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A plausible involvement of $GABA_A$/benzodiazepine receptor in the anxiolytic-like effect of ethyl acetate fraction and quercetin isolated from *Ricinus communis* Linn. leaves in mice

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ABSTRACT

Ricinus communis Linn. (Euphorbiaceae) leaves are used in Indian traditional medicine to treat inflammatory and central nervous disorders. The polyphenolic compounds like quercetin (QUR), gallic acid, and rutin display an anxiolytic-like activity. These constituents are also present in *Ricinus communis* leaves; however there are no scientific investigations conducted to verify the anxiolytic-like effects of ethyl acetate fraction of *Ricinus communis* (RCLEA) leaves. HPLC technique was used to quantify the polyphenols contained in RCLEA fraction. It revealed the contents (% w/w): gallic acid (0.63), rutin (4.36), QUR (1.62) and Pyrogallol (PYR) (14.07). The present study investigated the anxiolytic-like effects of RCLEA fraction and two major polyphenols in *Ricinus communis* in experimental models of anxiety compared with a positive control diazepam (DZP) (1 mg/kg, i.p.). In order to investigate the anxiolytic-like effect, doses of RCLEA fraction (25, 50 and 100 mg/kg, i.p), QUR and PYR (1, 5 and 10 mg/kg respectively, i.p.) were administered to mice and subjected to open field test (OFT), elevated plus maze (EPM) or rota-rod test. The results of OFT revealed the significant increase in time spent in central area with treatments of RCLEA fraction (50 and 100 mg/kg, $p < 0.05$ and $p < 0.01$ respectively), QUR (10 mg/kg, $p < 0.05$) and PYR (10 mg/kg, $p < 0.05$). The reduction in rearings was observed with doses of RCLEA fraction, QUR and PYR. Significant reductions were found in defecation after treatments of RCLEA fraction and QUR and comparable to DZP. The results of OFT were further validated using EPM, showed that RCLEA fraction and QUR treatments (50 and 10 mg/kg, respectively) produced a significant increase in the time spent and entry into the open arms of elevated plus maze, with a profile comparable to that of DZP. No significant changes were observed in the rota-rod test, suggesting that the RCLEA fraction, QUR and PYR did not cause neurotoxicity, sedation and muscle relaxation commonly related to benzodiazepines. RCLEA fraction and QUR presented anxiolytic-like effect on the EPM, which was partially reversed by flumazenil suggested involvement of $GABA_A$/benzodiazepine receptor. These results suggest that RCLEA fraction and QUR exerts anxiolytic-like effects, and its mechanism of action appears to be modulated by $GABA_A$/benzodiazepine receptor also supported by molecular docking study.

1. Introduction

Anxiety is a behavioral process to deal with challenging situations. Fear and anxiety share common physical and mental signs, like escaping, hypervigilance, and an increased awareness level to avoid injury (Pires et al., 2013). Reports suggest that anxiety disorders are considered as high burden and prevalence, affecting around 10% of the world's population. World Health Organization estimated that, between 1990 and 2013, the number of people suffering from anxiety disorders increased by nearly 50% (World Health Organization (WHO), 2016). Anxiety disorders are highly co-morbid conditions that have been treated with Herbal medicine and Complementary and Alternative Medicine (CAM) since ancient times (Sarris et al., 2011). Thus, it is enviable to look for fast acting, better-tolerated, more efficient and fewer side effects of anxiolytics. Several reports have confirmed the use of CAM among

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psychiatric disorders, particularly depression and anxiety as a general phenomenon (Liu et al., 2015a).

Central nervous system (CNS) disorders such as anxiety, insomnia, pain and depression are frequently related with down function of the GABAergic system. $GABA_A$ positive modulators display anxiolytic (Rupprecht et al., 2006), antidepressant (Möhler, 2012), antiepileptic (Treiman, 2001), sedative hypnotic (Winsky-Sommerer, 2009) and analgesic (Munro et al., 2013) actions by improving GABA inhibitory transmission. Many compounds interact with GABA receptor complex at benzodiazepines sites (Manayi et al., 2016). Benzodiazepines are associated with side effects, such as sedation, myorelaxation, ataxia, amnesia, and ethanol and barbiturate potentiation and tolerance (Girish et al., 2013). It redirects desire towards avoiding side effects and at the same time choice of comparatively safe and effective herbal or natural molecules (Huerta-Reyes et al., 2013).

Ricinus communis Linn. (RC) is a member of Euphorbiaceae family, commonly known as castor, or *Palma Christi*, which is spread across the world (Ross, 2001). RC has African origin and now distributed and cultivated in India (Kirtikar and Basu, 2005). Since ancient times this plant has been exploited medicinally, mainly for the laxative properties of the extracted castor oil (Darmanin et al., 2009; Ribeiro et al., 2016). In India RC leaves is traditionally used for treating pain and CNS disorders (Rashid et al., 2015; Sharma et al., 2013; Sreekeesoon and Mahomoodally, 2014). Preclinical studies on RC leaves reported neuroprotective, analgesic, anti-inflammatory, anticancer and antioxidant activities (Darmanin et al., 2009; Ilavarasan et al., 2006; Lee et al., 2012; Murade et al., 2017; Singh et al., 2009; Taur and Patil, 2011). It primarily constitutes polyphenols: quercetin (QUR), rutin, gallic acid, gentisic acid, epicatechin, ellagic acid, kaempferol, tannins, essential oils: β-caryophyllene, 1, 8-cineole, camphor, α-pinene and alkaloids: ricinine, ricicomin A and *N*-demethylricinine reported from RC leaves (Darmanin et al., 2009; Pham et al., 2020; Singh et al., 2009; Tan et al., 2009). Fatty acids and ricinine, found in the crude hexane, dichloromethane and methanol leaf extracts, showed toxicity to pests in crop protection studies (Pham et al., 2020). In the present study we have used polyphenol enriched ethyl acetate fraction from crude hydroethanolic extract of RC leaves.

Polyphenolic compounds constitute one of the most abundant groups of plant secondary metabolites. It is estimated that more than 8000 structures are known (Bravo, 1998). These compounds exhibit broad range of biological activities, antioxidant, antiviral, anticancer including actions in the CNS (Hanrahan et al., 2011; Liu et al., 2021). Several polyphenolic compounds reported to display anxiolytic actions without the associated myorelaxant, sedative, and ataxic effects (Almeida et al., 2009; Wang et al., 2002). Many authors reported an affinity of flavonoids towards benzodiazepine site of $GABA_A$ receptors (Hanrahan et al., 2011; Medina et al., 1997; Wasowski and Marder, 2012). It is widely accepted that flavonoid compounds, including flavones isolated from herbs exert neuroprotective effects and permeable across the blood–brain (Kavvadias et al., 2004; Liu et al., 2021; Youdim et al., 2004, 2003). For that reason, flavonoids appear to be feasible alternative to benzodiazepines for future anxiolytic drug search and development.

QUR (3,3',4',5,7-pentahydroxyflavon), a natural source of polyphenol and flavonoid, is widely distributed in vegetables and fruits in the form of glycone or carbohydrate conjugates and is abundant in the daily diet (Zhang et al., 2020a). Recent studies have shown that QUR plays multiple roles in inhibition of inflammation, oxidative damage, platelet aggregation, and capillary permeability; it also exhibits anti-viral, anti-inflammatory, anti-cancer, antiobesity, cardiovascular protective, hepatoprotective, anxiety, as well as depression-related disorders and neuroprotective activities(Davoodvandi et al., 2020; Deng et al., 2020; Ebrahimpour et al., 2020; Wang et al., 2019; Zhang et al., 2020a; Zhang et al., 2020b). It is also reported that free radical scavenging activity of QUR, isoquercitrin, quercitrin and rutin is linked with suppressing anxiety and depression symptom (Lee et al., 2001). Pyrogallol (PYR) (1,2,3-benzenetriol) is a polyphenol found in various fruits and vegetables such as avocado and apricot (Ozturk Sarikaya, 2015). It is reported as anti-inflammatory agent in cystic fibrosis (Nicolis et al., 2008), possess acetylcholinesterase inhibitory activity in Alzheimer's disease and antioxidant (Ozturk Sarikaya, 2015), antimicrobial potential, its mechanism of action occurs through enzymatic inhibition by oxidized compounds (Lima et al., 2016).

Flavonoids and phenolic acids demonstrated a part in the mitigation of anxiety. A wide range of natural flavonoid compounds are found to be ligands for $GABA_A$ receptors in the CNS which led to the contemplation that they act as benzodiazepine-like molecules (Girish et al., 2013; Hanrahan et al., 2011). Likewise, anxiolytic, antidepressant, neuroprotective and antiepileptic activities of QUR (Aguirre-Hernández et al., 2010; Bhutada et al., 2010), gallic acid (Dhingra et al., 2012; Mansouri et al., 2014), and rutin (Hernandez-Leon et al., 2017) have been reported. Similarly, phytoconstituents, viz. gallic acid, rutin, and QUR from MeOH:water extract of RC leaves have been documented as potent antioxidants (Singh et al., 2009).

On the other hand, chromatographic quantification of polyphenolic compounds from MeOH extract (Babu et al., 2017; Wafa et al., 2014), aqueous extract (Upasani et al., 2003) and ethanol extract (Ghramh et al., 2019) of RC leaves were reported. Here in our study, for the first time ethyl acetate fraction (RCLEA) was chromatographically quantified for the presence of polyphenolic compounds viz. QUR, PYR, rutin and gallic acid. Further, based on ethnopharmacological findings and related associations, it motivated us to investigate putative anxiolytic-like effect of RCLEA fraction, QUR and PYR using the elevated plus-maze test (EPM), open field test (OFT) and rota-rod test. Moreover, the molecular docking was performed to examine *in-silico* correlation of QUR and PYR with $GABA_A$ receptor at benzodiazepine site. In order to have insights into the mechanism of anxiolytic effect, flumazenil (FLU) (3 mg/kg) with antagonism on $GABA_A$/benzodiazepine receptor was co-administrated with RCLEA fraction, QUR and PYR to examine the involvement of GABAergic nervous system.

2. Materials and methods

2.1. Plant material

The leaves of *Ricinus communis* Linn. (Family: Euphorbiaceae) were collected from Sangamner, Maharashtra state, India (GPS coordinates 19°34′ 37.7004″ N and 74°12′ 28.6632 E) in February 2016. The plant was identified, authenticated by the Botanical Survey of India, Pune, Maharashtra, India (*Voucher Specimen Number: VDM-01/ 2016*).

2.2. Extraction, isolation and characterization of compounds

The crude extract of RC leaves was obtained by maceration with absolute ethanol at 40–45 °C for 7 days, filtered and concentrated under reduced pressure using a rotary evaporator (Heidolph Labrota 4000 Efficient, Germany). The crude alcoholic extract (40 g) was then suspended in 20 ml distilled water and successively fractionated with n-hexane (3 × 450 ml), $CHCl_3$ (3 × 400 ml) and EtOAc (3 × 400 ml) to obtain RCLH (8.23% w/w), RCLC (9.32% w/w), RCLEA (7.54% w/w) fractions and residual hydro-alcoholic fraction RCLA (19.60 w/w %), respectively. The process was repeated to obtain more yields of fractions.

RCLEA fraction (10 g) was saponified with 6% alcoholic KOH followed by preparative TLC (Silica gel-H, Merck, India) of unsaponified fraction to isolate Compound-1 (Toluene: EtOAc: MeOH, 5:3:2, R_f 0.70, 21 mg) as a yellow amorphous powder and Compound-2 (Toluene: EA: AcOH: formic acid, 2.5:5:2:0.5, R_f 0.76, 37 mg) as a white amorphous powder (Murade, 2017). The isolated compounds 1 and 2 were further characterized using physicochemical and analytical techniques viz. melting point, UV, IR, ^{1}H NMR, ^{13}C NMR, and mass spectra to confirm their identity.

2.3. Preliminary phytochemical screening

The RCLEA fraction was subjected to preliminary phytochemical screening (Gurav et al., 2007, 2013, 2020; Murade, 2017) to confirm the presence of different secondary metabolites viz. alkaloids, glycosides, steroids, saponins, phenolic compounds, tannins, flavonoids, and carbohydrates.

2.4. Quantification of fraction using HPLC

The HPLC analysis was performed on a Younglin Acme 9000 series HPLC system equipped with isocratic pump and UV–Vis detector (190–600 nm), and the separation was carried out with a Hypersil^{TM} C_{18} column (250 × 10 mm, 5μm particle size). The RCLEA fraction was dissolved in methanol to achieve 1000 μL/mL concentration and further quantified using HPLC analysis. Stock solution of pure standards viz. gallic acid, rutin, QUR and PYR were prepared in methanol at concentration of 100–1000 µl/ml and stored at 4 °C in dark for further analysis. All sample solutions were filtered through a 0.45 µm membrane filter (Millipore) and injected directly. The HPLC mobile phase was prepared fresh and consisted of a mixtures of acetonitrile (ACN): 0.05% orthophosphoric acid (OPA) in H_2O (10:90), ACN: 0.05% OPA in H_2O (50:50), ACN: 0.05% OPA in H_2O (50:50) and methanol: 0.05% OPA in H_2O (90:10) for gallic acid, rutin, QUR and PYR, respectively using isocratic mode of analysis with flow rate 0.7 ml/min. with an injection volume of RCLEA fraction 20 μl. The HPLC analysis was performed at ambient temperature and data was analyzed using Autochro-3000 software. The chromatogram was monitored at a wavelength between 256–280 nm during the experiment. The peaks were identified by comparing its retention time with that of standards markers and quantified with the help of calibration curves that were separately constructed with pure standards.

