The animals of three test groups (B, C and D) received doses of 100, 200 and 300 mg/kg of IS.CR (100 mg in 1 ml of normal saline), respectively. The animals of group A-D were administered with respective treatments once daily via oral gavage for four weeks (day 1 to 28) and continued till completion of behavioral studies (day 48).

During behavior studies for anxiety, group E (n = 10) was added as a positive control to which diazepam (2 mg/kg, i.p.) was administered 60 min before each test. For the behavioral tests employed to evaluate memory and learning, a new group F (n = 10) was included to work as a positive control treated with piracetam (200 mg/kg, i.p.) for one week (day 30 to 36). During behavioral tests for memory, another group G (n = 10) was included in which rats were administered with scopolamine (1 mg/kg, i.p.) only. In all behavioral tests for memory, the animals of groups B,C,D,F and G were treated with scopolamine (1 mg/kg, i.p.) about 0.5 h before experimentation to induce amnesia. The animal grouping with specified treatments and experimental layout for behavioral studies is given in detail in Fig. 1.

2.6. Behavioral tests for anxiety

2.6.1. Open field test (OFT)

The open-field test is a widely adopted paradigm to assess the animal’s anxiety and general locomotion under the influence of test treatments (MakoriArika et al., 2019). The open areas tend to prove anxiogenic in rodents but they simultaneously have an innate curiosity to explore these places. Before testing each animal, the open field apparatus (80 × 80 cm) was thoroughly cleaned with ethanol to avoid any lingering stimuli. On the experimental day, after one hour of treatments, each animal was individually exposed to the open field and their behavior was examined for 5 min. The animal’s anxiety was evaluated by monitoring preference for open areas of field. Other parameters i.e. number of rearings and number of line crossings were also taken into account and assessed via ANY-maze version 6.1, Stoelting Co. USA to assess the influence of IS.CR on general locomotion of rats.

2.6.2. Light/Dark (L/D) test

This test was used as another tool to explore the anxiolytic capacity of IS.CR according to the method described (Bano et al., 2014). The rats were tested in an apparatus comprising two compartments. Both cabins were made up of white and dark acrylic sheets and designated as light and dark compartments respectively, interconnected through a slit (10 × 10 cm). The dimensions of the light compartment were 40 cm × 40 cm while the dark compartment was 40 cm × 33 cm. On the test day, the animals of each group were administered with designated treatments and behavioral testing was performed after 60 min. Each animal was monitored for 5 min for their preference of light and dark compartments as the increased preference for the dark compartment is the feature of anxiety in rodents.

2.6.3. Elevated plus-maze (EPM) test

This test was performed by testing the rats in a customized plus-shaped maze (110 × 10 cm) comprising two open and two closed arms and elevated to the height of 50 cm. In this test, the animal’s anxiety is depicted by their increased stay in the closed arms of the maze (Latha et al., 2015). On the experimental day, after 1 h of designated group-wise treatments, the rats were individually tested in the arena of elevated plus-maze for 5 min. The parameters i.e. entries in the open arms of maze as well as the duration of stay there were recorded to assess the impact of IS.CR on anxiety-like behavior. The animal’s behavior of increased preference for open arms is taken as a representation of anxiolytic-like behavior.

2.7. Behavioral tests for learning and memory

2.7.1. Y-maze test (spontaneous alternation behavior)

The Y-maze test was used to evaluate the impact of IS.CR on short-term memory in scopolamine-amnesic rats (Ionita et al., 2017). The test apparatus comprised a Y-shaped maze (50 × 10 × 15 cm) that included three consecutive arms (A, B and C) arranged at 120°. On the experiment day, the animals were administered with scopolamine after 30 min of administration of
specified group-wise treatments. Later, 30 min after scopolamine administration, each animal was introduced in the Y-maze and allowed to explore it for 5 min. The rodents have the innate curiosity to explore the novel environment, thus the rat with efficient memory tends to enter the arm that was not previously explored. However, the scopolamine-amnesic rats retain reduced memory of the previously explored arm and revisit the same arm that results in reduced spontaneous alternation behavior. % SAP was calculated through the formula given:

\[ \% \text{SAP} = \frac{\text{Time spent with old object}}{\text{Time spent with old object} + \text{Time spent with new object}} \times 100 \]

2.7.2. Novel object recognition (NOR) test

This test is widely employed to estimate the rodent's cognition in different models of CNS illnesses as healthy rodents show the tendency to interact with novel surroundings (Jilani et al., 2018; Retinasamy et al., 2019). The capacity of IS.CR to affect the animal's short-term memory was examined in the scopolamine-amnesic rat model by exposing them to familiar and new objects in an apparatus of 80 × 80 cm dimensions. On experiment day, the rats were initially habituated to the test apparatus for 1 h. The test comprised two sessions. During the first session, the rats were individually exposed to two geometrically identical objects for 10 min. Later, one object was replaced by a new object (second session) and the animal's interest in the new and old object was monitored for 5 min. From these observed parameters, the discrimination index was calculated that if increased, shows better working memory.

\[ DI = \left[ \frac{\text{Time spent with new object} - \text{Time spent with old object}}{\text{Time spent with new object} + \text{Time spent with old object}} \right] \]

2.7.3. Passive avoidance test (PAT Step-through)

The impact of IS.CR on learning and memory was further validated through this widely employed test. The animals were tested for long-term memory by testing them during two consecutive days in an apparatus consisting of two inter-connected light and dark compartments (Kim et al., 2020). The lightened compartment was mounted with a light source (100 W) while the dark compartment was equipped with a stainless-steel grid floor to provide an aversive stimulus i.e. electric shock of 0.5 mA. On the first day of PAT (acquisition trial), the animals were individually placed in the lightened compartment from where they tend to exit due to their innate preference for dark places. On entering the dark compartment through the connecting slit, the animals were delivered with an electric shock 0.5 mA for 2 sec through Coulbourn Instruments Animal Shocker (USA) so that they could make a memory of this aversive stimulus. The animals were treated with respective group-wise treatments 1 h before the acquisition trial. After 24 h of the acquisition trial, the animals were subjected to a retention trial without applying foot shock. Each animal was individually noted for step-through latencies for 5 min during acquisition and retention trials to evaluate the effect of IS.CR on animal's memory.

