Anxiolytic, antidepressant and antioxidant activity of the methanol extract of *Canarium resiniferum* leaves

Md. Shahin Shah [1,2,#], Mohammed Abu Tayab [1,#], Anisur Rahman [1], Muhammad Nazmul Hasan [1], Md. Saddam Hossain Talukder [1], A. M. Kafil Uddin [1], Md. Jabed [1], Md. Nazim Uddin Chy [1,2], Arkajyoti Paul [2,3], Md. Masudur Rahman [1,*], Talha Bin Emran [3,*], Veronique Seidel [4,*]

[1]Department of Pharmacy, International Islamic University Chittagong, Chittagong 4318, Bangladesh (shahiiuc@gmail.com) (abutayab.me@gmail.com) (anisaneen@gmail.com) (nazmulsagar86@gmail.com) (hzowelctg@gmail.com) (kafilprincebd@gmail.com) (faisalahammed855@gmail.com) (nazim107282@gmail.com)

[2]Drug Discovery, GUSTO A Research Group, Chittagong 4203, Bangladesh

[3]Department of Pharmacy, BGC Trust University Bangladesh, Chittagong 4381, Bangladesh (arka.bgctub@gmail.com)

[4]Natural Products Research Laboratory, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom

#Equal Contribution

***Corresponding authors:**

Veronique Seidel (veronique.seidel@strath.ac.uk)

Talha Bin Emran (talhabmb@bgctub.ac.bd)

Md. Masudur Rahman (masud@iiuc.ac.bd)

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

List of Abbreviations

MECR: Methanol extract of *Canarium resiniferum*

EPM: Elevated plus-maze test

HBT: hole-board test

LDB: Light-dark box test.

TST: Tail suspension test

FST: Forced swim test

TPC: Total phenolic content

TFC: Total flavonoid content

FRAP: Ferric reducing antioxidant power assay

QE: Quercetin equivalents

GAE: Gallic acid equivalents

Keywords: Animal behavioral tests; Biological activity; Medicinal plants; Oxidative stress; Phytochemicals

Highlights:

- MECR leaves (400 mg/kg) showed significant anxiolytic activity in mice
- MECR (200,400 mg/kg) displayed significant antidepressant activity in mice

- MECR displayed high total phenolic/flavonoid contents and antioxidant activity

Novelty: Although the genus *Canarium* has been extensively previously studied for its biological activity, our findings are the first to report on the pharmacological activity of *C. resiniferum.* The potential to alleviate anxiety and depression disorders exhibited by this species warrants further investigation as a safe alternative treatment for anxiety and depression.

Type of article: Original Research article

Length of the Manuscript: Title: 94 characters; Abstract: 245 words; Text: 5441 words; References: 50; Figures: 4. Tables: 2.

Section: 1. Natural Products

Taxonomy: Traditional Herbal Medicine; Mental Disorder; Antioxidant studies; Depression; Oxidative stress; Anxiety Disorder

Canarium resiniferum (CR) leaves

Methanol extract (MECR)

- Total phenolic content (TPC)
- Total flavonoid content (TFC)
- Rapid phytochemical analysis

- High TPC and TFC
- Presence of alkaloids, tannins, phenols, and flavonoids

In vivo study

Anxiolytic activity

- Elevated plus-maze (EPM) test
- Hole-board test (HBT)
- Light-dark box (LDB) test

- Dose-dependent activity with oral administration of MECR (100, 200, 400 mg/kg) in all tests
- Significant activity at 400 mg/kg compared to diazepam

Antidepressant activity

- Tail suspension test (TST)
- Forced swim test (FST)

- Dose-dependent activity with oral administration of MECR (100, 200, 400 mg/kg) in all tests
- Significant activity for all doses (except for 100 mg/kg in the FST) compared to imipramine

In vitro study

Antioxidant activity

- DPPH radical scavenging assay
- Ferric Reducing Antioxidant Power (FRAP) assay

High activity in the DPPH and FRAP assays

MECR has the potential to alleviate anxiety and depression disorders and may represent a safe alternative treatment for anxiety and depressive disorders

Abstract

Background and Aim: This study evaluated the anxiolytic, antidepressant, and antioxidant activity of the methanol extract of *Canarium resiniferum* (MECR) leaves, and determined the total phenolic and flavonoid contents in this extract.

Experimental procedure: The anxiolytic effect of MECR (100, 200, 400 mg/kg, p.o.) was tested in mice using the elevated plus-maze (EPM) test, the hole-board test (HBT), and the light-dark box (LDB) test. Its antidepressant effect was evaluated in the tail suspension (TST) and the forced swim (FST) tests. The total phenolic (TPC) and flavonoid (TFC) content was measured using standard colorimetric assays. Antioxidant activity was determined using the DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays.