2.5. Molecular docking study

The X-ray crystal structure of $GABA_A$ receptor containing two α1, two β2 and one γ2 subunits (PDB ID: 6D6T) with resolution 3.86 Å was retrieved from protein data bank (Zhu et al., 2018a; Zhu et al., 2018b). The protein structure was curated by protein preparation wizard of Schrödinger maestro 2018-1 MM share version and subsequently water molecules and other nonstandard residues were removed. Partial charges were assigned by using the OPLS-2005 force field. The correct protonation states were assigned through PROPKA and the resulting structure was subjected to restrained minimization with 0.3 Å RMSD. The site at which FLU is bound was explored in docking study as a benzodiazepine binding site. The 2D ligand structures were drawn with Marvin Sketch application and subsequently with Schrödinger LigPrep module the lowest energy 3D conformers were generated. The most stable conformer was used in docking studies. A grid box of dimension 20Å^3 was placed around the binding site and docking simulation was carried out with Schrödinger Glide extra precision (XP) module. The binding pose with the lowest docking score was retained and the docking results were analyzed using glide XP visualize (Sinha et al., 2020).

2.6. Drugs and treatments

Analytical markers for HPLC study viz. gallic acid, rutin, QUR and PYR were purchased from TCI Chemicals (India) Pvt. Ltd, Hyderabad, India. The solvents (Merck, India) used in the study were of analytical grade. For the study drugs were administered i.p. DZP (1 mg/kg, Ranbaxy Laboratories Ltd, Baddi, Solan, India) was used as a standard drug (positive control) (Diniz et al., 2019; Mansouri et al., 2014). The doses of RCLEA fraction (25, 50 and 100 mg/kg), isolated compounds QUR (Aguirre-Hernández et al., 2010; Grundmann et al., 2009) and PYR (1, 5 and 10 mg/kg) and negative control (0.9% saline containing 0.5% Tween 80) were acutely administered in mice. The

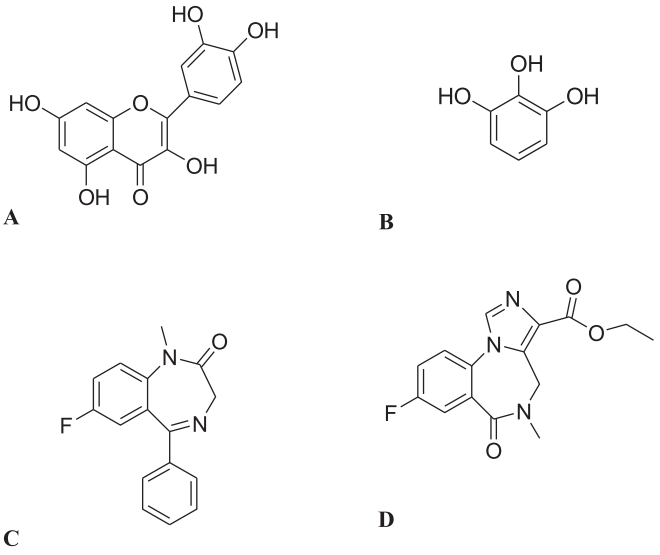

Fig. 1. The structures of the isolated compounds, QUR (A) and PYR (B); positive control DZP (C) and $GABA_A$/benzodiazepine receptor antagonist flumazenil (D).

$GABA_A$/benzodiazepine receptor antagonist flumazenil (3 mg/kg, Usan Pharmaceuticals Pvt. Ltd, Mumbai, India) was injected i.p. 15 min prior to administration of test and reference drug to evaluate possible mechanism of action (Lin et al., 2021; Mansouri et al., 2014). All solutions were prepared freshly on test days and administered in a volume of 10 ml/kg of the animal´s body weight. The structures of the isolated compounds, positive control and $GABA_A$/benzodiazepine receptor antagonist flumazenil are presented in Fig. 1.

2.7. Animals

The animal experiments were performed using Swiss albino mice of either sex weighing 25–30 g. The animals were housed under controlled conditions of temperature 23 ±2 °C; humidity: 45–65% and 12 h light/dark cycle and received pellet diet and water *ad libitum*. The animals were accustomed to the laboratory atmosphere for at least 24 h and randomly assigned to experimental groups. All procedures were carried out to minimize the number of animals used (n = 6 per group) and their sufferings. The experiments were performed as per the guidelines of CPCSEA, Govt. of India after approved protocol by Institutional Animal Ethical Committee, Amrutvahini College of Pharmacy, Sangamner, Maharashtra, India (Registration No. 1153/PO/ac/08/CPCSEA) under number protocol number 1153/AVCOP/IAEC/2016-17/03. The experimental schematic representation of behavioral test shown in Fig. 2.

2.8. Acute oral toxicity study

The acute oral toxicity study of RCLEA fraction was conducted as per the OECD guideline 423 (OECD, 2002; Gurav et al., 2020) with modifications. Briefly, the female mice (n=6 mice, 3 at preliminary and 3 at the confirmatory stage) were used for acute toxicity. The mice were fasted overnight with free access to water. The single dose of RCLEA fraction of 300 mg/kg, suspended in normal saline containing 0.5% Tween 80, was administered orally to 3 mice. After that, the animals were observed for any signs of toxicity and mortality for 24 h. Then the test was repeated in other 3 mice for confirmation. Based on zero mortality at 300 mg/kg, next higher dose of 2000 mg/kg was tested in similar fashion using another set of 6 mice (3 at preliminary and 3 at the confirmatory stage). The animals were observed frequently at 15, 30, and 60 min and every 4 h in the first 12 h, and thereafter up to 14 days for cage side clinical observations, mortality or moribund stage and weekly body weight.

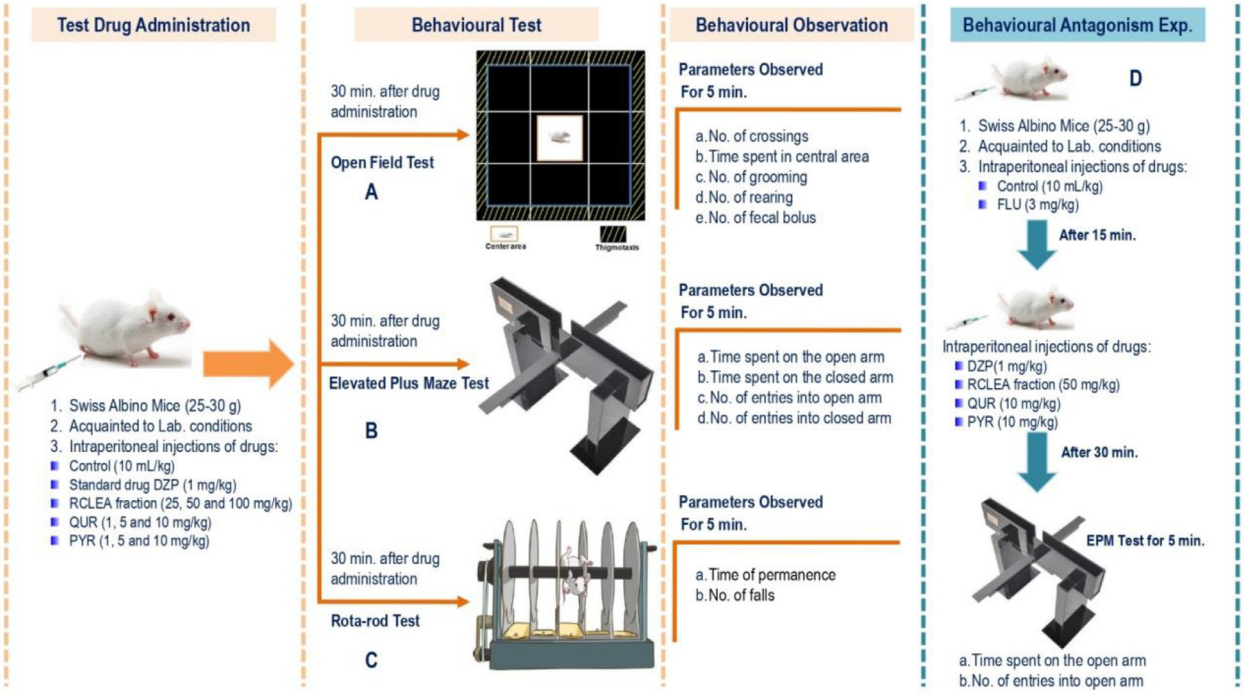

Fig. 2. Schematic representation of the behavioral test. Assessment of anxiolytic-like activity using open-field test (A); elevated plus-maze (B); rota-rod test (C) and antagonism experiment to assess involvement $GABA_A$/benzodiazepine receptors (D).

2.9. Assessment of anxiolytic-like activity

2.9.1. OFT

OFT is a behavioral test to investigate the emotionality and exploratory activity of the mice (Archer, 1973). OFT was accomplished on the acrylic arena (transparent walls and black floor, 30 × 30 × 15 cm). The area of the arena was distributed into nine equal squares. All the mice were individually placed into the center of the arena and permitted to explore. Ambulation (the number of squares crossed with all four paws), numbers of grooming, rearing, and defecation were recorded for the last 5 min of the 6 min testing period (Murade, 2017; Murade et al., 2017). The details of animal treatment schedule of OFT are represented in Fig. 2**A.**

2.9.2. EPM test

EPM test apparatus comprised of two perpendicular open arms (30 × 5 cm), two perpendicular closed arms (30 × 5 cm), extending from a central platform (5 × 5 cm) that were open on the top. The maze was constructed of black painted wood and was elevated 38 cm above the floor level. The mouse was placed on the central platform of the EPM, and the number of entries into and time spent on the open and closed arms entries were recorded for 5 min (Ham et al., 2020; Murade, 2017). The details of animal treatment schedule of EPM are represented in Fig. 2**B.** In separate set of experiment, antagonism study was conducted to investigate the involvement of $GABA_A$/benzodiazepine. The dose of $GABA_A$/benzodiazepine antagonist FLU and pretreatment period of the antagonist was chosen based on earlier studies (Grundmann et al., 2009; Lin et al., 2021; Mansouri et al., 2014). The details of animal treatment schedule of antagonism study using EPM are represented in Fig. 2**D.**

2.9.3. Rota-rod test

Rota-rod test is extensively used to assess muscle coordination, balance, and neurotoxicity in rodents (Arora et al., 2020; Galdino et al., 2012). The animals were trained on rota-rod apparatus (Omega Scientific, Bangalore, India) 24 h before performance of the test. Animals were individually placed on rota-rod apparatus on four paws (Conditions: diameter of bar: 2.5 cm; height: 25 cm above the floor; speed: 12 rpm; Time: 60 s.). The number of falls and the time of permanence on the rotating bar were recorded automatically (Galdino et al., 2012; Murade et al., 2017). The details of animal treatment schedule of rota-rod test are represented in Fig. 2**C.**

2.10. Statistical analysis

All results are expressed as mean ± standard error of the mean. Data were analyzed using one-way ANOVA followed by the Tukey-Kramer *post-hoc* multiple comparison test. Differences between the experimental groups were considered significant when $p < 0.05$. All statistical analyses were carried out by using Graph-Pad Prism (GraphPad Prism Software version 6, San Diego, CA, USA).

3. Results

3.1. Preliminary phytochemical screening

The preliminary phytochemical screening of RCLEA fraction of RC leaves revealed the presence of tannins, glycosides, saponins, flavonoids, phenolic compounds, carbohydrates (data not shown).