2.7.4. Morris water maze (MWM) test

This hippocampus-based memory task is a widely employed tool to assess spatial learning in rodents (Andre et al., 2019; Wang et al., 2017). In the swimming arena of the circular water tank (150 × 50 cm), the animal used visual cues to navigate and locate the hidden platform (10 × 10 cm). The water comprising (25 °C) tank was divided into four quadrants (NW, SW, SE, NE) on the basis of four poles (N, S, E, W). The proximal cues (geometrical signs ▲, □, ○, △) were displayed on the internal surface of the tank and the same distal cues were displayed on standees placed in the surroundings of the tank to help the animal to navigate (Malik et al., 2020; Shakeel et al., 2020). The test consisted of the duration of six consecutive days and the position of the platform was kept constant throughout the experiment. During the first two training days, the animals were trained for 2 min (four trials/animal daily) to locate the visible platform. During training trials, the rats were slightly pushed towards the platform and allowed to stay there for 30 s, if they fail to locate it. During the next three trial days, the water was opacified by using any non-toxic opacifying agent (ink, milk, etc.) to hide the platform, and animals were tested daily to locate the platform within 2 min and their escape latencies were noted. On probe day, the animals were tested without placing the platform in the maze and their number of entries in the platform quadrant and duration of stay there were recorded to evaluate their memory. The videos of test were recorded and later evaluated through behavioral tracking software i.e. ANY-maze version 6.1, Stoelting Co. USA.

2.8. Biochemical assays for AChE, MDA, GPx and SOD

The rats (n = 4) were randomly chosen from each group after completion of the Morris water maze test on the 48th day of study.
The animals were decapitated for brain extraction after anesthetizing them with 5% isoflurane v/v mixed with synthetic air (Lab-Gas, Multan, Pakistan) by Rodent anesthesia Vaporizing System (Kent Scientific Corporation USA). For the preparation of homogenate, 0.3 g of brain was mixed with 3 ml of phosphate buffer (pH 7.4) and subjected to centrifugation (12000 rpm) for 20 min at reduced temperature (Alqahtani et al., 2020; Imran et al., 2021). The homogenate was evaluated for the levels of acetylcholinesterase (AChE), malondialdehyde (MDA), glutathione peroxidase (GPx) and superoxide dismutase (SOD) according to the method described (Samad et al., 2019).

2.9. Docking studies

1) System preparation of the crystal structure of acetylcholinesterase enzyme in complex with a non-chiral donepezil-like inhibitor

To develop the docking calculations the crystal structure of acetylcholinesterase enzyme in complex with a non-chiral donepezil-like inhibitor 17 (PBD code: 5NAP) (Caliandro et al., 2013) was used. This structure was optimized using Schrödinger Suite 2017-1’s Protein Preparation Wizard module (Friesner et al., 2004) using the following standard protocol:

i) The protein structure was refined, and the hydrogen bond (H-bond) network was optimized.

ii) Protonation states at physiological pH were identified using PropKa utility.

iii) An important step is the restrained molecular minimization.

   It was performed using the Impact Refinement (Impref) module by restraining the heavy atoms restrained to remain within a low root-mean-square deviation (RMSD) from the initial coordinates (Friesner et al., 2004; Madhavi Sastry et al., 2013).

Finally, the molecular structures of the detected compounds were built using Maestro Editor (Maestro, version 11.1, Schrödinger, LLC), after which the 3D conformations were optimized using LigPrep module. Since there can be structures with zwitterion characters, the ionization/tautomeric states at physiological pH conditions were predicted using Epik, Schrödinger, after that the energy minimization was performed utilizing Macromodel using the OPLS2005 force field.

2) Molecular docking analysis

The Glide, Schrödinger, LLC (Madhavi Sastry et al., 2013; Moon et al., 2017) was used to determine and obtain the docking studies with Standard Precision (SP) mode and default parameters. The docking grid was generated with default settings centered at the co-crystallized ligand. To allow the binding of larger ligands, a scaling factor of 0.8 for the van der Waals radii of non-polar protein atoms was used. A protocol based on Induced Fit Docking (IFD) was also used to generate alternative conformations of the receptor suitable to bind with ligands having strange orientations. This (IFD) method allows the protein to undergo backbone movements, side-chain movements, or both, upon ligand docking.

Specifically, the docking procedure comprises of four steps where accuracy is ensured by Glide's scoring function and Prime's advanced conformational refinement: (i) initial Glide docking into the rigid receptor to generate an ensemble of poses; (ii) sampling of the protein using the Prime module side-chain prediction module followed by a structure minimization of each protein/ligand complex pose; (iii) redocking of the ligand into low energy induced-fit structures from the previous step using default Glide settings (no vdW scaling); and (iv) estimating the binding energy (IFDScore) by accounting for the docking energy (GScore), as well as receptor strain and solvation terms (Prime energy). Finally, we compared all the poses of the most and least active ligands for each along the molecular set, taking into account the extent of residue movement generated by the IFD calculation.

3) ADMET study

The best conformations generated from the docking results were analyzed using the SMILES (Simplified Molecular Input Line Entry System) and further studied with the aid of admetSAR program (Moon et al. 2017). The SMILES is an online server for predicting the key roles in the drug discovery and give all important information about the drug parameters following the Lipinski Rule (Moon et al. 2017).

2.10. Statistical analysis

The data's normality was first validated through the Kolmogorov-Smirnov test and outcomes of all behavioral (n = 8) and biochemical studies (n = 4) were evaluated by parametric one-way ANOVA followed Dunnett's multiple comparison test. However, two-way ANOVA followed by a Tukey's test was employed to evaluate the escape latencies observed in MWM, respectively. All data were expressed as Mean ± SEM and P ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. In-vitro studies

3.1.1. Total flavonoids and phenolic content

The methanol extract of the Indigofera sessiliflora was used in the study as alcohols are widely used solvents that allow the maximum extraction of phenols and flavonoids from plants (Houghton and Raman 1998). The results revealed that per gram of IS.CR extract is equivalent to 71.4 ± 5.3 mg of gallic acid in estimation of total phenolic content and 29.2 ± 3.8 mg of quercetin in estimation of the total flavonoid content (Table 1).

3.1.2. Antioxidant assay through ABTS and DPPH methods

The antioxidative properties of plants are often directly related to their capacity to manage the oxidative stress related diseases. The antioxidant potential of the IS.CR was explored through the well known ABTS and DPPH assays. The methanolic extract of Indigofera sessiliflora demonstrated a notable radical scavenging potential in a dose-dependent manner with IC50 672.10 ± 21.18 μg/ml in ABTS and IC50 767.68 ± 18.04 μg/ml in DPPH assay as depicted in Table 1.

3.1.3. AChE inhibitory activity

The Ellman’s method is employed for in-vitro characterization of enzymatic hydrolysis of acetylcholine. The inhibitors of AChE have been explored for the discovery of most effective approach to treat the cognitive symptoms of AD. The results of this test depicted the concentration-dependent inhibition of AChE by IS. CR with IC50 of 225.82 ± 11.21 μg/ml (Table 1).