Results and Conclusion: MECR, at all doses, showed dose-dependent anxiolytic activity. At 400 mg/kg, it significantly increased the time spent and number of entries in the open arms (EPM test), the number of head-dips (HBT), and the time spent into the light compartment (LDB) test compared to the control. In the TST and FST, MECR dose-dependently reduced the duration of immobility compared to untreated animals. This was significant for all doses except for 100 mg/kg in the FST model. MECR showed high TPC and TFC (90.94 ± 0.75 mg GAE/g and 51.54 ± 0.78 mg QE/g of dried extract, respectively) and displayed potent activity in the DPPH radical scavenging (IC_{50} = 177.82 µg/mL) and FRAP assays. These findings indicate that *C. resiniferum* has the potential to alleviate anxiety and depression disorders, which merits further exploration.

Keywords: Animal behavioral tests; Biological activity; Medicinal plants; Oxidative stress; Phytochemicals

1. Introduction

Canarium resiniferum Bruce ex King (Burseraceae) is a large evergreen tree native to Bangladesh and the Assam state of India.[1] In Bangladesh, the plant known as Dhup, is used by traditional medicinal healers for its resin which is commonly applied for the topical treatment of eczema.[2] Extracts or phytoconstituents of *Canarium* species have demonstrated a wide range of biological effects including hepatoprotective, analgesic, antimicrobial, antihypercholesterolemic, antioxidant, vasorelaxant, antiviral, anti-obesity, antidiabetic, antipyretic, anti-inflammatory, anticancer, α-amylase and α-glucosidase inhibitory activity.[3-13] To the best of our knowledge, *C. resiniferum* has yet to be explored for its phytoconstituents and pharmacological activity.

Anxiety disorders and depression are common mental disorders with symptoms that range from mild to severe. Anxiety disorders are characterised by a feeling of fear, often chronic, in response to the presence of threatening or unfamiliar situations. Depressive disorders are characterized by symptoms such as loss of interest, sadness, sleeplessness, poor appetite, the inability to perform daily tasks, and in severe cases a tendency to commit suicide.[14] Many of the current anxiolytic and antidepressant drugs exhibit undesirable side effects that contribute to poor patient compliance with the treatments.[15,16] This has been associated with an increase in the demand for medicinal plants as safer alternative therapies. Many plants have anxiolytic and/or antidepressant potential and contain diverse phytoconstituents which may be exploited for the development of new drugs to treat these disorders, particularly in cases where patients do not respond to current medications.[17,18] The present study was undertaken to investigate the anxiolytic and antidepressant activity of the methanol extract of *C. resiniferum* (MECR) leaves using behavioral models in mice. As reactive oxygen species play an important role in the pathophysiology of depression and

anxiety,[19,20] we sought to further determine the levels of total phenolics and flavonoids in MECR and investigate the antioxidant/free-radical scavenging potential of this extract.

2. Materials & methods

2.1. Drugs and chemicals

Methanol (MeOH), ferric chloride ($FeCl_3$), aluminum chloride ($AlCl_3$), potassium ferricyanide, sodium carbonate (Na_2CO_3), potassium acetate and phosphate buffer were obtained from Merck (Darmstadt, Germany). Ascorbic acid (AC) and quercetin were obtained from BDH Chemicals Ltd. (Poole, UK). Gallic acid (GA), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), trichloro-acetic acid (TCA) and Folin-Ciocalteu reagent (FCR) were procured from Sigma Chemicals Co. (St. Louis, MO, USA). Diazepam and imipramine hydrochloride were purchased from Gonoshasthaya Pharmaceuticals Ltd (Dhaka, Bangladesh). All residual reagents were of analytical grade.

2.2. Plant material and extract preparation

The leaves of *Canarium resiniferum* (CR) were obtained from the Forest Research Institute, Chittagong, Bangladesh, in September 2019. The proper identification of the plant material was made by Prof. Dr. Shaikh Bokhtear Uddin, Herbarium Department of Botany, University of Chittagong, Bangladesh (accession number: CTGUH SR7925). Fresh and disease-free leaves were washed thoroughly and then left to dry naturally at 25 °C. The dried powdered leaves (500 g) were macerated in 100% MeOH (1.5 L) for 15 days with occasional shaking. Following filtration through cotton and Whatman no. 1 filter paper, the resulting solution was concentrated under

reduced pressure to yield a gummy extract (3.4 g). An aliquot of this extract (10 g) was stored at 4 ºC for further analysis.