3.2. Isolation of phytoconstituents

3.2.1. Characterization of Compound-1 (QUR)

Compound-1: Yellow amorphous powder (0.025 g); melting point: 315.6 °C; **UV** λ_{max} (methanol): 283 nm; ***FTIR*** *(KBr,* cm^{-1}*)*: 3288.29, 3012.91, 1662.48, 1562.54, 1514.70, 1359.83, 1164.66, 1093.30, 767.94, and 679.78; Molecular formula: $C_{15}H_{10}O_7$; ***ESI-MS*** *(m/z)*: 301, 273, 257, 229, 193, 179, 121, 107; ***^{1}H-NMR*** *(400 MHz, DMSO-d_6)*: 6.2 (s, 1H, H-6), 6.4 (s, 1H, H-8), 7.8 (s, 1H, H-2'), 7.6 (d, J= 8.44 Hz, 1H, H-6'), 6.9 (d, J= 8.48 Hz, 1H, H-5'), 0.5 (bs, 5-OH), 8.7 (s, 7-OH), 9.3 (s, 3'-OH), 9.1 (s, 4'-OH), 12.3 (s, 3-OH); ***^{13}C NMR*** *(400 MHz, DMSO-d_6)*: 175.40, 135.55 (C-3), 160.58 (C-5), 163.61 (C-7), 144.59 (C-3'), 146.28 (C-4'), 147.19, 98.15, 93.25, 156.1, 102.93, 122.15, 147.15, 115.28,

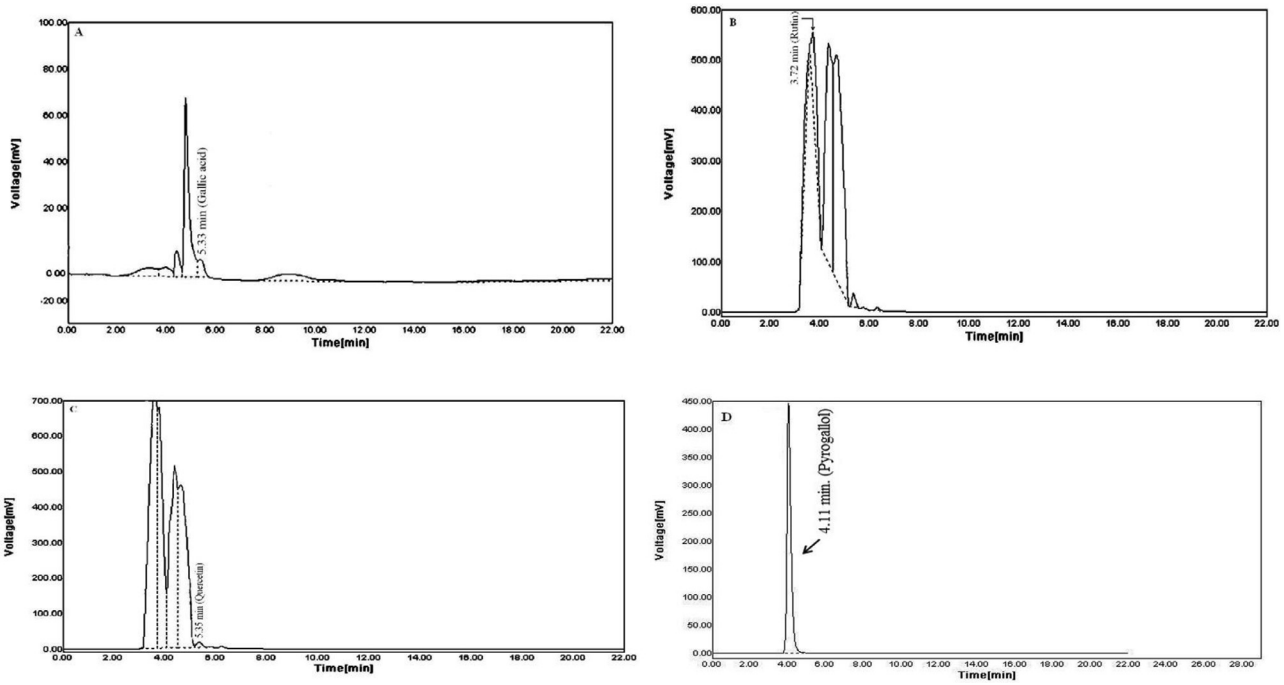

Fig. 3. RP-HPLC chromatogram of the quantified constituents from RCLEA fraction: (A) gallic acid, (B) rutin, (C) QUR, and (D) PYR from RCLEA fraction.

120.15. The compound-1 was identified as QUR and it was in accordance with literature (Saaby et al., 2009; Savic et al., 2014).

3.2.2. Compound-2 (PYR)

Compound-2: White amorphous powder (0.037 g); melting point: 130.4 °C; UV λ_{max} (methanol): 272 nm; ***FTIR*** *(KBr, cm^{-1})*: 3534.21, 3230.62, 1401.97, 1483.29, 1519.53, 1618.35, 3024.54, 1210.24, 865.61, 828.10, 759.05 and 708.50; molecular formula: $C_6H_6O_3$ ***ESI-MS*** *(m/z)*: 126.1, 108, 97, 80, 63 and 52; ***^{1}H NMR*** *(400 MHz, DMSO-d_6)*: 6.30 (d, 1H, J=7.88 Hz, H-4 & H-6), 6.44 (t, 1H, J=8.24 Hz and 7.88 Hz, H-5), 8.47 (bs, 3 -OH); ***^{13}C NMR*** *(400 MHz, DMSO-d_6)*: 145.95 (C-1 & C-3), 132.81 (C-2), 107.12 (C-4 & C-6), 118.56 (C-5). The compound-2 was identified as a 1, 2, 3- benzenetriol commonly known as PYR (Khan et al., 2002; Oladimeji and Igboasoiyi, 2014).

3.3. Quantification of fraction using HPLC analysis

Chromatographic analysis using HPLC was executed for the quantitative estimation of gallic acid, rutin, QUR and PYR from the RCLEA fraction. The chromatograms are presented in Fig. 3**.** The contents of gallic acid, rutin, QUR and PYR found in RCLEA fraction were 0.63, 4.36, 1.62 and 14.07% w/w, respectively. The retention time of quantified gallic acid (t_R = 5.33 min), rutin (t_R = 3.72 min), QUR (t_R = 5.35 min) and PYR (t_R = 4.11 min). The HPLC analysis resulted in calibration curves of the standards: gallic acid, Y = 66.074x + 190.27 (r^2 = 0.9955), rutin, Y = 52.29x + 3.651 (r^2 = 0.9991), QUR, Y = 25.441x – 32.734 (r^2 = 0.9970) and PYR, Y = 7.6746x – 2.786 (r^2 = 0.9935).

3.4. Molecular docking studies

The $GABA_A$ receptors are the major inhibitory neurotransmitter receptors often targeted in drug design in various CNS disorders (Sigel and Steinmann, 2012). The $GABA_A$ receptors are pentameric ligand-gated ion channels containing α, β, and γ subunits. Typically, the major isoform of $GABA_A$ contains two α subunits, two β subunits, and one γ subunit (Elgarf et al., 2018). The classical benzodiazepines bind at the benzodiazepine binding site at the interface of α and γ subunits (Puthenkalam et al., 2016). There are some reports in which the homopentameric structure of $GABA_A$ containing α1 subunits have been used as a template and the homology model of the heteropentameric structure containing α, β and γ subunits have been used in docking studies (Negi et al., 2018; Sahila et al., 2015). However, now the crystal structure of the heteropentameric structure of $GABA_A$ in complex with FLU is available (PDB ID: 6D6T). The resolution of this crystal structure is slightly low (3.86 Å) may be due to the presence of five different chains. But, our interest was only in D and E chains which were α1 and γ2 subunits, respectively. Molecular docking study showed that the co-crystallized FLU binds at the interface of α1 and γ2 subunits. The subunit α1 (chain D) residues Ala161, Tyr160, Ser159, Tyr160, Thr207, Phe100, Tyr210, Val212, Ser206, Ser205 and His102 within 5 Å from the FLU may be important in the binding interactions. While from subunit γ2 (chain E) the residues Met130, Thr142, Ala79, Phe77, Phe78, Asp56, Tyr58, Met57 and Glu189 within 5 Å may be likewise important in the key binding interactions (Fig. 4). A non-bonded hydrogen bond interaction is one of the key interactions which decide the binding affinity and consequent lower and favorable binding free energy. In the case of co-crystallized FLU, the residue Thr142 from chain E and residue Thr160 from chain D forms two such hydrogen bond interactions. The other non-bonded interactions such as hydrophobic π-π stacking interactions and π-cation interactions with the residues Met130 and Ala79 from chain E and Ala161, Ser159, His102 and Tyr210 from chain D were also equally important in the favorable binding of FLU at the binding site. The results of Schrödinger Glide XP docking are given in Table 1.

From the docking results, it is evident that QUR has the lowest docking score. Lower the docking scores higher is the binding energy and more favorable binding interactions at the binding site (Fig. 5). The chromen-4-one core nucleus accommodates in a binding site and the hydroxyl group at 7th position makes a key hydrogen bond interaction with Ala161. This hydrogen bond interaction is also formed with FLU with the carbonyl oxygen on the diazepine ring. Both the hydrogen bonds are between the backbone donor –NH of ala161 and acceptor oxygen of QUR and FLU. The hydrogen bond interaction with Thr142 which is found in FLU carbonyl oxygen of ethyloxycarbonyl substituent on imidazole ring was not found to occur with other compounds. Interestingly, DZP forms

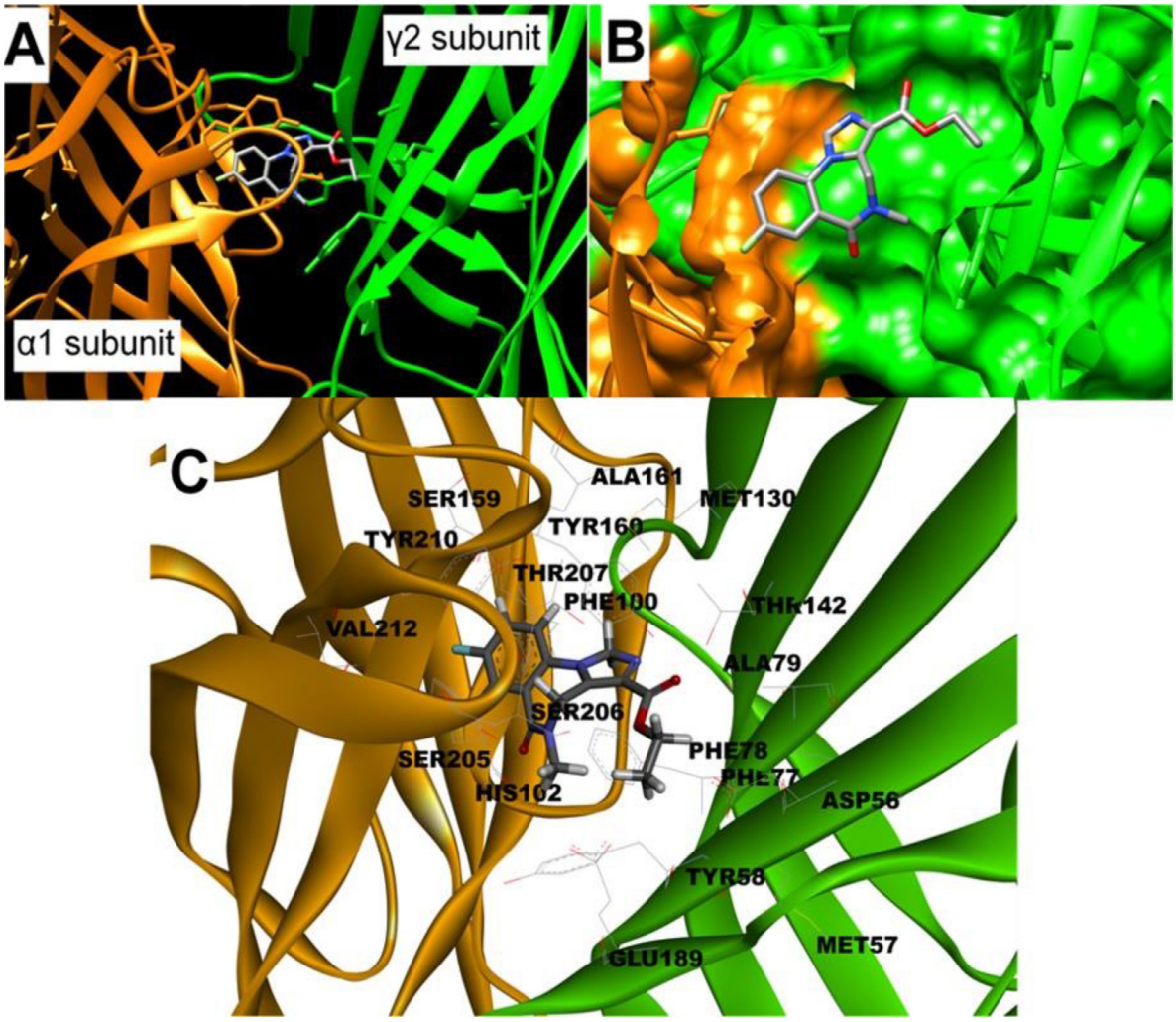

Fig. 4. FLU at the binding site of $GABA_A$. A) FLU bound at the interface of the $\alpha 1$ subunit (orange color ribbon) and $\gamma 2$ subunit (green color ribbon); B) FLU poses at the binding site, and C) The interacting residues at the binding site.

Table 1
Binding interaction of ligands with the $GABA_A$/benzodiazepine receptor site (PDB ID: 6D6T).