3.1.4. UHPLC-MS secondary metabolites analysis

The total ion chromatogram resulting from UHPLC-MS analysis of IS.CR is shown in Fig. 2. The UHPLC-MS analysis, revealed the tentative identification of eighteen different phytochemicals (Table 2). Most of these tentatively identified chemical constituents were belonging to flavonoid group of secondary metabolites and these were artocommunol CA, cadabicine methyl ether,
Table 1

| Test Sample | TPC mg GAE/g | TFC mg QUE/g | ABTS Assay IC₅₀ (µg/ml) | DPPH Assay IC₅₀ (µg/ml) | AChE Assay IC₅₀ (µg/ml) |
|-------------|--------------|--------------|-------------------------|-------------------------|-------------------------|
| IS.CR       | 71.4 ± 5.3   | 29.2 ± 3.8 mg| 672.10 ± 21.18          | 767.68 ± 18.04          | 225.82 ± 11.21          |

Table 2

Secondary metabolites present in IS.CR.

| S. No | RT (min) | Mol. Mass     | Tentative identification                  | Mol. Formula                  | Compound class          | B. peak (m/z) |
|-------|----------|---------------|-------------------------------------------|-------------------------------|-------------------------|---------------|
| 1     | 0.639    | 180.0626 C₇H₁₀O₆ | L-Galactose                               | C₇H₁₀O₆                      | Glucose                 | 179.0626      |
| 2     | 0.667    | 508.1747 C₇H₁₀O₃ | 4,7-Didehydroxyphysalin B                | C₇H₁₀O₃                      | Steroid                 | 507.1747      |
| 3     | 0.698    | 300.1197 C₁₂H₁₆O₆ | Salidroside                               | C₁₂H₁₆O₆                      | Steroid                 | 299.1197      |
| 4     | 1.193    | 310.1439 C₁₀H₁₄O₄ | Methyl 6-O-digalloyl-beta-D-glucopyranoside | C₁₀H₁₄O₄                      | Galloyl ester           | 309.1439      |
| 5     | 2.395    | 316.0947 C₁₇H₂₀O₇ | Pendulone                                  | C₁₇H₂₀O₇                      | Glucoside               | 315.0947      |
| 6     | 7.566    | 432.1586 C₁₃H₁₈O₇ | Artomuncomul CA                           | C₁₃H₁₈O₇                      | Glucoside               | 431.1586      |
| 7     | 7.753    | 449.229 C₁₈H₂₂O₈ | Cadabicine methyl ether                   | C₁₈H₂₂O₈                      | Flavonoid               | 448.229       |
| 8     | 8.263    | 756.2486 7,4'-Dihydroxy-5-methoxyflavonone 7-neohesperidene-4'-glucoside | > limit                       | C₁₈H₂₂O₉                      | Flavonoid               | 755.2486      |
| 9     | 8.508    | 574.1316 C₁₇H₂₀O₄ | 6,7,3'-Tetrahydroxyaurone 6-(2',4',6'-triacyethylglucose) | C₁₇H₂₀O₄                      | Glucoside               | 573.1316      |
| 10    | 8.933    | 464.0954 C₁₉H₂₂O₆ | 8-Hydroxyfluteolin 8-glucoside           | C₁₉H₂₂O₆                      | Fatty acid              | 463.0954      |
| 11    | 8.945    | 624.1699 C₁₉H₂₂O₇ | Tricetin 7-methyl ether 3'-glucose-5'-rhamnoside | C₁₉H₂₂O₇                      | Fatty acid              | 623.1699      |
| 12    | 8.99     | 464.1299 C₂₀H₂₄O₁₂ | Homoeriodictyolchalcone 2'-glucoside     | C₂₀H₂₄O₁₂                      | Flavonoid               | 463.1299      |
| 13    | 9.286    | 448.138 C₁₇H₁₆O₆ | Poriolin                                  | C₁₇H₁₆O₆                      | Fatty acid              | 447.138       |
| 14    | 11.757   | 288.2306 9,16-dihydroxy-palmitic acid | Desmethoxysudachitin      | C₁₇H₁₆O₆                      | Fatty acid              | 287.2306      |
| 15    | 12.549   | 330.072 8-Hydroxyfluteolin 8-glucoside | Homoeriodictyolchalcone 2'-glucoside | C₁₇H₁₆O₆                      | Fatty acid              | 329.072       |
| 16    | 12.879   | 374.0997 8-Methoxycurcuminol | Desmethoxysudachitin | C₁₇H₁₆O₆                      | Fatty acid              | 373.0997      |
| 17    | 14.856   | 378.2395 1,9-dideoxyforskolin | Poriolin                                  | C₁₇H₁₆O₆                      | Fatty acid              | 377.2395      |
| 18    | 19.165   | 280.2408 6,8,9E-octadecadienonic acid | Poriolin                                  | C₁₇H₁₆O₆                      | Fatty acid              | 279.2408      |

Fig. 2. Total ion chromatogram (TIC) of IS.CR in UHPLC-MS analysis (negative ionization mode) revealing the presence of secondary metabolites 1) L-Galactose, 2) 4,7-Didehydroxyphysalin B, 3) Salidroside, 4) Methyl 6-O-digalloyl-beta-D-glucopyranoside, 5) Pendulone, 6) Artomuncomul CA, 7) Cadabicine methyl ether, 8) 7,4'-Dihydroxy-5-methoxyflavonone 7-neohesperidene-4'-glucoside, 9) 6,7,3'-Tetrahydroxyaurone 6-(2',4',6'-triacyethylglucose), 10) 8-Hydroxyfluteolin 8-glucoside, 11) Tricetin 7-methyl ether 3'-glucose-5'-rhamnoside, 12) Homoeriodictyolchalcone 2'-glucoside, 13) Poriolin, 14) 9,16-dihydroxy-palmitic acid, 15) Desmethoxysudachitin, 16) 8-Methoxycurcuminol, 17) 1,9-dideoxyforskolin and 18) 6,8,9E-octadecadienonic acid.