2.3. Experimental animals

Swiss albino adult mice of both genders (each weighing ca. 23–30 g) were obtained from Jahangirnagar University, Dhaka, Bangladesh. The animals were acclimatized for a period of 14 days under controlled conditions (temperature: 25 ± 2 ºC; relative humidity: 55–60 %; 12 h light/dim cycle) and were given standard feed and water *ad libitum*. All tests were conducted from 9.00 am to 5.00 pm. Ethical approval for the investigation (Pharm-P&D-147/14-19/P153006) was obtained from by the Ethical Survey Panel and the P&D Board of the Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

2.4. Experimental design

The mice were divided into groups (I -V) (n = 6) containing both male and female animals. Group-I was administrated the vehicle (1% Tween 80 in distilled water, p.o.). Group-II received the standard drug diazepam (1 mg/kg, i.p.) in the elevated plus-maze (EPM) test, the hole-board test (HBT), the light-dark box (LDB) test [21-24] and the standard drug imipramine (1 mg/kg, i.p.) - a tricyclic antidepressant - in the tail suspension test (TST) and the forced swim test (FST). [25,26] The remaining groups III, IV, V were given MECR (100, 200, 400 mg/kg, p.o.), respectively. These doses were selected based on the acute oral toxicity results and were similar to those reported in previous studies examining the anxiolytic and antidepressant of plant extracts. [22,27]

2.5. Acute oral toxicity study

The animals were separated randomly into 4 groups (n = 6) and were kept fasted overnight prior to the experiment. On the day of the experiment, the treated groups were administered MECR (1000, 2000, and 4000 mg/kg, p.o) while the control group received the vehicle orally. The mice were monitored for possible signs and symptoms of toxicity (e.g. sedation, allergic syndromes, motor impairment) over a short period (3 h) followed by a longer period (72 h). The mortality rate was recorded for each group up to 24 h after treatment. [28,29]

2.6. Evaluation of the anxiolytic activity

2.6.1. Elevated plus-maze (EPM) test

The EPM apparatus consisted of four arms, including two open (5 × 35 cm) and two closed (5 × 15 × 30 cm) arms, joint together with a central platform (5 × 5 cm). The maze was placed 60 cm above ground level. The animals from groups I-V were treated 30 min before the test was begun by placing individual animals on the central platform. The time spent in the open arms and the number of entries in the open arms were recorded over a period of 5 min.[30]

2.6.2. Hole-board test (HBT)

The hole-board (HB) test apparatus is a wooden compartment (40 × 40 × 25 cm) with 16 holes each 3 cm in diameter. The animals from groups I-V were treated 30 min before being placed individually on the HB apparatus. The number of head-dips was counted over a 5 min period of observation.[21]

111

112 *2.6.3. Light-dark box (LDB) test*

113 The LDB apparatus was a Plexiglas box with two compartments (each 25 × 25 cm) joint together.
114 One of the compartments was dark and covered with a lid, the other one was brightly lit and open.
115 The two compartments were connected by a 3 cm hole. The animals from groups I-V were treated
116 60 min before being placed individually in the light compartment of the apparatus and allowed to
117 move around. The time that the animals spent in the light and the dark compartments was recorded
118 for a period of 5 min. [22]

119

120 *2.7. Evaluation of the antidepressant activity*

121 *2.7.1. Tail suspension test (TST)*

122 Animals in group I-V were treated 30 min prior to being individually hanged 50 cm above the
123 ground using adhesive tape placed about 1 cm from the tip of their tail and for a period of 6 min.
124 The duration of immobility (in seconds) was recorded for the suspended animals within each
125 group. [25]

126

127 *2.7.2. Forced swim test (FST)*

128 Animals in group I-V were treated 30 min prior to being placed individually for a period of 6 min
129 inside a glass cylindrical chamber (25 cm high ×10 cm diameter) filled with water (up to 19 cm)
130 at a temperature of 23 ± 1 ºC. The duration of immobility (in seconds) of animals that stopped
131 swimming was assessed during the last 4 minutes of the test. [26]

132

133 *2.8 Statistical analysis*

134 The results obtained from the behavioral tests were expressed as the means ± SEM of experiments
135 run in triplicate. One-way analysis of variance (ANOVA), followed by Dunnett's multiple
136 comparisons test, was used to analyse the differences between control and treated groups. *P* values
137 < 0.05 were considered as statistically significant. All statistical analyses were performed using
138 SPSS v. 16.0 and GraphPad Prism v 8.0 (GraphPad Software Inc., San Diego, CA).

139

140 *2.9. Qualitative phytochemical analysis*

141 MECR was subjected to a qualitative phytochemical analysis to identify phytoconstituents such as
142 alkaloids, carbohydrates, proteins, glycosides, phenols, tannins, flavonoids and terpenoids as per
143 standard protocols. [31]

144

145 *2.10. Quantitative phytochemical analysis*

146 *2.10.1. Total phenolic content (TPC)*

147 The total phenolic content of MECR was measured following a standard procedure. [32] An aliquot
148 (0.5 mL) of MECR (1 mg/mL) was mixed with 2.5 mL of FCR (10%, w/v) and 2 mL of Na_2CO_3
149 (7.5%, w/v). The mixture was incubated for 5 min at 50 ºC and then left to cooled down. The
150 absorbance was measured at 760 nm against distilled water as a blank. A standard calibration curve
151 was generated using six concentrations of gallic acid (15.62–500 µg/mL) and TPC was expressed
152 as mg of gallic acid equivalents (GAEs) per g of dried MECR. The test was performed in triplicate.