Pubchem CID	Ligand	Binding affinity (k.cal.mol^{-1})	The non-bonded interactions with the amino acid residues at the binding site	
			H-Bond interaction	π-π stacking interaction
5280343	QUR	-9.982	Ser206, Asp56, Ala161	Tyr210, Tyr160, Ala79
1057	PYR	-6.001	Ser205, His102, Ser159	Tyr210
3016	DZP	-7.299	Ser205	Phe77, Ser206, Tyr210, His102, Ser159, Phe100, Tyr160
3373	FLU	-8.725	Ala161, Thr142	Tyr210, His102, Ser159, Tyr160, Met130, Ala79

the hydrogen bond interactions with Ser205 where one of the acceptor nitrogen atoms of diazepine ring forms a hydrogen bond with the serine side chain oxygen atom of hydroxyl group. QUR on the other hand forms hydrogen bond interaction with adjacent residue Ser206 but with the side chain oxygen atom of a hydroxyl group and oxygen atom of hydroxyl group on phenyl ring substituted on 2nd position of flavones ring. Contrary to all these results, the compound PYR was found to make key hydrogen bonds and hydrophobic interactions. The hydroxyl groups in this compound make hydrogen bond interactions with Ser205, His102, and Ser159 residues. Here the hydrogen bond interaction with Ser205 is similar to the hydrogen bond interaction with DZP. However, the hydrophobic interaction is not found with PYR whereas only the π-π stacking interaction was observed with Tyr210. Thus, the docking study suggests that the QUR has the most favorable binding pose and the best docking score compared to the drugs DZP and FLU.

3.5. Acute toxicity study

The acute oral toxicity of RCLEA fraction showed no mortality or moribund stage and there was no toxicity of any nature observed up to the dose of 2000 mg/kg during the observation period of 14 days. Thus, the estimated LD_{50} of RCLEA fraction was more than 2500 mg/kg.

3.6. Assessment of anxiolytic-like activity

3.6.1. Effect of fraction and isolated compounds on the OFT

To examine the effect of RCLEA fraction, QUR and PYR on anxiolytic-like behavior, we performed the OFT. The administration of DZP, RCLEA fraction, QUR and PYR showed insignificant effect [$F(10, 55) = 1.345$, $p = 0.2308$] on number of crossings (Fig. 6**A**). The time spent in central area (Fig. 6**B**) [$F(10, 55) = 3.965$, $p < 0.0004$] was significantly in-

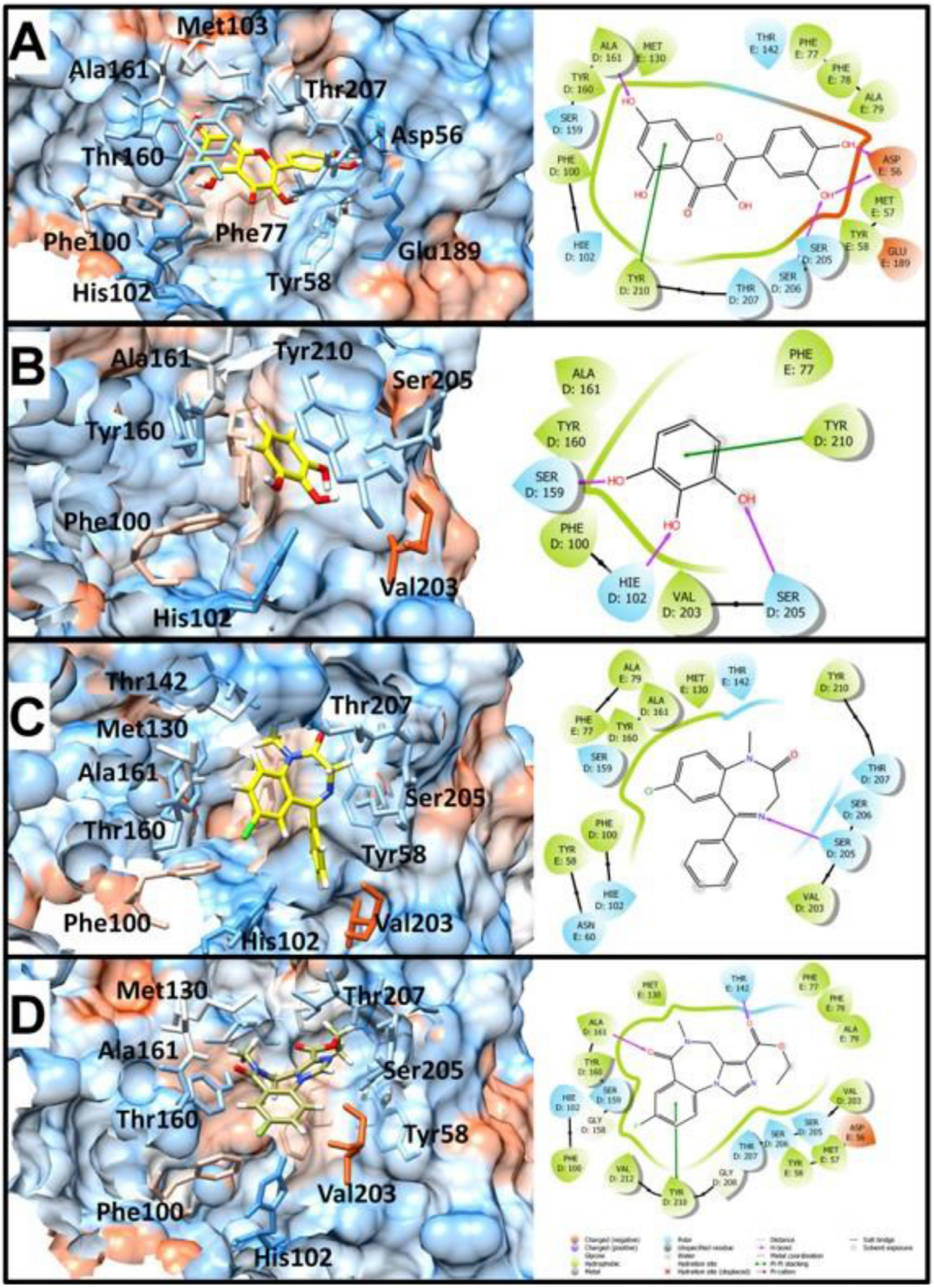

Fig. 5. Binding poses of compounds and interaction diagrams. A) QUR binding pose at the binding site and interaction diagrams; B) Binding pose of PYR and interaction diagrams; C) Binding pose of DZP and interaction diagrams; and D) Binding pose of FLU and the interaction diagrams.

creased by treatment with DZP (1 mg/kg; $p < 0.01$), RCLEA fraction (50 and 100 mg/kg; $p < 0.05$ and $p < 0.01$, respectively), QUR (10 mg/kg; $p < 0.05$) and PYR (10 mg/kg; $P < 0.05$). Treatments showed insignificant differences in grooming behavior [$F(10, 55) = 0.4211$, $p = 0.9304$)] (Fig. 6**C**). DZP ($p < 0.01$), RCLEA fraction (50 and 100 mg/kg; $p < 0.05$ and $p < 0.01$, respectively), QUR and PYR (10 mg/kg each; $p < 0.05$ and $p < 0.05$) showed significant effect on the number of rearing [$F(10, 55) = 3.903$, $p < 0.0005$] (Fig. 6**D**). The fecal bolus count [$F(10, 55) = 5.261$, $p < 0.0001$] was reduced by treatments with DZP ($p < 0.001$), RCLEA (25, 50 and 100 mg/kg; $p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively), and QUR (10 mg/kg, $p < 0.05$) while PYR showed insignificant effect (Fig. 6**E**).

3.6.2. *Effects of fraction and isolated compounds on the EPM*

EPM test is based on the principle that the experience on an EPM evokes an approach -escaping conflict. This conflict is substantially

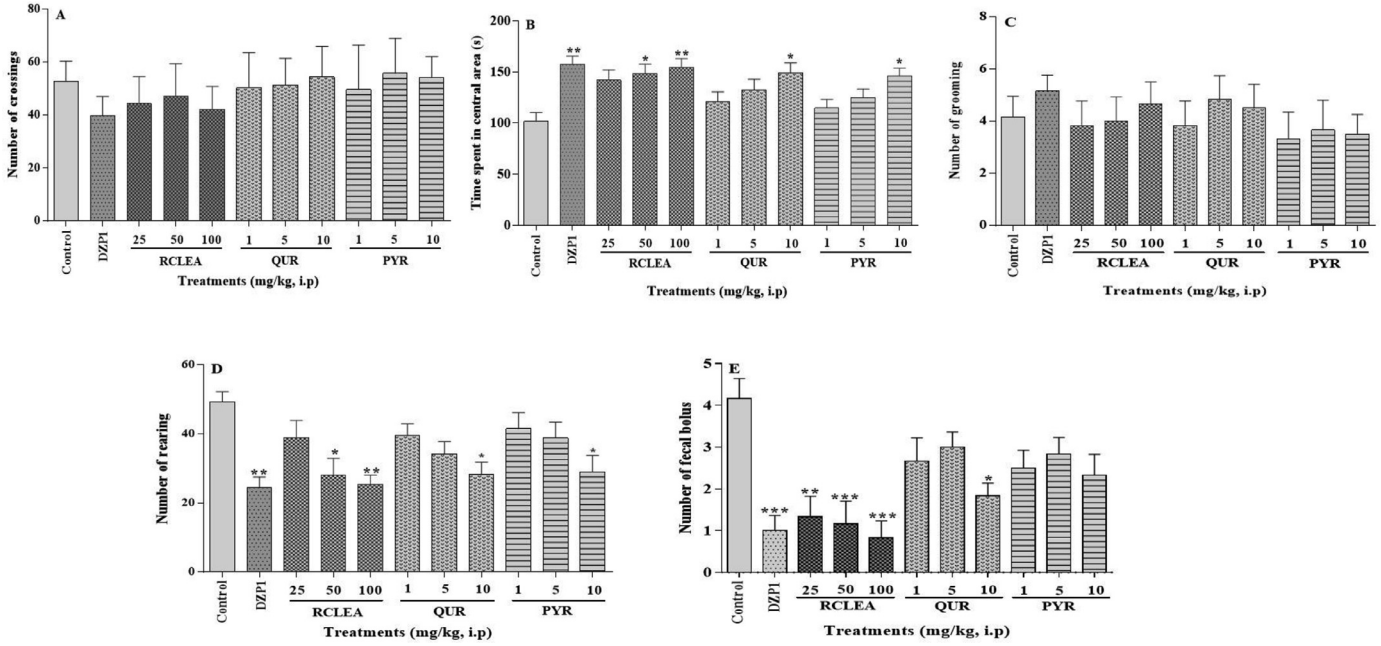

Fig. 6. Effect of acute treatment of RCLEA fraction of RC on number of crossings (A); time spent in a central area (B); number of grooming (C); number of rearings (D) and fecal bolus (E) evaluated in the open-field test in mice. Animals received i.p. treatments of control (0.5 % Tween 80 in normal saline, 10 mL/kg); DZP (1 mg/kg); RCLEA fraction (25, 50 and 100 mg/kg) and QUR or PYR (1, 5 and 10 mg/kg). Data are expressed as mean ±SEM (n= 6 mice/group), $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to control group (One-way ANOVA followed by Tukey-Kramer multiple comparison test).

brawny than that evoked by an entry into enclosed arms. The reduced aversion to the open arms as a result of anxiolytic-like effect. An anxiolytic compound increases frequency of entries into open arms and increases time spent in open arms without any change in locomotor activity. While, the anxiogenic drugs increases time spent and number of closed arms entries (Mansouri et al., 2014; Lin et al., 2021). One way ANOVA showed that DZP (1 mg/kg; $p < 0.001$), RCLEA fraction (25, 50 and 100 mg/kg; $p < 0.01$, $p < 0.001$ and $p < 0.01$, respectively) and QUR (1, 5 and 10 mg/kg; $p < 0.01$, $p < 0.05$ and $p < 0.001$, respectively) significantly increased time spent in open arms of EPM [$F(10, 55) = 16.36$, $p < 0.0001$] **(**Fig. 7**A)**. The treatments with DZP (1 mg/kg; $p < 0.001$), RCLEA fraction (25, 50 and 100 mg/kg; $p < 0.001$, $p < 0.001$ and $p < 0.01$ respectively), QUR (1, 5, and 10 mg/kg; $p < 0.001$, $p < 0.05$ and $p < 0.001$ respectively) and PYR (10 mg/kg; $p < 0.05$) decreased the time spent in closed arms [$F(10, 55) = 16.02$, $p < 0.0001$] **(**Fig. 7**B)**. Further, administration of DZP (1 mg/kg; $p < 0.001$), RCLEA fraction (25, 50 and 100 mg/kg; $p < 0.05$, $p < 0.001$ and $p < 0.05$) and QUR (1 and 10 mg/kg; $p < 0.05$ and $p < 0.001$) increased number of entries on open arms parameter *[*$F(10, 55) = 5.236$, $p < 0.0001$] **(**Fig. 7**C)**. The treatment with DZP (1 mg/kg; $p < 0.001$), RCLEA fraction (50 and 100 mg/kg; $p < 0.01$ and $p < 0.05$) and QUR (10 mg/kg; $p < 0.01$ vs. control) [$F(10, 55) = 3.766$, $p < 0.0007$] **(**Fig. 7**D)** showed significant decrease in number of entries in closed arms of EPM.