3.2. Behavioral tests for anxiety

3.2.1. Open field test

For behavioral tests, rats were divided into separate groups and administered orally with 100, 200 and 300 mg/kg of IS.CR in normal saline till the completion of the study. On the 29th day of the study, the animals were exposed to the arena of open field to evaluate the effect of IS.CR on general locomotion and anxiety-like behavior. The one-way ANOVA revealed the significant overall inter-group variation for time spent in the centre of the field [F(4,35) = 37.85, P < 0.0001] (Fig. 3A) and number of entries in central zone [F(4,35) = 11.11, P < 0.001] (Fig. 3B). The post-hoc multiple comparison test revealed that animals of the control group preferred the central zone significantly less (P < 0.001) as compared to diazepam treated rats. Similarly, the rats treated with IS.CR showed reduced anxiety-like behavior in a dose-dependent manner. The rats treated with 200 and 300 mg/kg of IS.CR revealed reduced anxiety as compared to control animals with P < 0.05 and P < 0.001 respectively. The number of line crossings (horizontal activity) and the number of rearings (vertical activity) were also monitored to evaluate the impact of IS.CR on general locomotion of the animals. The animals of all treated groups significantly varied for the number of rearings [F(4,35) = 28.79, P < 0.0001] and number of line crossings [F(4,35) = 11.73, P < 0.001]. The rats treated with 200 and 300 mg/kg of IS.CR showed an increased number of rearings (P < 0.05) as compared to control animals (Fig. 3C). Likewise, the IS.CR at 300 mg/kg led to increased line crossings (P < 0.05) as shown in Fig. 3D. However, the outcomes of all observed parameters were non-significant at the lowest used dose of IS.CR (100 mg/kg).
3.2.2. Light/dark box test

This test was employed on the 31st day of study to evaluate the anxiety resolving capacity of IS.CR by exploiting the conflict among animal’s inclination to discover the new milieu and its distress towards lit areas. The one-way ANOVA revealed the prominent variation among all groups for duration of animal’s stay in dark box \( F(4,35) = 15.89, P < 0.0001 \) and in lightbox \( F(4,35) = 23.13, P < 0.0001 \). The animals treated with 200 and 300 mg/kg of IS.CR revealed reduced anxiety-like behavior as the animals spent less time in dark box as compared to controls animals with \( P < 0.01 \) and \( P < 0.001 \), respectively (Fig. 4A). Similarly, the duration of stay in lightbox was significantly increased (\( P < 0.001 \)) in animals treated with 200 and 300 mg/kg of IS.CR (Fig. 4B). The diazepam was used as a standard anxiolytic drug that prominently reduced (\( P < 0.0001 \)) animals’ anxiety towards lit areas as compared to the control rats. However, IS.CR did not show any significant outcomes at 100 mg/kg unveiling the dose-dependent anxiolytic-like potential of the extract.

3.2.3. Elevated plus-maze test

The animals were tested for anxiety-like behavior by exposing them to the arena of the elevated plus-maze on the 33rd day of the study. The analysis of results revealed that rats of all treated groups showed significant variation for their entries in open arms of maze \( F(4,35) = 15.76, P < 0.0001 \) and for their duration of stay there \( F(4,35) = 22.89, P < 0.0001 \). The 200 and 300 mg/kg of IS.CR reduced anxiety-like behavior in animals as their entries in open arms of the maze were significantly increased (\( P < 0.01 \)) as compared to the control group. (Fig. 4C). Similarly, the animals of these groups (200 and 300 mg/kg of IS.CR) spent significantly more time in open arms (\( P < 0.001 \)) as compared to control rats, a sign of reduced anxiety-like behavior in rodents (Fig. 4D).

3.3. Behavioral tests for learning and memory

3.3.1. Y-maze test

On the 36th day of the study, the rats were tested by y-maze for spontaneous alternation behavior (SAP) to evaluate the impact of IS.CR on short-term memory. The one-way ANOVA revealed the prominent inter-group difference for % SAP \( F (5,42) = 58.38, P < 0.0001 \). The post-hoc test revealed that scopolamine treated rats showed significantly reduced % SAP (\( P < 0.0001 \)) as compared to the control group. However, this amnesic effect was prominently reversed by piracetam (\( P < 0.0001 \)). Likewise, the IS.CR 200 and 300 mg/kg reversed the amnesic effects of scopolamine satisfactorily with \( P < 0.01 \) and \( P < 0.0001 \), respectively (Fig. 5A). The IS.CR caused improved short-term memory in a dose-dependent manner as the outcomes remained non-significant at 100 mg/kg dose.

3.3.1.1. Novel object recognition test

On the 38th day of study, the animals were tested for their capacity to discriminate the novel object from familiar ones. The significant overall variation among all groups was noted for their time spent with novel object
[F(5,42) = 15.53, P < 0.0001] and discrimination index [F(5,42) = 4.47, P < 0.0001]. The post-hoc test revealed that scopolamine administered animals had poor memory to differentiate the novel object from familiar ones (P < 0.01) that resulted in reduced discrimination index (P < 0.05) and these effects were prominently reversed by piracetam (P < 0.05). Similarly, these amnesic effects were significantly reversed by IS.CR dose-dependently as the animals treated with 200 and 300 mg/kg had sufficient memory of familiar objects and they spent more time in exploration of the novel object (P < 0.05) as shown in Fig. 5B. Likewise, the discrimination index was also increased in animals treated with 200 and 300 mg/kg of IS.CR (P < 0.05) as compared to the scopolamine treated group (Fig. 5C).

3.3.1.2. Passive avoidance test. The animals of all treated groups were tested on two consecutive days during acquisition and retention trials. The analysis of results revealed no significant inter-group variation during the acquisition trial [F(5,42) = 0.14, P = 0.98] that was conducted after one hour of treatments on the 40th day of study. However, a prominent variation among animals' behavior was noted during the retention trial [F(5,42) = 7.12, P < 0.0001], carried out 24 h after the acquisition trial on the 41st day. The animals treated with scopolamine showed reduced latency to enter the dark compartment as compared to the control group (P < 0.001). However, this amnesic behavior was reversed by IS.CR the way as piracetam did (P < 0.0001). The administration of 200 and 300 mg/kg of IS.CR caused longer step-through latencies as compared to scopolamine treated rats with P < 0.05 and P < 0.01, respectively (Fig. 6).

3.3.1.3. Morris water maze test. The results of the Morris water maze test depicted the memory-improving effects of IS.CR. A notable inter-group difference for escape latencies of animals during three training days (45th – 47th days) was revealed by the two-way ANOVA [F(5,84) = 34.33, P < 0.0001]. The scopolamine administration caused poor remembrance of platform quadrant and animals remained thigmotaxic in the maze which resulted in longer escape latencies as compared to the control animals [F(1,14) = 82.08, P < 0.0001]. The administration of piracetam led to reduced straying behavior and shorter escape latencies as compared to the scopolamine treated group [F(1,14) = 61.90, P < 0.0001]. Similarly, the IS.CR treatment caused improved remembrance of the platform positioned quadrant as depicted by shorter escape latencies of animals treated with 200 [F(1,14) = 29.80, P < 0.0001] and 300 mg/kg [F(1,14) = 35.56, P < 0.0001] dose in comparison to the scopolamine group (Fig. 7A). On probe day (day 48), the animals were evaluated for their long-term memory by monitoring their number of entries in the platform comprising quadrant and duration of stay there. The scopolamine treated rats showed reduced remembrance of that quadrant as their number of visits and duration of stay were significantly reduced (P < 0.05) as compared to the control group [F(1,14) = 61.90, P < 0.0001]. However, these signs of impaired memory were notably reversed by test extract as animals treated with 200 and 300 mg/kg of IS.CR showed an increased...
number of entries (P < 0.05) in the quadrant where the platform was positioned previously as well as a prolongation of their stay there (P < 0.05).