3.6.3. *Effect of $GABA_A$/benzodiazepine receptor antagonist*

In order to understand the anxiolytic-like mechanism of test drugs RCLEA fraction, QUR and PYR either via GABAergic or non-GABAergic involvement, RCLEA fraction (50 mg/kg), QUR and PYR (10 mg/kg respectively) were co-administered with FLU (benzodiazepine antagonist). FLU alone was unable to increase time spent in open arms ($p > 0.05$ vs. control; $p < 0.001$ vs. DZP group) while its co-administration of DZP reversed the anxiolytic-like effect of DZP (FLU+DZP; $p < 0.001$ vs. DZP). Likewise, co-administration of FLU with RCLEA fraction (FLU+RCLEA; $p < 0.001$ vs. DZP), QUR (FLU+QUR; $p < 0.01$ vs. control, $p < 0.001$ vs. DZP and $p < 0.01$ vs. FLU) partially reversed the time spent in open arms with no significant effect with PYR ($p > 0.05$ vs. control and FLU, $p < 0.001$ vs. DZP) [$F(9, 50) = 25.64$, $p < 0.0001$]. **(**Fig. 8**A)**. One way ANOVA [$F(9, 50) = 10.90$, $p < 0.0007$] that the co-administration of FLU with DZP (FLU+DZP; $p > 0.05$ vs. control and $p < 0.001$ vs. DZP) reversed the number of open arms entries. FLU co-administration with RCLEA fraction (FLU+RCLEA; $p > 0.05$ vs. control and FLU, $p < 0.05$ vs. DZP) and QUR ($p < 0.05$ vs. control and FLU, $p > 0.05$ vs. DZP) partially reversed the number of entries in open arms with no significant effect on PYR ($p > 0.05$ vs. control and FLU, $p < 0.001$ vs. DZP) [$F(9, 50) = 10.90$, $p < 0.0007$]. **(**Fig. 8**B)**.

Table 2
Effect of acute treatment of RCLEA fraction, QUR and PYR on number of falls in rota-rod test in mice.

Treatment	Dose (mg/kg, i.p.)	Number of falls
Control	10 mL/kg	0.2 ± 0.07
DZP	1	0.8 ± 0.07 ^{ns}
RCLEA	25	0.3 ± 0.07 ^{ns}
	50	0.5 ± 0.08 ^{ns}
	100	0.6 ± 0.09 ^{ns}
QUR	1	0.2 ± 0.10 ^{ns}
	5	0.5 ±0.11 ^{ns}
	10	0.5 ± 0.13 ^{ns}
PYR	1	0.3 ± 0.16 ^{ns}
	5	0.6 ± 0.21 ^{ns}
	10	0.8 ± 0.30 ^{ns}

The results are expressed as mean ±SEM (n= 6 mice/group) ^{ns} $P > 0.05$ as compared to control group (One-way ANOVA followed by Tukey-Kramer multiple comparison test).

3.6.4. *Effect of fraction and isolated compounds on the rota-rod test*

Rota-rod test was conducted to verify neurotoxicity and muscle relaxation effects of the RCLEA fraction, QUR and PYR from the leaves of RC. Treatments of positive control DZP, and RCLEA fraction, QUR and PYR exhibited no significant difference in the number of falls [$F(10, 55) = 0.9565$, $p = 0.4908$] **(see** Table 2**)** as well as time of perma-

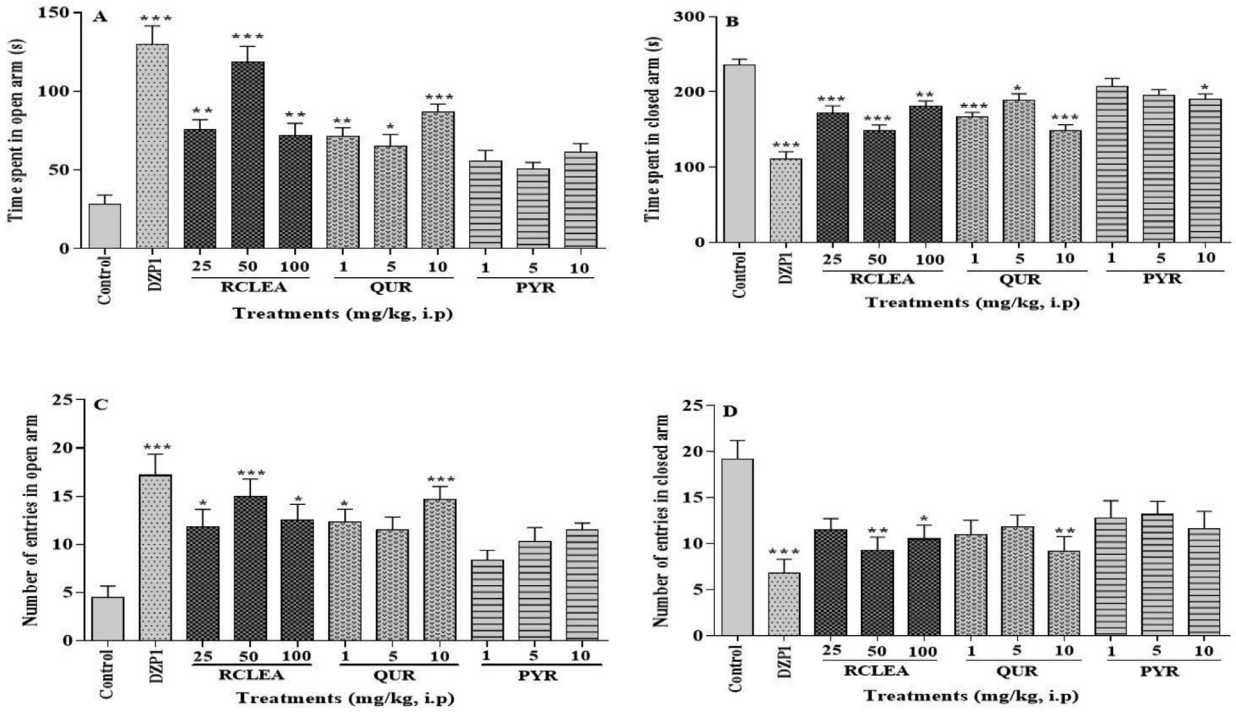

Fig. 7. Effect of acute treatment of RCLEA fraction of RC on the EPM test in mice. Animals were treated i.p. with control (0.5 % Tween 80 in normal saline, 10 mL/kg); DZP (1 mg/kg); RCLEA fraction (25, 50 and 100 mg/kg) and QUR or PYR (1, 5 and 10 mg/kg) expressed by the time spent in open arms (A), time spent in the closed arm (B), number of entries in open arms (C) and number of entries in the closed arms (D). Data are expressed as mean ±SEM (n= 6 mice/group). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group (One-way ANOVA followed by Tukey-Kramer multiple comparison test).

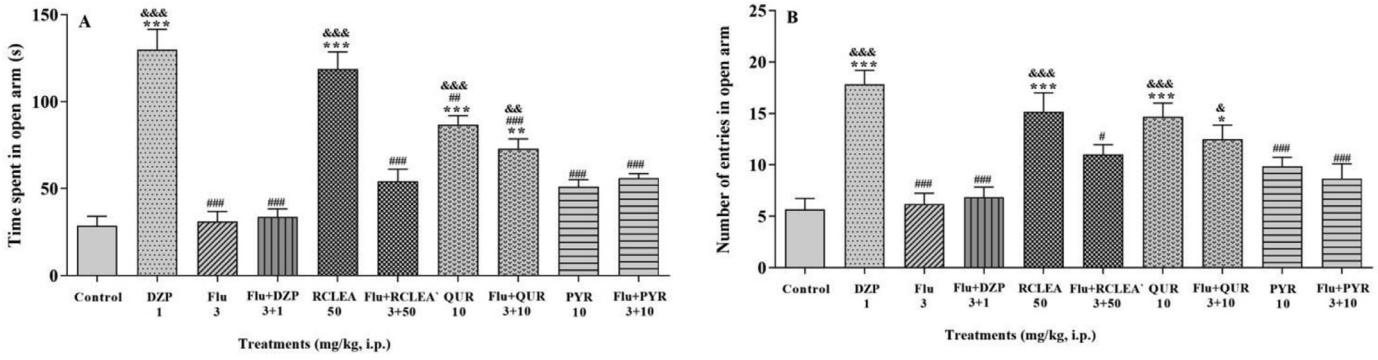

Fig. 8. Effect of FLU (3 mg/kg, i.p., 15 min before administration of DZP, RCLEA, QUR or PYR) on the EPM test. The animals received i.p. treatments of control (0.5 % Tween 80 in normal saline, 10 mL/kg), DZP (1 mg/kg), FLU+DZP (3+1 mg/kg), FLU+RCLEA (3+50 mg/kg), FLU+QUR (3+10 mg/kg) or FLU+PYR (3+10 mg/kg) expressed by the time spent in open arms (A) and number of entries in open arms (B). Results are expressed as mean ±SEM (n= 6 mice/group), $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. DZP group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ vs. FLU group (One-way ANOVA followed by Tukey-Kramer multiple comparison test).

nence [$F(10, 55) = 1.894$, $p = 0.0658$] at any dose level as shown in Fig. 9.

4. Discussion

RC is single-species genus in the Euphorbiaceae family (Kole, 2011). The results presented in this work, add to the stock of knowledge on RC leaves and emphasize the relevance of the information of the traditional medicine. In the present study, the anxiolytic-like effect of the RCLEA fraction of RC as well as its two isolated metabolites, identified as QUR, PYR and further plausible involvement of GABAergic system in different behavioral models.

The polyphenolic metabolites isolated QUR and PYR further quantified using HPLC from RCLEA fraction, also gallic acid and rutin quantified for the first time. The earlier studies revealed the presence of flavonoids, phenolic compounds, saponins, and glycosides in RC leaves (Singh et al., 2009; Wafa et al., 2014). These polyphenolic compounds present in the fraction may be contributed to their anxiolytic-like activity. Several reports have linked presence of polyphenolic and flavonoids with anxiolytic effect (Estrada-Camarena et al., 2019; Girish et al., 2013; Grundmann et al., 2009; Herrera-Ruiz et al., 2008; Mansouri et al., 2014; Noguerón-Merino et al., 2015).

Animal study using the rota-rod test, OFT and EPM are simple, standardized, efficient and consistent tools to screen prospective anxiolytic-like drugs (Garlet et al., 2019; Robinson et al., 2018).

OFT imitates cerebral activation and anxious animals freeze in a corner. Conversely, benzodiazepine receptor agonists show behavioral changes which are in covenant with decreased anxiety while promoting exploratory activities (Murtala and Akindele, 2020). Thigmotaxis is connected to challenges to escape from a novel aversive situation and is

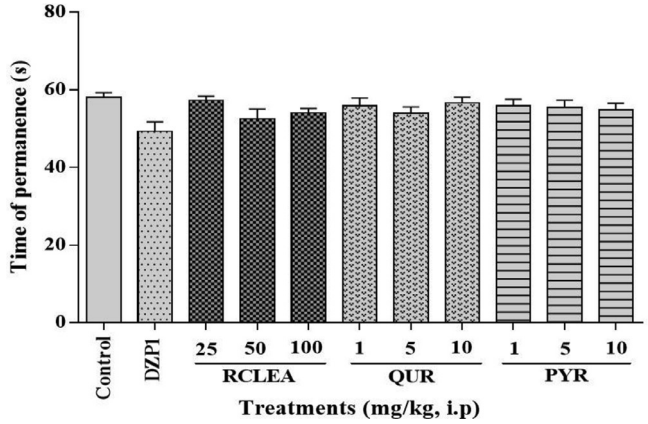

Fig. 9. Effect of acute treatment of RCLEA fraction of RC on time of permanence on rota-rod test in mice. Animals received i.p. treatments of control (0.5 % Tween 80 in normal saline, 10 mL/kg); DZP (1 mg/kg); RCLEA fraction (25, 50 and 100 mg/kg) and QUR or PYR (1, 5 and 10 mg/kg). The results are expressed as mean ±SEM (n= 6 mice/group), $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to control group (One-way ANOVA followed by Tukey-Kramer multiple comparison test).

an important anxiety-related behavior in OFT, which is sensitive to the action of benzodiazepines (Shaw et al., 2007). An increase in the number of rearings and the number of center square crossings in the OFT reported for the medicinal plant extract (Barua et al., 2009). Likewise, DZP showed an increase in the number of crossings and time spent in the central arena at low doses which is in agreement with the previous studies (Galdino et al., 2012; Murtala and Akindele, 2020). Similar to DZP, RCLEA fraction, QUR and PYR also demonstrated increase in time spent in the central area of open field which suggested anxiolytic-like effect. Exposure to novel surrounding in OFT causes aversion and increases grooming behavior in animal to resolve unknown fear or aversive stimulus (Lalonde and Strazielle, 2009). Contrary to this, in the present study, the grooming behavior remained unaltered upon acute administration of DZP, RCLEA fraction, QUR, and PYR, which is beyond understanding and needs more studies. Similar results were reported for sesquiterpenoids isolated from *Nectandra grandiflora* leaves. It may be due to behavioral struggle between grooming and vertical exploration with consequently reduction of occasions to groom (Garlet et al., 2019). The rearing is thought to be a novelty-evoked exploratory behavior that contains components of both stereo-activity and anxiety (Eisener-Dorman et al., 2010). However, the rearing behavior appeared to be independent of crossings as evidenced by the observation of significantly decreased rearing along with no change in the number of crossings (Shaw et al., 2007). In agreement with other studies, the treatment with DZP and RCLEA, QUR, and PYR significantly suppressed the rearing behavior indicating their anxiolytic-like effect may be due to sedation (Chaves et al., 2018; Garlet et al., 2019). Emotional behavior displayed by rodents in the form of higher number of fecal bolus (Kulesskaya and Voikar, 2014). In accordance to this, DZP, RCLEA, and QUR attenuated defecation which further strengthens their anxiolytic-like effect (Archer, 1973; Galdino et al., 2012).

An anxiolytic compounds increases the frequency of entries and time spent in open arms, while the anxiogenic drugs increase time spent and a number of entries into closed arms (Ham et al., 2020; Mansouri et al., 2014). In the present investigation, administration of RCLEA (25, 50, and 100 mg/kg) QUR (1, 5, and 10 mg/kg) significantly increased the number of entries as well as the time spent in the open arms on EPM comparable to DZP suggesting their anxiolytic-like effect. Our findings are in agreement with previous studies of anxiolytic-like effects presented by secondary metabolites such as QUR (Aguirre-Hernández et al., 2010; Bhutada et al., 2010), 5-methoxyflavone (Shanmugasundaram et al., 2020), 6-bromoflavanone (Ognibene et al., 2008), gallic acid (Mansouri et al., 2014), ellagic acid (Girish et al., 2013) and berberine (Peng et al., 2004), spinosin (Liu et al., 2015b).

It is well known that the actions of benzodiazepines are based on the enhancement of GABAergic transmission at benzodiazepine-sensitive GABA receptors (Han et al., 2009). $GABA_A$ receptor mediates most of the inhibitory synaptic transmissions in the central nervous system (Girish et al., 2013). Conventional hypnotics and anxiolytic drugs, such as barbiturates and benzodiazepines, enhance GABAergic transmission via the $GABA_A$ receptor (Sieghart et al., 2012). However, typical $GABA_A$/benzodiazepine agonists have side effects such as muscle relaxation, sedation, and depressive mood (Girish et al., 2013; Sieghart et al., 2012). FLU is commonly reported in the studies to demonstrate and confirm the specific involvement of $GABA_A$/benzodiazepine receptors in the mechanism of action of substances or a plant extract with anxiolytic activity (Garlet et al., 2019; Lin et al., 2021). In the present study, to rule out the involvement of $GABA_A$ receptors, the anxiolytic effect of RCLEA, QUR and PYR was assessed in FLU treated mice. Results of our study are in agreement with others studies, the pretreatment of FLU (3 mg/kg) showed partial but significant reversal in the time spent and the number of entries into open arms of EPM after administration of RCLEA fraction (50 mg/kg) and QUR (10 mg/kg), whereas insignificant change in PYR (10 mg/kg) treated mice (Garlet et al., 2019; Girish et al., 2013; Mansouri et al., 2014). Therefore, it can be speculated that RCLEA fraction and QUR possibly interacted with $GABA_A$/benzodiazepine receptor mediated anxiolytic-like effect.

Rota-rod test employed to access motor coordination evolves complex behavioral domain and reflects balance, muscle strength, neurotoxicity, and patterned gait as well as sensory competence (Galdino et al., 2012; Murade et al., 2017). The administration of DZP, RCLEA fraction, QUR, and PYR did not produce any change in number of falls and the time of permanence at any dose level, devoid of motor incoordination, muscle relaxation or sedation typical side effects of benzodiazepines. Most of the flavonoid compounds do not exhibit benzodiazepine related side effects (Almeida et al., 2009; Medina et al., 1998, 1997; Wasowski and Marder, 2012). Some authors reported benzodiazepines at relatively high dose causes loss of motor coordination possibly due to a blockage of neuromuscular transmission (Costa et al., 2014; Monteiro et al., 2020).

To confirm the *in-vivo* anxiolytic-like effect of QUR isolated from RCLEA fraction, docking studies were performed in the present study and the interaction of QUR with the binding sites on human $\alpha 2$ subunit-comprising $GABA_A$ receptors was analyzed using molecular docking (Shanmugasundaram et al., 2020). These studies are imperative in drug design and utilized to predict possible binding affinity of drugs at target binding sites (Karim et al., 2017). In the present study, the docking of $GABA_A$ at benzodiazepine binding site suggests non-selective agonist- DZP and competitive antagonist-FLU have strong interactions (Zhu et al., 2018). While non-benzodiazepine molecule- QUR has the most favorable binding pose and the best docking score compared to DZP and FLU (Negi et al., 2018). As reported previously, flavonoids show high affinity for benzodiazepine binding site of the $GABA_A$ receptors and act as rational pharmacophore (Dekermendjian et al., 1999; Hanrahan et al., 2011). In clinical domain benzodiazepines exhibited pharmacological effects (anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic) make them the most important $GABA_A$ receptor-modulating agents (Sieghart et al., 2012). On the other hand, PYR had displayed fewer interactions with $GABA_A$ at benzodiazepine site (in line with the *in-vivo* studies). As reported in similar study, flavonoids like rosmarinic acid and kaempferol are showed good binding affinity towards $GABA_A$ receptors (Negi et al., 2018).

In summary, our results suggest that acute administration of anxiolytic-like effect on mice exerted by RCLEA fraction may be attributed mainly due to presence of gallic acid, rutin and QUR (Aguirre-Hernández et al., 2010; Murtala and Akindele, 2020). However, more studies on neuropharmacological, biochemical and phytochemical aspects will be needed in order to better understand the mechanisms re-

sponsible for anxiolytic-like effect and to identify other active secondary metabolites present in the RCLEA fraction.

5. Conclusion

Our findings suggest that the RCLEA fraction and QUR isolated from leaves of RC offered significant anxiolytic-like effect could be due to the interaction of these compounds on GABAergic system at benzodiazepine receptor site. These findings are in agreement with the ethnopharmacological use of this plant in CNS disorders. Considering behavioral test and good interactions of QUR with $GABA_A$/benzodiazepine receptor complex in molecular docking study suggest potential target for drug discovery of anxiolytic agents.

6. CRediT authorship contribution statement

VM performed the experiments and contributed to the acquisition and analysis of data; AW, DP and SD contributed to the analytical and physico-chemical characterization; RP and SG contributed to molecular docking experiments; MW contributed to pharmacological studies and commented on the manuscript; VM, DH and SG designed the experiments and wrote the manuscript; VM, DH contributed equally to this study.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phyplu.2021.100041.

References

Aguirre-Hernández, E., González-Trujano, M.E., Martínez, A.L., Moreno, J., Kite, G., Terrazas, T., Soto-Hernández, M., 2010. HPLC/MS analysis and anxiolytic-like effect of quercetin and kaempferol flavonoids from Tilia americana var. mexicana. J. Ethnopharmacol. 127, 91–97. doi:10.1016/j.jep.2009.09.044.

Almeida, E.R.De, Rafael, K.R.D.O., Couto, G.B.L., Ishigami, A.B.M., 2009. Anxiolytic and anticonvulsant effects on mice of flavonoids, linalool, and x03B1; -tocopherol presents in the extract of leaves of cissus sicyoides L. (vitaceae). J. Biomed. Biotechnol. 2009, 1–7. doi:10.1155/2009/274740.

Archer, J., 1973. Tests for emotionality in rats and mice: a review. Anim. Behav. 21, 205–235. doi:10.1016/S0003-3472(73)80065-X.

Arora, I., Behl, T., Grover, M., Sachdeva, M., Pal, G., Khan, N., 2020. Study of anxiolytic and motor co-ordination activity of Cucurbita moschata and its possible mechanism through GABA receptors. Obes. Med. 18, 100204. doi:10.1016/j.obmed.2020.100204.

Babu, P.R., Bhuvaneswar, C., Sandeep, G., Ramaiah, C.V., Rajendra, W., 2017. Hepatoprotective role of Ricinus communis leaf extract against D-galactosamine induced acute hepatitis in albino rats. Biomed. Pharmacother. 88, 658–666. doi:10.1016/j.biopha.2017.01.073.

Barua, C.C., Roy, J.D., Buragohain, B., Barua, A.G., Borah, P., Lahkar, M., 2009. Anxiolytic effect of hydroethanolic extract of Drymaria cordata L Willd. Indian J. Exp. Biol. 47, 969–973.

Bhutada, P., Mundhada, Y., Bansod, K., Ubgade, A., Quazi, M., Umathe, S., Mundhada, D., 2010. Reversal by quercetin of corticotrophin releasing factor induced anxiety- and depression-like effect in mice. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 34, 955–960. doi:10.1016/j.pnpbp.2010.04.025.

Bravo, L., 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr. Rev. 56, 317–333. doi:10.1111/j.1753-4887.1998.tb01670.x.

Chaves, E.M.C., Honório-Júnior, J.E.R., Sousa, C.N.S., Monteiro, V.S., Nonato, D.T.T., Dantas, L.P., Lúcio, A.S.S.C., Barbosa-Filho, J.M., Patrocínio, M.C.A., Viana, G.S.B., Vasconcelos, S.M.M., 2018. The anxiolytic-like effect of 6-styryl-2-pyrone in mice involves GABAergic mechanism of action. Metab. Brain Dis. 33, 139–149. doi:10.1007/s11011-017-0139-5.

Costa, J.P., De Oliveira, G.A.L., De Almeida, A.A.C., Islam, M.T., De Sousa, D.P., De Freitas, R.M., 2014. Anxiolytic-like effects of phytol: possible involvement of GABAergic transmission. Brain Res. 1547, 34–42. doi:10.1016/j.brainres.2013.12.003.

Darmanin, S., Wismayer, P.S., Camilleri Podesta, M.T., Micallef, M.J., Buhagiar, J.A., 2009. An extract from Ricinus communis L. leaves possesses cytotoxic properties and induces apoptosis in SK-MEL-28 human melanoma cells. Nat. Prod. Res. 23, 561–571. doi:10.1080/14786410802228579.

Davoodvandi, A., Shabani Varkani, M., Clark, C.C.T., Jafarnejad, S., 2020. Quercetin as an anticancer agent: focus on esophageal cancer. J. Food Biochem. 44, 1–10. doi:10.1111/jfbc.13374.

Dekermendjian, K., Kahnberg, P., Witt, M.R., Sterner, O., Nielsen, M., Liljefors, T., 1999. Structure-activity relationships and molecular modeling analysis of flavonoids binding to the benzodiazepine site of the rat brain GABA(A) receptor complex. J. Med. Chem. 42, 4343–4350. doi:10.1021/jm991010h.

Deng, Q., Li, X.X., Fang, Y., Chen, X., Xue, J., 2020. Therapeutic potential of quercetin as an antiatherosclerotic agent in atherosclerotic cardiovascular disease: a review.. Evidence-based Complement. Altern. Med. 2020. doi:10.1155/2020/5926381.

Dhingra, D., Chhillar, R., Gupta, A., 2012. Antianxiety-like activity of gallic acid in unstressed and stressed mice: possible involvement of nitriergic system. Neurochem. Res. 37, 487–494. doi:10.1007/s11064-011-0635-7.

Diniz, T.C., de Oliveira Júnior, R.G., Miranda Bezerra Medeiros, M.A., Gama e Silva, M., de Andrade Teles, R.B., dos Passos Menezes, P., de Sousa, B.M.H., Abrahão Frank, L., de Souza Araújo, A.A., Russo Serafini, M., Stanisçuaski Guterres, S., Pereira Nunes, C.E., Salvador, M.J., da Silva Almeida, J.R.G., 2019. Anticonvulsant, sedative, anxiolytic and antidepressant activities of the essential oil of Annona vepretorum in mice: Involvement of GABAergic and serotonergic systems. Biomed. Pharmacother. 111, 1074–1087. doi:10.1016/j.biopha.2018.12.114.

Ebrahimpour, S., Zakeri, M., Esmaeili, A., 2020. Crosstalk between obesity, diabetes, and alzheimer's disease: introducing quercetin as an effective triple herbal medicine. Ageing Res. Rev. 62, 101095. doi:10.1016/j.arr.2020.101095.

Eisener-Dorman, A.F., Grabowski-Boase, L., Steffy, B.M., Wiltshire, T., Tarantino, L.M., 2010. Quantitative trait locus and haplotype mapping in closely related inbred strains identifies a locus for open field behavior. Mamm. Genome 21, 231–246. doi:10.1007/s00335-010-9260-z.

Elgarf, A.A., Siebert, D.C.B., Steudle, F., Draxler, A., Li, G., Huang, S., Cook, J.M., Ernst, M., Scholze, P., 2018. Different benzodiazepines bind with distinct binding modes to GABA a receptors. ACS Chem. Biol. 13, 2033–2039. doi:10.1021/acschembio.8b00144.