3.4. Biochemical studies of isolated rat brains

After behavioral tests, the experimental animals were sacrificed to evaluate the levels of malondialdehyde (MDA), glutathione peroxidase (GPx), superoxide dismutase (SOD) and acetylcholinesterase (AChE) in brain homogenate. High levels of MDA in brain homogenates indicates increased oxidative stress in neuronal tissues. In our results, one-way ANOVA revealed that levels of MDA fluctuated significantly among animals of all groups [F(5,18) = 32.76, P < 0.0001]. The post-hoc test demonstrated that the administration of scopolamine caused increased oxidative stress in the rat brain (P < 0.0001). However, these effects were prominently less in animals treated with piracetam (P < 0.0001) and 200 (P < 0.001) and 300 mg/kg of IS.CR (P < 0.0001) as shown in Fig. 8A.

GPx is an endogenous defense enzyme that provides protection against the destructive consequences of peroxides. The levels of GPx varied prominently in animals of all treated groups [F(5,18) = 7.96, P < 0.001]. The animals treated with scopolamine had significantly reduced levels of GPx as compared to control animals (P < 0.01). However, these worsenings were improved by IS.CR at 200 and 300 mg/kg as enzyme levels were increased (P < 0.05) as compared to scopolamine treated rats (Fig. 8B).

SOD is known to protect against ROS-mediated pathogenesis of various ailments by combating oxidative stress via the dismutation of superoxide radicals. The results of this biochemical assay demonstrated a prominent difference for SOD levels among animals of all groups [F(5,18) = 46.34, P < 0.0001]. The scopolamine administration caused reduced levels of this defensive enzyme as compared to control animals (P < 0.001). The treatment with IS.CR showed significant reversal of this deterioration, like piracetam, as the dose of 200 (P < 0.01) and 300 mg/kg (P < 0.0001) caused ele-
vated levels of SOD as compared to the scopolamine treated group (Fig. 8C).

The brain levels of acetylcholinesterase revealed prominent inter-group variation \( [F(5,18) = 148.2, \ P < 0.0001] \). The levels of AChE were increased in animals treated with scopolamine as compared to control animals (\( P < 0.0001 \)). However, these increased levels of AChE were prominently reduced in the brains of animals, like piracetam, treated with IS.CR at dose 200 (\( P < 0.01 \)) and 300 mg/kg (\( P < 0.0001 \)) as compared to the scopolamine treated group (Fig. 8D). However, the outcomes remained non-significant at dose 100 mg/kg of IS.CR.

3.5. Docking studies

The docking calculations were used to predict the interaction of secondary metabolites with the target binding site for further validation of the observed pharmacological potential of detected phytoconstituents in neurological disorder.

The ligand Artocommunol shows four \( \pi-\pi \) stacking interactions with the residues TYR70, TRP279, TYR334 (Table 3). This ligand has stabilization with H\(_2\)O on the carbonyl group (Fig. 9A). In this ligand, these \( \pi-\pi \) stacking interactions show an attractive, noncovalent interaction between aromatic rings of the ligand with the residue TYR70, TRP279, TYR334. This kind of interaction is very good for the stabilization of the ligand into the target. Furthermore, \( \pi-\pi \) stacking interactions and the –H bond with H\(_2\)O together provide a very good coupling environment for this ligand with the acetylcholinesterase. In other ligands such as Pendulone, 8-Methoxycirsilineol and Desmethoxysudachitin, similar \( \pi-\pi \) stacking interactions between the ligand and enzyme were observed along with the –H bond with H\(_2\)O which facilitates interaction with the target protein. Overall, these results validate the importance of including water molecules in a ligand–protein docking simulation, whenever it is possible.

While the ligand 1,9-dideoxyforskolin shows H-bond with residues TYR121: 2.35 Å, 2.38 Å, 2.21 Å and 2.23 Å (Fig. 9B). Similarly, 6E,9E-octadecadienoic acid (Fig. 9C) and Methyl 6-O-digalloyl-bet α-D-glucopyranoside and L-Galactose also show H-bonds with acetylcholinesterase residues which stabilize these ligands into the target molecule.

The ligand 6E-9E-Octadecadienoic acid (Fig. 9C) shows interactions with the residues PHE 331 with a distance of 2.29 Å, TRP84: 250 Å, TYR70: 2.31 Å, and PHE230: 2.53 Å (Table 3). Also, it is possible to see good interactions with the residues GLH199 and GLY.
Fig. 8. Biochemical evaluation of isolated brains of rats pre-treated with IS.CR at doses of 100, 200 and 300 mg/kg. The levels of (A) malondialdehyde (B) glutathione peroxidase (C) superoxide dismutase and (D) acetylcholinesterase were noted and compared to scopolamine-treated animals, using piracetam as standard. Data is evaluated by one-way ANOVA followed by Dunnet's test and articulated as mean ± SEM (n = 8). ** P < 0.01, *** P < 0.001 **** P < 0.0001 comparisons between control and scopolamine treated animals, #### P < 0.0001 comparisons between scopolamine and piracetam treated animals, b P < 0.05, bb P < 0.01, bbb P < 0.001 comparisons between scopolamine and 200 mg/kg IS.CR treated animals, c P < 0.01 ccc P < 0.0001 comparisons between scopolamine and 300 mg/kg IS.CR treated animals.