Estrada-Camarena, E., Sollozo-Dupont, I., Islas-Preciado, D., González-Trujano, M.E., Carro-Juárez, M., López-Rubalcava, C., 2019. Anxiolytic- and anxiogenic-like effects of Montanoa tomentosa (Asteraceae): dependence on the endocrine condition. J. Ethnopharmacol. 241, 112006. doi:10.1016/j.jep.2019.112006.

Galdino, P.M., Nascimento, M.V.M., Florentino, I.F., Lino, R.C., Fajemiroye, J.O., Chaibub, B.A., de Paula, J.R., de Lima, T.C.M., Costa, E.A., 2012. The anxiolytic-like effect of an essential oil derived from Spiranthera odoratissima A. St. Hil. leaves and its major component, β-caryophyllene, in male mice. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 38, 276–284. doi:10.1016/j.pnpbp.2012.04.012.

Garlet, Q.I., Rodrigues, P., Barbosa, L.B., Londero, A.L., Mello, C.F., Heinzmann, B.M., 2019. Nectandra grandiflora essential oil and its isolated sesquiterpenoids minimize anxiety-related behaviors in mice through GABAergic mechanisms. Toxicol. Appl. Pharmacol. 375, 64–80. doi:10.1016/j.taap.2019.05.003.

Ghramh, H.A., Khan, K.A., Ibrahim, E.H., Setzer, W.N., 2019. Synthesis of gold nanoparticles (AuNPs) using ricinus communis leaf ethanol extract, their characterization, and biological applications. Nanomaterials 9. doi:10.3390/nano9050765.

Girish, C., Raj, V., Arya, J., Balakrishnan, S., 2013. Involvement of the GABAergic system in the anxiolytic-like effect of the flavonoid ellagic acid in mice. Eur. J. Pharmacol. 710, 49–58. doi:10.1016/j.ejphar.2013.04.003.

Grundmann, O., Nakajima, J.-I., Kamata, K., Seo, S., Butterweck, V., 2009. Kaempferol from the leaves of Apocynum venetum possesses anxiolytic activities in the elevated plus maze test in mice. Phytomedicine 16, 295–302. doi:10.1016/j.phymed.2008.12.020.

Gurav, S., Gulkari, V., Duragkar, N., Sakharwade, S., Itankar, P., Patil, A., 2007. Analgesic and anti-inflammatory activity of Flacourtia ramontchi L. Herit. Pharmacologyonline 2, 20–31.

Gurav, S.S., Deshkar, N.S., Tilloo, S.K., Duragkar, N.J., Burade, K., 2013. Antimicrobial and antioxidant evaluation of flacourtia ramontchi L. Herit. J. Herbs, Spices Med. Plants 19, 76–95. doi:10.1080/10496475.2012.743107.

Gurav, S.S., Gurav, N.S., Patil, A.T., Duragkar, N.J., 2020. Effect of explant source, culture media, and growth regulators on callogenesis and expression of secondary metabolites of Curcuma longa. J. Herbs, Spices Med. Plants 26 (2), 172–190. doi:10.1080/10496475.2019.1689542.

Gurav, N., Gurav, S., Wanjari, M., Prasad, S., Wayal, S., Rarokar, N., 2020. Development and evaluation of aphrodisiac potential of a classical ayurvedic formulation,'Kaamdev ghrita' in rat model. J. Ayurveda Integr Med. doi:10.1016/j.jaim.2020.09.007.

Ham, H.J., Lee, Y.S., Yun, J., Han, S.B., Son, D.J., Hong, J.T., 2020. Anxiolytic-like effects of the ethanol extract of Magnolia obovata leaves through its effects on GABA-benzodiazepine receptor and neuroinflammation. Behav. Brain Res. 383, 112518. doi:10.1016/j.bbr.2020.112518.

Han, H., Ma, Y., Eun, J.S., Li, R.H., Hong, J.T., Lee, M.K., Oh, K.W., 2009. Anxiolytic-like effects of sanjoinine A isolated from Zizyphi Spinosi Semen: possible involvement of GABAergic transmission. Pharmacol. Biochem. Behav. 92, 206–213. doi:10.1016/j.pbb.2008.11.012.

Hanrahan, J.R., Chebib, M., Johnston, G.A.R., 2011. Flavonoid modulation of GABA A receptors. Br. J. Pharmacol. 163, 234–245. doi:10.1111/j.1476-5381.2011.01228.x.
Hernandez-Leon, A., González-Trujano, M.E., Fernández-Guasti, A., 2017. The anxiolytic-like effect of rutin in rats involves GABA A receptors in the basolateral amygdala. Behav. Pharmacol. 28, 303–312. doi:10.1097/FBP.0000000000000290.
Herrera-Ruiz, M., Román-Ramos, R., Zamilpa, A., Tortoriello, J., Jiménez-Ferrer, J.E., 2008. Flavonoids from Tilia americana with anxiolytic activity in plus-maze test. J. Ethnopharmacol. 118, 312–317. doi:10.1016/j.jep.2008.04.019.
Huerta-Reyes, M., Herrera-Ruiz, M., González-Cortazar, M., Zamilpa, A., León, E., Reyes-Chilpa, R., Aguilar-Rojas, A., Tortoriello, J., 2013. Neuropharmacological in vivo effects and phytochemical profile of the extract from the aerial parts of Heteropterys brachiata (L.) DC. (Malpighiaceae). J. Ethnopharmacol. 146, 311–317. doi:10.1016/j.jep.2012.12.049.
Ilavarasan, R., Mallika, M., Venkataraman, S., 2006. Anti-inflammatory and free radical scavenging activity of Ricinus communis root extract. J. Ethnopharmacol. 103, 478–480. doi:10.1016/j.jep.2005.07.029.
Karim, N., Khan, I., Abdelhalim, A., Abdel-Halim, H., Hanrahan, J.R., 2017. Molecular docking and antiamnesic effects of nepitrin isolated from Rosmarinus officinalis on scopolamine-induced memory impairment in mice. Biomed. Pharmacother. 96, 700–709. doi:10.1016/j.biopha.2017.09.121.
Kavvadias, D., Sand, P., Youdim, K.A., Qaiser, M.Z., Rice-Evans, C., Baur, R., Sigel, E., Rausch, W.D., Riederer, P., Schreier, P., 2004. The flavone hispidulin, a benzodiazepine receptor ligand with positive allosteric properties, traverses the blood-brain barrier and exhibits anticonvulsive effects. Br. J. Pharmacol. 142, 811–820. doi:10.1038/sj.bjp.0705828.
Khan, M., Lampronti, I., Martello, D., Bianchi, N., Jabbar, S., Choudhuri, M., Datta, B., Gambari, R., 2002. Identification of pyrogallol as an antiproliferative compound present in extracts from the medicinal plant Emblica officinalis: Effects on in vitro cell growth of human tumor cell lines. Int. J. Oncol. 21, 187–192. doi:10.3892/ijo.21.1.187.
Kirtikar, K.R., Basu, B.D., 2005. Indian Medicinal Plants, Second. ed. International Book Distributors, Dehradun, India.
Kole, C., 2011. Wild Crop Relatives: Genomic and Breeding Resources, Wild Crop Relatives: Genomic and Breeding Resources: Oilseeds. Springer Berlin Heidelberg, Berlin, Heidelberg doi:10.1007/978-3-642-14871-2.
Kulesskaya, N., Voikar, V., 2014. Assessment of mouse anxiety-like behavior in the light-dark box and open-field arena: role of equipment and procedure. Physiol. Behav. 133, 30–38. doi:10.1016/j.physbeh.2014.05.006.
Lalonde, R., Strazielle, C., 2009. The relation between open-field and emergence tests in a hyperactive mouse model. Neuropharmacology 57, 722–724. doi:10.1016/j.neuropharm.2009.07.010.
Lee, E., Eom, J.E., Kim, H.L., Kang, D.H., Jun, K.Y., Jung, D.S., Kwon, Y., 2012. Neuroprotective effect of undecylenic acid extracted from Ricinus communis L. through inhibition of μ-calpain. Eur. J. Pharm. Sci. 46, 17–25. doi:10.1016/j.ejps.2012.01.015.
Lee, M.H., Lin, R.D., Shen, L.Y., Yang, L.L., Yen, K.Y., Hou, W.C., 2001. Monoamine oxidase B and free radical scavenging activities of natural flavonoids in Melastoma candidum D. Don. J. Agric. Food Chem. 49, 5551–5555. doi:10.1021/jf010622j.
Lima, V.N., Oliveira-Tintino, C.D.M., Santos, E.S., Morais, L.P., Tintino, S.R., Freitas, T.S., Geraldo, Y.S., Pereira, R.L.S., Cruz, R.P., Menezes, I.R.A., Coutinho, H.D.M., 2016. Antimicrobial and enhancement of the antibiotic activity by phenolic compounds: Gallic acid, caffeic acid and pyrogallol. Microb. Pathog. 99, 56–61. doi:10.1016/j.micpath.2016.08.004.
Lin, Y.S., Peng, W.H., Shih, M.F., Cherng, J.Y., 2021. Anxiolytic effect of an extract of Salvia miltiorrhiza Bunge (Danshen) in mice. J. Ethnopharmacol. 264, 113285. doi:10.1016/j.jep.2020.113285.
Liu, L., Liu, C., Wang, Y., Wang, P., Li, Y., Li, B., 2015a. Herbal medicine for anxiety, depression and insomnia. Curr. Neuropharmacol. 13, 481–493. doi:10.2174/1570159x1304150831122734.
Liu, Z., Silva, J., Shao, A.S., Liang, J., Wallner, M., Shao, X.M., Li, M., Olsen, R.W., 2021. Flavonoid compounds isolated from Tibetan herbs, binding to GABAA receptor with anxiolytic property. J. Ethnopharmacol. 267, 113630. doi:10.1016/j.jep.2020.113630.
Liu, J., Zhai, W.-M., Yang, Y.-X., Shi, J.-L., Liu, Q.-T., Liu, G.-L., Fang, N., Li, J., Guo, J.-Y., 2015b. GABA and 5-HT systems are implicated in the anxiolytic-like effect of spinosin in mice. Pharmacol. Biochem. Behav. 128, 41–49. doi:10.1016/j.pbb.2014.11.003.
Manayi, A., Nabavi, S.M., Daglia, M., Jafari, S., 2016. Natural terpenoids as a promising source for modulation of GABAergic system and treatment of neurological diseases. Pharmacol. Rep. 68, 671–679. doi:10.1016/j.pharep.2016.03.014.
Mansouri, M.T., Soltani, M., Naghizadeh, B., Farbood, Y., Mashak, A., Sarkaki, A., 2014. A possible mechanism for the anxiolytic-like effect of gallic acid in the rat elevated plus maze. Pharmacol. Biochem. Behav. 117, 40–46. doi:10.1016/j.pbb.2013.12.011.
Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1998. Neuroactive flavonoids: new ligands for the Benzodiazepine receptors. Phytomedicine 5, 235–243. doi:10.1016/S0944-7113(98)80034-2.
Medina, J.H., Viola, H., Wolfman, C., Marder, M., Wasowski, C., Calvo, D., Paladini, A.C., 1997. Overview - Flavonoids: a new family of benzodiazepine receptor ligands. Neurochem. Res. 22, 419–425. doi:10.1023/A:1027303609517.
Möhler, H., 2012. The GABA system in anxiety and depression and its therapeutic potential. Neuropharmacology 62, 42–53. doi:10.1016/j.neuropharm.2011.08.040.
Monteiro, Á.B., Kelly de Souza Rodrigues, C., Petícia do Nascimento, E., Sales, V., dos, S., de Araújo Delmondes, G., Nogueira da Costa, M.H., Pereira de Oliveira, V.A., Pereira de Morais, L., Boligon, A.A., Barbosa, R., Martins da Costa, J.G., Alencar de Menezes, I.R., Bezerra Felipe, C.F., Kerntopf, M.R., 2020. Anxiolytic and antidepressant-like effects of Annona coriacea (Mart.) and caffeic acid in mice. Food Chem. Toxicol. 136. doi:10.1016/j.fct.2019.111049.
Munro, G., Hansen, R.R., Mirza, N.R., 2013. GABAa receptor modulation: potential to deliver novel pain medicines? Eur. J. Pharmacol. 716, 17–23. doi:10.1016/j.ejphar.2013.01.070.
Murade, V., 2017. Isolation and Characterization of Naturally Occuring Neuropharmacological Compounds. Savitribai Phule Pune University, Pune.
Murade, V., Deshmukh, K., Murade, R., Raut, D., Chavan, M., Hase, D., 2017. Involvement of opioid receptors in antinociceptive activity of semi purified fraction and β-amyrin isolated from Ricinus communis Linn. leaves in mice. Orient. Pharm. Exp. Med. 17, 355–364. doi:10.1007/s13596-017-0285-7.
Murtala, A.A., Akindele, A.J., 2020. Anxiolytic- and antidepressant-like activities of hydroethanol leaf extract of Newbouldia laevis (P.Beauv.) Seem. (Bignoniaceae) in mice. J. Ethnopharmacol. 249, 112420. doi:10.1016/j.jep.2019.112420.
Negi, A., Singh, P., Taneja, N., Mani, S., 2018. Molecular-docking study of anti-stress natural compounds against GABAa receptor portends the novel approach to stress treatment. J. Appl. Pharm. Sci. 8, 38–43. doi:10.7324/JAPS.2018.81205.
Nicolis, E., Lampronti, I., Dechecchi, M.C., Borgatti, M., Tamanini, A., Bianchi, N., Bezzerri, V., Mancini, I., Grazia Giri, M., Rizzotti, P., Gambari, R., Cabrini, G., 2008. Pyrogallol, an active compound from the medicinal plant Emblica officinalis, regulates expression of pro-inflammatory genes in bronchial epithelial cells. Int. Immunopharmacol. 8, 1672–1680. doi:10.1016/j.intimp.2008.08.001.
Noguerón-Merino, M.C., Jiménez-Ferrer, E., Román-Ramos, R., Zamilpa, A., Tortoriello, J., Herrera-Ruiz, M., 2015. Interactions of a standardized flavonoid fraction from Tilia americana with Serotoninergic drugs in elevated plus maze. J. Ethnopharmacol. 164, 319–327. doi:10.1016/j.jep.2015.01.029.
OECD, 2002. Test No. 423: acute oral toxicity - acute toxic class method, OECD guideline for testing of chemicals. OECD Guidelines for the Testing of Chemicals. OECD Section 4 doi:10.1787/9789264071001-en.
Ognibene, E., Bovicelli, P., Adriani, W., Saso, L., Laviola, G., 2008. Behavioral effects of 6-bromoflavanone and 5-methoxy-6,8-dibromoflavanone as anxiolytic compounds. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 32, 128–134. doi:10.1016/j.pnpbp.2007.07.023.
Oladimeji, O.H., Igboasoiyi, A., 2014. Isolation, characterization and antimicrobial analysis of ethyl gallate and pyrogallol from Acalypha wilkesiana var. lace-acalypha (Muell &Arg.). Afr. J. Pharmacol. Ther. 3, 79–84.
Ozturk Sarikaya, S.B., 2015. Acethylcholinesterase inhibitory potential and antioxidant properties of pyrogallol. J. Enzyme Inhib. Med. Chem. 30, 761–766. doi:10.3109/14756366.2014.965700.
Peng, W.-H., Wu, C.-R., Chen, C.-S., Chen, C.-F., Leu, Z.-C., Hsieh, M.-T., 2004. Anxiolytic effect of berberine on exploratory activity of the mouse in two experimental anxiety models: interaction with drugs acting at 5-HT receptors. Life Sci. 75, 2451–2462. doi:10.1016/j.lfs.2004.04.032.
Pham, N.K.T., Tran, T.T.L., Duong, T.H., Trung, N.T., Phan, D.C.T., Mai, D.T., Nguyen, V.K., Huynh, B.L.C., Nguyen, T.A.T., Tran, T.D., Tran, T.N.M., Nguyen, T.P., 2020. Ricicomin A, a new alkaloid from the leaves of Ricinus communis Linn. Nat. Prod. Res. 0, 1–7. doi:10.1080/14786419.2020.1839456.
Pires, L.F., Costa, L.M., Silva, O.A., De Almeida, A.A.C., Cerqueira, G.S., De Sousa, D.P., De Freitas, R.M., 2013. Anxiolytic-like effects of carvacryl acetate, a derivative of carvacrol, in mice. Pharmacol. Biochem. Behav. 112, 42–48. doi:10.1016/j.pbb.2013.09.001.
Puthenkalam, R., Hieckel, M., Simeone, X., Suwattanasophon, C., Feldbauer, R.V., Ecker, G.F., Ernst, M., 2016. Structural studies of GABAA receptor binding sites: which experimental structure tells us what? Front. Mol. Neurosci. 9, 1–20. doi:10.3389/fnmol.2016.00044.
Rashid, S., Ahmad, M., Zafar, M., Sultana, S., Ayub, M., Khan, M.A., Yaseen, G., 2015. Ethnobotanical survey of medicinally important shrubs and trees of Himalayan region of Azad Jammu and Kashmir, Pakistan. J. Ethnopharmacol. 166, 340–351. doi:10.1016/j.jep.2015.03.042.
Ribeiro, P.R., de Castro, R.D., Fernandez, L.G., 2016. Chemical constituents of the oilseed crop Ricinus communis and their pharmacological activities: a review. Ind. Crops Prod. 91, 358–376. doi:10.1016/j.indcrop.2016.07.010.
Robinson, L., Spruijt, B., Riedel, G., 2018. Between and within laboratory reliability of mouse behaviour recorded in home-cage and open-field. J. Neurosci. Methods 300, 10–19. doi:10.1016/j.jneumeth.2017.11.019.
Ross, I.A., 2001. Medicinal Plants of the World, Plant Science. Humana Press, Totowa, NJ doi:10.1007/978-1-59259-237-1.
Rupprecht, R., Eser, D., Zwanzger, P., Möller, H.J., 2006. GABAA receptors as targets for novel anxiolytic drugs. World J. Biol. Psychiatry 7, 231–237. doi:10.1080/15622970600868525.
Saaby, L., Rasmussen, H.B., Jäger, A.K., 2009. MAO-A inhibitory activity of quercetin from Calluna vulgaris (L.) Hull. J. Ethnopharmacol. 121, 178–181. doi:10.1016/j.jep.2008.10.012.
Sahila, M., Babitha, P.P., Bandaru, S., Nayarisseri, A., Doss, V.A., 2015. Molecular docking based screening of GABA (A) receptor inhibitors from plant derivatives. Bioinformation 11, 280–289. doi:10.6026/97320630011280.
Sarris, J., Panossian, A., Schweitzer, I., Stough, C., Scholey, A., 2011. Herbal medicine for depression, anxiety and insomnia: a review of psychopharmacology and clinical evidence. Eur. Neuropsychopharmacol. 21, 841–860. doi:10.1016/j.euroneuro.2011.04.002.
Savic, Ivan, Nikolic, V., Savic, Ivana, Nikolic, L., Jovic, M., Jovic, M., 2014. The qualitative analysis of the green tea extract using ESI-MS method. Savrem. Tehnol. 3, 30–37. doi:10.5937/savteh1401030s.
Shanmugasundaram, J., Subramanian, V., Nadipelly, J., Kathirvelu, P., Sayeli, V., Cheriyan, B.V., 2020. Anxiolytic–like activity of 5–methoxyflavone in mice with involvement of GABAergic and serotonergic systems - in vivo and in silico evidences. Eur. Neuropsychopharmacol. 1–11. doi:10.1016/j.euroneuro.2020.05.009.

Sharma, J., Gairola, S., Gaur, R.D., Painuli, R.M., Siddiqi, T.O., 2013. Ethnomedicinal plants used for treating epilepsy by indigenous communities of sub-Himalayan region of Uttarakhand, India. J. Ethnopharmacol. 150, 353–370. doi:10.1016/j.jep.2013.08.052.

Shaw, D., Annett, J.M., Doherty, B., Leslie, J.C., 2007. Anxiolytic effects of lavender oil inhalation on open-field behaviour in rats. Phytomedicine 14, 613–620. doi:10.1016/j.phymed.2007.03.007.

Sieghart, W., Ramerstorfer, J., Sarto-Jackson, I., Varagic, Z., Ernst, M., 2012. A novel GABA A receptor pharmacology: drugs interacting with the $\alpha +\beta$ - interface. Br. J. Pharmacol. 166, 476–485. doi:10.1111/j.1476-5381.2011.01779.x.

Sigel, E., Steinmann, M.E., 2012. Structure, function, and modulation of GABAA receptors. J. Biol. Chem. 287, 40224–40231. doi:10.1074/jbc.R112.386664.

Singh, P.P., Ambika, Chauhan, S.M.S., 2009. Activity guided isolation of antioxidants from the leaves of Ricinus communis L. Food Chem. 114, 1069–1072. doi:10.1016/j.foodchem.2008.10.020.

Sinha, S.K., Shakya, A., Prasad, S.K., Singh, S., Gurav, N.S., Prasad, R.S., Gurav, S.S., 2020. An in-silico evaluation of different Saikosaponins for their potency against SARS-CoV-2 using NSP15 and fusion spike glycoprotein as targets. J. Biomol. Struct. Dyn. doi:10.1080/07391102.2020.1762741, 0, 000.

Sreekeesoon, D.P., Mahomoodally, M.F., 2014. Ethnopharmacological analysis of medicinal plants and animals used in the treatment and management of pain in Mauritius. J. Ethnopharmacol. 157, 181–200. doi:10.1016/j.jep.2014.09.030.

Tan, Q.G., Cai, X.H., Dua, Z.Z., Luo, X.D., 2009. Three terpenoids and a tocopherol-related compound from Ricinus communis. Helv. Chim. Acta 92, 2762–2768. doi:10.1002/hlca.200900105.

Taur, D.J., Patil, R.Y., 2011. Antiasthmatic activity of Ricinus communis L. roots. Asian Pac. J. Trop. Biomed. 1, S13–S16. doi:10.1016/S2221-1691(11)60113-5.

Treiman, D.M., 2001. GABAergic mechanisms in epilepsy. Epilepsia 42, 8–12. doi:10.1046/j.1528-1157.2001.042Suppl.3008.x.

Upasani, S.M., Kotkar, H.M., Mendki, P.S., Maheshwari, V.L., 2003. Partial characterization and insecticidal properties of Ricinus communis L foliage flavonoids. Pest Manag. Sci. 59, 1349–1354. doi:10.1002/ps.767.

Wafa, G., Amadou, D., Larbi, K.M., Héla, E.F.O., 2014. Larvicidal activity, phytochemical composition, and antioxidant properties of different parts of five populations of Ricinus communis L. Ind. Crops Prod. 56, 43–51. doi:10.1016/j.indcrop.2014.02.036.

Wang, H., Hui, K.M., Chen, Y., Xu, S., Wong, J.T.F., Xue, H., 2002. Structure-activity relationships of flavonoids, isolated from Scutellaria baicalensis, binding to benzodiazepine site of GABAA receptor complex. Planta Med. 68, 1059–1062. doi:10.1055/s-2002-36357.

Wang, Y.S., Shen, C.Y., Jiang, J.G., 2019. Antidepressant active ingredients from herbs and nutraceuticals used in TCM: pharmacological mechanisms and prospects for drug discovery. Pharmacol. Res. 150, 104520. doi:10.1016/j.phrs.2019.104520.

Wasowski, C., Marder, M., 2012. Flavonoids as GABAA receptor ligands: the whole story? J. Exp. Pharmacol. 4, 9–24. doi:10.2147/JEP.S23105.

Winsky-Sommerer, R., 2009. Role of GABAA receptors in the physiology and pharmacology of sleep. Eur. J. Neurosci. 29, 1779–1794. doi:10.1111/j.1460-9568.2009.06716.x.

World Health Organization (WHO), 2016. Investing in treatment for depression and anxiety leads to fourfold return [WWW Document]. https://www.who.int/news-room/detail/13-04-2016-investing-in-treatment-for-depression-and-anxiety-leads-to-fourfold-return (accessed 5.10.20).

Youdim, K.A., Dobbie, M.S., Kuhnle, G., Proteggente, A.R., Abbott, N.J., Rice-Evans, C., 2003. Interaction between flavonoids and the blood-brain barrier: in vitro studies. J. Neurochem. 85, 180–192. doi:10.1046/j.1471-4159.2003.01652.x.

Youdim, K.A., Qaiser, M.Z., Begley, D.J., Rice-Evans, C.A., Abbott, N.J., 2004. Flavonoid permeability across an in situ model of the blood-brain barrier. Free Radic. Biol. Med. 36, 592–604. doi:10.1016/j.freeradbiomed.2003.11.023.

Zhang, J.liang, Liu, M., Cui, W., Yang, L., Zhang, C.nuan, 2020. Quercetin affects shoaling and anxiety behaviors in zebrafish: involvement of neuroinflammation and neuron apoptosis. Fish Shellfish Immunol. 105, 359–368. doi:10.1016/j.fsi.2020.06.058.

Zhu, S., Noviello, C.M., Teng, J., Walsh Jr, R.M., Kim, J.J., Hibbs, R.E., 2018. Human GABA-A receptor alpha1-beta2-gamma2 subtype in complex with GABA and flumazenil, conformation B [WWW Document]. https://www.rcsb.org/structure/6D6T (accessed 6.18.20).

Zhang, J., Ning, L., Wang, J., 2020. Dietary quercetin attenuates depressive-like behaviors by inhibiting astrocyte reactivation in response to stress. Biochem. Biophys. Res. Commun. 533, 1338–1346. doi:10.1016/j.bbrc.2020.10.016.

Zhu, S., Noviello, C.M., Teng, J., Walsh, R.M., Kim, J.J., Hibbs, R.E., 2018. Structure of a human synaptic GABAA receptor. Nature 559, 67–88. doi:10.1038/s41586-018-0255-3.