Table 3
Table showing the AdmetSAR analysis and docking prediction about interaction of secondary metabolites with the target in the crystal structure of acetylcholinesterase enzyme in complex with a non-chiral donepezil-like inhibitor 17 (PDB code: SNAP).

| Compound                        | Mol. wt | Log P | HBA | HBD | BBB (Probability) | Carcinogen | Residue interaction: H-Bond                  | Residue with π-π stacking interactions | H₂O-Complex |
|--------------------------------|---------|-------|-----|-----|-------------------|------------|---------------------------------------------|----------------------------------------|-------------|
| L-Galactose                    | 180.07  | -3.38 | 6   | 5   | −(0.9028)         | Non-carcinogen | ASP72: 2.23 Å, TYR: 2.20 Å, 2.35 Å                  |                                       |             |
| Methyl 6-O-digalloyl-beta-D-glucopyranoside | 310.14 | -1.59 | 10  | 6   | −(0.6841)         | Non-carcinogen | TRP64: 2.27 Å, TYR70: 2.25 Å, 2.24 Å, GLY118: 2.21 Å, SER200: 2.19 Å |                                       |             |
| Pendulone                      | 316.09  | 1.53  | 6   | 1   | −(0.3990)         | Non-carcinogen | TRP84: 2.27 Å, TYR70: 2.25 Å, 2.24 Å, GLY118: 2.21 Å, SER200: 2.19 Å |                                       |             |
| Artocoomunol CA                | 432.16  | 5.76  | 6   | 1   | +(0.8523)         | Non-carcinogen | TYR334 X                                           |                                       |             |
| Poriolin                       | 448.14  | 0.29  | 10  | 6   | −(0.9429)         | Non-carcinogen | TYR70: 2.25 Å, TRP279: 2.24 Å, TYR334: 2.22 Å, TYR334 |                                       |             |
| Desmethoxyxudacitin            | 330.07  | 2.59  | 7   | 3   | −(0.3998)         | Non-carcinogen | ARG289: 2.22 Å, SER286: 2.23 Å, TYR121: 2.29 Å       |                                       |             |
| 8-Methoxyxursilene             | 374.09  | 2.91  | 8   | 2   | −(0.4399)         | Non-carcinogen | TRP64: 2.29 Å, TRP121: 2.58 Å                     |                                       |             |
| 1,9-dideoxyforskolin           | 378.24  | 3.43  | 5   | 1   | +(0.8696)         | Non-carcinogen | TYR121: 2.35 Å, 2.38 Å, 2.21 Å, 2.23 Å             |                                       |             |
| 6E,SE-octadecadienoic acid     | 280.24  | 5.88  | 1   | 1   | +(0.9646)         | Non-carcinogen | GLH199, GLY 118.                                   |                                       |             |
These kinds of interactions are non-covalent. These interactions of secondary metabolites are an important factor in the stabilization into target of the phytoconstituents in neurological disorders.

4. Discussion

In this study, an array of experiments was used to assess characteristic features and the neuropharmacological potential of *Indigofera sessiliflora*. The *in-vitro* phytochemical screening of IS.CR for total phenols and flavonoids was carried out in an effort to reveal the presence of phytoconstituents that are claimed to be directly correlated with the antioxidant capacity of the plants (Maqsood et al., 2014; Zargoosh et al., 2019). In addition, the employed antioxidant assays exposed the radical scavenging capacity of IS. CR that might be manifested due to its phenol and flavonoid content. The functional hydroxyl groups owned by flavonoids perform antioxidant functions through the chelation of metal ions and/or scavenging the generated free radicals (Kumar and Pandey 2013).

![Docking interactions for the ligand](image-url)
Their metal chelating property provides pronounced protection from the generation of free radicals, detrimental to certain biomolecules (Kumar et al., 2013). Moreover, the use of naturally existing antioxidants is increasingly advocated to avoid the hazards associated with synthetic antioxidants (Li et al., 2014). Due to their crucial role in preventing and delaying cell damage, the natural antioxidants are also considered as “lead” structures for devising new therapeutic options to treat and inhibit the progression of AD (Perry et al., 1999).

Furthermore, the chemical characterization of IS.CR was performed through UHPLC-MS to identify the secondary metabolites responsible for the neuroprotective potential of the plant. The outcomes revealed the occurrence of eighteen different phytoconstituents from the flavonoid group of secondary metabolites and these were artemisinin CA, cadabine methyl ether, 7,4′-diidihydroxy-5-methoxyflavanone 7-neohesperidoside-4′-glucoside, 8-hydroxyxyleton 8-glucoside, tricentin 7-methyl ether 3′-glucoside-5′-rhamnoside, porilolin, desmethoxyxudacinatin, and 8-methoxyxirinoline. The remaining compounds were glucose (L-galactose), steroid (4,7-didehydroxyephysalin B), glucoside (salidroside and 6,7,3′-tetra hydroxyaurone 6′-2′,4′,6′-triacetylglucoside), galloyl ester (methyl 6-O-digalloyl-beta-D-glucopyranoside), isoflavonanones (pendulone), chalcone (homeoriodyctylchalcone 2′-glucoside), fatty acids (9,16-di-hydroxyl-palmitic acid and 6E,9E-octadecadienoic acid), and diterpenoid (1,9-dideoxyforskolin) derivatives of secondary metabolites.

During in-vivo experimentation, the effect of chronically administered IS.CR on anxiety-like behavior was evaluated through OFT, L/D and EPM behavioural tests. The animals treated with IS.CR demonstrated a dose-dependent increase in anxiolytic-like behaviour as their preference for the innately aversive open area was increased in OFT. Furthermore, these animals spent more time in innately frightening zones i.e. light compartment and elevated open arms in L/D and EPM tests, respectively. However, the animals treated with IS.CR did not reduce the number of rearing and line-crossings in OFT unveiling the anxiolytic-like capacity of IS.CR without having any sedative effect on the brain. The outcomes were contradicting to diazepam which revealed a significant sedative effect, in addition to anxiolytic effects, as animal’s rearing and line crossings (index of locomotion) were reduced as compared to the control group. The anxiolytic drugs are known to potentiate GABAergic neuro-inhibition. The flavonoids are the phytoconstituents that modulate the GABA receptors, the most important inhibitory receptors in the brain, thus exert anxiolytic-like activity via GABA-mediated neuronal inhibition (Hanrahan et al., 2011).

Moreover, the effect of flavonoids enriched IS.CR on learning and memory was investigated in a scopolamine-induced amnesic model that imitates the cognitive scarcity seen in AD. Scopolamine is a non-selective antimuscarinic drug that induces memory impairment by interrupting the cholinergic signalling in both animals and humans (Kouémou et al., 2017; Rahimzadegan and Soodi, 2018). By interfering with acetylcholine in the brain, it can result in increased oxidative stress and consequent cognitive deterioration (Rahnama et al., 2015). In this study, the IS.CR pre-treated rats showed dose-dependent reversal of scopolamine-lessened %SAP and discrimination index in y-maze and NOR tests, revealing the valuable effects of IS.CR on animal’s recognition memory. Additionally, the rats of these groups also depicted the longer step-through latencies and shorter escape latencies in PAT and MWM tests revealing the long-term memory enhancement impact of IS.CR. Flavonoids are ubiquitous components of plants that have been consumed for health-boosting purposes since the beginning of life on earth. These phytoconstituents are known to cause modulation and upregulation of CREB (cAMP response element-binding protein) expression which acts to increase the expression of neurotrophins crucial for memory (Spencer, 2009).

Oxidative stress plays an offending role in the pathogenesis of a range of disorders specially NDs including anxiety (Bouayed et al., 2009) and memory (De Lima et al., 2005). The brain requires a substantial amount of oxygen and is abnormally more vulnerable to ROS-mediated damage (Uttara et al., 2009). The scopolamine administration is associated with increased ROS that may consequently lead to depletion of endogenous antioxidants and lipid peroxidation (Rahimzadegan and Soodi, 2018). The outcomes of biochemical assays verified the scopolamine-induced reduction in GPx and SOD and elevation of MDA levels in rat brains. Furthermore, the biochemical assays also revealed the increased brain acetylcholinesterase activity after scopolamine administration and this finding is in line with previous investigations (Kouémou et al., 2017; Lian et al., 2017). AChE is an enzyme that hydrolyzes acetylcholine and terminates the cholinergic neurotransmission. The inhibition of this enzyme has been considered fruitful in the management of AD as AChE inhibitors lead to an accumulation of acetylcholine causing increased stimulation of cholinergic receptors and resulting in therapeutic relief for the memory deficits. The precise mechanism of scopolamine-induced increased acetylcholinesterase activity is not fully understood. However, Hārtl et al reported an increased expression of this protein as a result of oxidative stress produced after scopolamine administration (Hārtl et al., 2011). Hence, the scopolamine-induced impairment of learning and memory might be the result of direct deterioration of neuronal components by ROS (Markesbery, 1997) or increased AChE expression, as AChE metabolizes the acetylcholine and weakens the cholinergic neurotransmission. However, these scopolamine-induced deteriorations shown in biochemical assays were significantly reversed by IS.CR. According to the literature, the flavonoids can suppress the free radical generation as well as can upregulate the in-built antioxidant defenses (Kumar and Pandey, 2013).

Among 18 detected secondary metabolites, admet SAR program provided an agreeable distribution profile for three secondary metabolites (Artocommunol, 1,9-dideoxyforskolin and 6E,9E-octadecadienoic acid) to cross the BBB. The docking studies to predict the interaction of secondary metabolites with the target binding site also revealed that prior optimization of the orientation of water molecules, in the absence of any bound ligand, exert no detrimental effect on the improved accuracy of ligand–protein docking (Morales-Bayuelo, 2017; Polo et al., 2021; Roberts and Mancera, 2008; Ul-Haq et al., 2020). In this sense, our docking methodology depicted a possible form to stabilize these ligands analyzed into the target related to phytoconstituents investigated in the current study.

The pharmacological potential of artocommuniol, a flavonoid, has been previously reported as it exerted inhibitory effects on platelet aggregation and xanthine-oxidase enzyme (Lin et al., 2009; Weng et al., 2006). Furthermore, its antioxidant effect has also been reported as it caused inhibition of oxidative DNA damage (Lin et al., 2009).

Excitotoxicity and/or mitochondrial dysfunctions leading to apoptosis have been the pathological reasons behind several NDs including AD (Uttara et al., 2009). 1,9-dideoxyforskolin is another secondary metabolite identified in IS.CR that belongs to the diterpenoid class of phytocompounds. Forskolin has been reported to bind and activate the adenylylate cyclase in previous study (insel and Ostrom, 2003). This interaction results in increased cAMP levels that are associated with better long-term memory (Barad et al., 1998) and improved cognition (Reneerkens et al., 2009). Additionally, the forskolin prevents the development of excitotoxicity-induced mitochondrial dysfunction (Ivanova et al., 2019). The neuroprotective role of forskolin might be due to its

M. Sajjad Haider, W. Ashraf, S. Javaid et al. Saudi Journal of Biological Sciences 28 (2021) 4384–4398
potential to block the calcium (Ca^{2+}) dysregulation by preventing the extreme Ca^{2+} influx into cerebral neurons (Ivanova et al., 2018). In a previously reported study, orally administered forskolin resulted in the reduced deposition of amyloid-beta plaques in the cortex and hippocampus of five months old mice. The study also exposed the forskolin-induced attenuation of the inflammatory response by brain immune cells (Owona et al., 2016).

The appropriate neuronal physiology requires well-controlled electrical gradients across the membrane and properly anchored receptors and ion channels to communicate neighboring cells. These crucial properties are determined by the mono and polyunsaturated fatty acid (PUFAs) composition of the neuronal membrane (Roberts and Mancera 2008). Being phospholipids in the neuronal membrane, these PUFAs also influence the signaling cascades directly thus encourage synaptic plasticity and neuroprotection (Yehuda et al., 2002). In a previous investigation, the researchers verified the neuroprotective and antioxidant potential of 8,13-dihydroxy-9,11-octadecadienoic acid significantly suppressed the cytotoxicity and apoptosis resulting from glutamate-induced oxidative stress (Kim, 2012).

5. Conclusion

The findings of the present study established the neuropharmacological role of phenols/flavonoids and the SMs present in the methanolic extract of Indigofera sessiliflora. The chronically administered IS.CR resulted in a dose-dependent reduction in anxiety-like behavior and reversal of scopolamine-induced amnesia in rats. The ex vivo biochemical analysis further validated the antioxidant and anti-cholinesterase capacity of IS.CR and supported the observed behavioral outcomes. The in-silico studies further predicted the BBB crossing characteristics of three SMs (Artocommunol, 1,9-dideoxyforskolin and 6E9octadecadienoic acid) of IS.CR and also demonstrated their ability to inhibit the activity of acetylcholinesterase. The antioxidant properties of the phytocompounds present in IS.CR along with their ability to participate in cholinergic neurromodulation can contribute to the neuroprotective potential of *Indigofera sessiliflora* identified in the traditional use of this plant.

6. Institutional review board statement

All animal studies were performed after availing authorization from “Ethical Committee of B.Z.U, Multan, Punjab, Pakistan (09/pec/2015 dated 20–05-2016) and were accomplished by following instructions of the “Institute of Laboratory Animal Resources” (ILAR), Commission on Life Sciences, National Research Council (NRC, 1996).

7. Data availability statement

All the raw datasets used for the generations of this study will be available on request to the corresponding author.

Funding

This work was funded by Distinguished Scientist Fellowship program at King Saud University, Riyadh, Saudi Arabia through research supporting project Number (RSP-2020/131).

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We are thankful to Mr. Muhammad Imran and Mr. Muhammad Alyas (Animal house Attendants) who devotedly worked for the welfare of laboratory animals. Furthermore, the authors extended their appreciation to Distinguished Scientist Fellowship program at King Saud University, Riyadh, Saudi Arabia for funding this work through Research Supporting Project Number (RSP-2020/131).

References

Adedayo, B.C., Oboh, G., Oyeleye, S.I., EjakpovI, I.L., Boligon, A.A., Athayde, M.L., 2015. Blanching alters the phenolic constituents and in vitro antioxidant and anticholinesterases properties of fireweed (*Crasocephalum crepidioides*). J. TaIlah Univ. Med. Sci. 10, 410–426.

Agatonovic-Kustrin, S., Kettle, C., Morton, D.W., 2018. A molecular approach in drug development for Alzheimer’s disease. Biomed. Pharmacothetaer. 106, 553–565.

Alqutnati, F., Assiri, M.A., Mohany, M., Imran, I., Javaid, S., et al., 2020. Coadministration of ketamine and perampanel improves behavioral function and reduces inflammation in acute traumatic brain injury mouse model. Biomed Res. Int. 2020, 3193725.

André, P., Zaccaroni, M., Fiorenazzi, P., Dellà Setà, D., Menzocchi, M., Farabollini, F., 2019. Offline consolidation of spatial memory: Do the cerebellar output circuits play a role? A study utilizing a Morris water maze protocol in male Wistar rats. Brain Res. 1718, 148–158.

Bano, F., Ahmed, A., Parveen, T., Hauder, S., 2014. Anxiolytic and hyperlocomotive effects of aqueous extract of *Nigella sativa* L. seeds in rats. Pak. J. Pharm. Sci. 27, 1547–1552.

Barad, M., Bourthouladze, R., Winder, D.G., Golan, H., Kandel, E., 1998. Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. Proc. Natl. Acad. Sci. U. S. A. 95, 15020–15025.

Bouajjali, J., Rammal, H., Soulaimani, R., 2009. Oxidative stress and anxiety Relationship and cellular pathways. Oxid. Med. Cell. Longev. 2, 63–67.

Calandrella, B., Pesaresi, A., Cariati, L., Piccopio, A., Oliverio, M., Lamb, D., 2018. Kinetic and structural studies on the interactions of Torpedo californica acetylcholinesterase with two donepezil-like rigid analogues. J. Enzyme Inhib. Med. Chem. 33, 794–803.

Christenhusz, M.J.M., Byng, I.W., 2016. The number of known plants species in the world and its annual increase. PLoS One. 261, 201–217.

Collaborators, G. 2016 N., 2019. Global, regional, and national burden of neurological disorders, 1950–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. Neurol. 18, 459.

Cuvelier, M.E., Berret, C., 1995. 4A Standard Calibration Techniques. Microflowin E-b. 28, 25–30.

Dhammadan, T., Tan, B.W.L., Liao, P., Ramanathan, S., Lim, G.K., Hassan, Z., 2018. *Clitoria ternatea* L. root extract ameliorated the cognitive and hippocampal long-term potentiation deficits induced by chronic hyperperfusion in the rat. J. Ethnopharmacol. 224, 381–390.

Davis, K.L., Moos, R.C., Trinkleberg, J.R., Pfefferbaum, A., Hollister, L.E., Kopell, B.S., 2017. Phytostrogenic: Improvement of long-term memory processes in normal humans. Science 80-). 201, 272–274.

De Andrade Teles, R.B., Diniz, T.C., Costa Pinto, T.C., De Oliveira, R.G., e Silva, M.G., et al., 2018. Flavonoids as therapeutic agents in Alzheimer’s and Parkinson’s diseases: A systematic review of preclinical evidences. Oxid. Med. Cell. Longev. 2018, 7043213.

De Lima, M.N.M., Polydoro, M., Laranj, A.D., Bonatto, F., Bromberg, E., et al., 2005. Recognition memory impairment and brain oxidative stress induced by postnatal iron administration. Eur. J. Neurosci. 21, 2521–2528.

Dhingra, D., Parle, M., Kulkarni, S.K., 2004. Memory enhancing activity of *Dhingra, D.*. De Andrade Teles, R.B., Diniz, T.C., Costa Pinto, T.C., De Oliveira, R.G., e Silva, M.G., et al., 2018. Flavonoids as therapeutic agents in Alzheimer’s and Parkinson’s diseases: A systematic review of preclinical evidences. Oxid. Med. Cell. Longev. 2018, 7043213.

De Lim, M.M.M., Polydoro, M., Laranjan, D.C., Bonatto, F., Bromberg, E., et al., 2005. Recognition memory impairment and brain oxidative stress induced by postnatal iron administration. Eur. J. Neurosci. 21, 2521–2528.

Dhingra, D., Parle, M., Kulkarni, S.K., 2004. Memory enhancing activity of *Dhingra, D.*. De Andrade Teles, R.B., Diniz, T.C., Costa Pinto, T.C., De Oliveira, R.G., e Silva, M.G., et al., 2018. Flavonoids as therapeutic agents in Alzheimer’s and Parkinson’s diseases: A systematic review of preclinical evidences. Oxid. Med. Cell. Longev. 2018, 7043213.

De Lima, M.M.M., Polydoro, M., Laranjan, D.C., Bonatto, F., Bromberg, E., et al., 2005. Recognition memory impairment and brain oxidative stress induced by postnatal iron administration. Eur. J. Neurosci. 21, 2521–2528.

Dhingra, D., Parle, M., Kulkarni, S.K., 2004. Memory enhancing activity of *Dhingra, D.*. De Andrade Teles, R.B., Diniz, T.C., Costa Pinto, T.C., De Oliveira, R.G., e Silva, M.G., et al., 2018. Flavonoids as therapeutic agents in Alzheimer’s and Parkinson’s diseases: A systematic review of preclinical evidences. Oxid. Med. Cell. Longev. 2018, 7043213.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Friess, R.A., Banks, J.L., Murphy, R.B., Halgren, T.A., Klicic, J.J., Mainz, D.T., et al., 2004. Glide: a new approach for rapid, accurate docking and scoring. 1. method and assessment of docking accuracy. J. Med. Chem. 47, 1739–1749.

Gomes, A., Pimpalo, R.C., Fortaleza, S., Figueira, I., Miguel, C., Aguilar, C., et al., 2015. Chemical characterization and bioactivity of phytochemicals from Iberian endemic Santolina semidentata and strategies for ex situ propagation. Ind. Crops Prod. 74, 505–513.