153

154 *2.10.2. Total flavonoid content (TFC)*

155 The total flavonoid content in MECR was determined using a colorimetric assay.[33] Aluminum
156 chloride (10% w/v, 0.2 mL), potassium acetate (1 M, 0.2 mL), MeOH (3 mL) and distilled water
157 (5.6 mL) were added to either 1 mL of MECR (1 mg/mL) or quercetin (12.5–100 µg/mL). The
158 resulting mixture was incubated for 30 min at 25 ºC and absorbance was measured in a
159 spectrophotometer at 420 nm against distilled water as a blank. The flavonoid content was
160 expressed as mg of quercetin equivalents (QEs) per g of dried MECR. The test was performed in
161 triplicate.

162

163 *2.11. Determination of the antioxidant effect*

164 *2.11.1. DPPH radical scavenging assay*

165 The DPPH assay was carried out according to a previously published protocol.[34] DPPH (0.004%,
166 w/v) (3 mL) was added to MeOH (3 mL) and various concentrations (500-15.625 µg/mL) of
167 MECR. The resulting mixture was left at 25 °C for 30 min, and absorbance was measured in a
168 spectrophotometer at 517 nm against distilled water as a blank. Ascorbic acid (500-15.625 µg/mL)
169 was used as a positive control. The test was performed in triplicate. The percentage of radical
170 scavenging activity was calculated using the following equation:

171 Scavenging % = $(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ = Only DPPH solution
172 and ABS_{sample} = sample (extract or standard) + DPPH solution

173

174 *2.11.2. Ferric reducing antioxidant power (FRAP) assay*

175 The reducing power capacity of MECR was evaluated using a previously described methodology.
176 [35] MECR (1 mL) was sequentially mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5
177 mL of potassium ferricyanide (1%, w/v). This mixture was incubated for 20 min at 50 ºC and then
178 mixed with 2.5 mL of trichloroacetic acid (10%, v/v). Following centrifugation for 10 min at 3000
179 rpm, the upper solution (2.5 mL) was transferred to a test tube and was subsequently mixed with
180 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%, w/v). The absorbance was measured
181 in a spectrophotometer at 700 nm against distilled water as a blank. Ascorbic acid (15.62–
182 500µg/mL) was used as a positive control. The FRAP values were expressed as content of Fe(II)
183 in µM/mg of extract using a standard curve with different concentrations of FeSO4. The test was
184 performed in triplicate.

185

186 **3. Results**

187 *3.1. Acute oral toxicity study*

188 Neither lethal effects nor evidence of behavioral toxicity (i.e. defecation, urination, lacrimation,
189 salivation, pilo-erection, aggressiveness, overactivity, convulsions, tremors, twitches) were
190 observed in animals following the oral administration of MECR at doses of 1000, 2000, and 4000
191 mg/kg. Therefore, MECR was deemed to be safe even at the highest dose level of 4000 mg/kg,
192 and its lethal dose (LD_{50}) was considered be > 4000 mg/kg. On that basis, the doses of extract
193 (100, 200 and 400 mg/kg) chosen for the subsequent in vivo experiments were considered as safe.

194

195 *3.2. Evaluation of the anxiolytic activity*

196 *3.2.1 Effects of MECR in the elevated plus maze (EPM) test*

197 The effects of MECR on the time spent and the number of entries in the open arms in the EPM test
198 are illustrated in Figure 1. Administration of MECR (100, 200, and 400 mg/kg) showed anxiolytic
199 activity by increasing both the time spent and the number of entries in the open arms in a dose-
200 dependent manner. The time spent and the number of entries in the open arms were significantly
201 increased in groups treated with MECR at 200 and 400 mg/kg. At 200 mg/kg, MECR showed a
202 moderate but significant anxiolytic effect in both the time spent (75 ± 2.98 s; $P < 0.001$) and the
203 number of entries (10.33 ± 0.92; $P < 0.01$). At 400 mg/kg, it greatly increased the time spent (97.33
204 ± 4.22 s; $P < 0.001$) and the number of entries (18.83 ± 1.40; $P < 0.001$). Mice treated with 100
205 mg/kg did not manifest significant improvement ($P > 0.05$) in the time spent and number of entries
206 in the open arms. As expected, diazepam at 1 mg/kg (positive control) significantly raised the time
207 spent (93 ± 2.42 s; $P < 0.001$ vs. control group) and number of entries (20 ± 0.82; $P < 0.001$) in
208 the open arms.
209

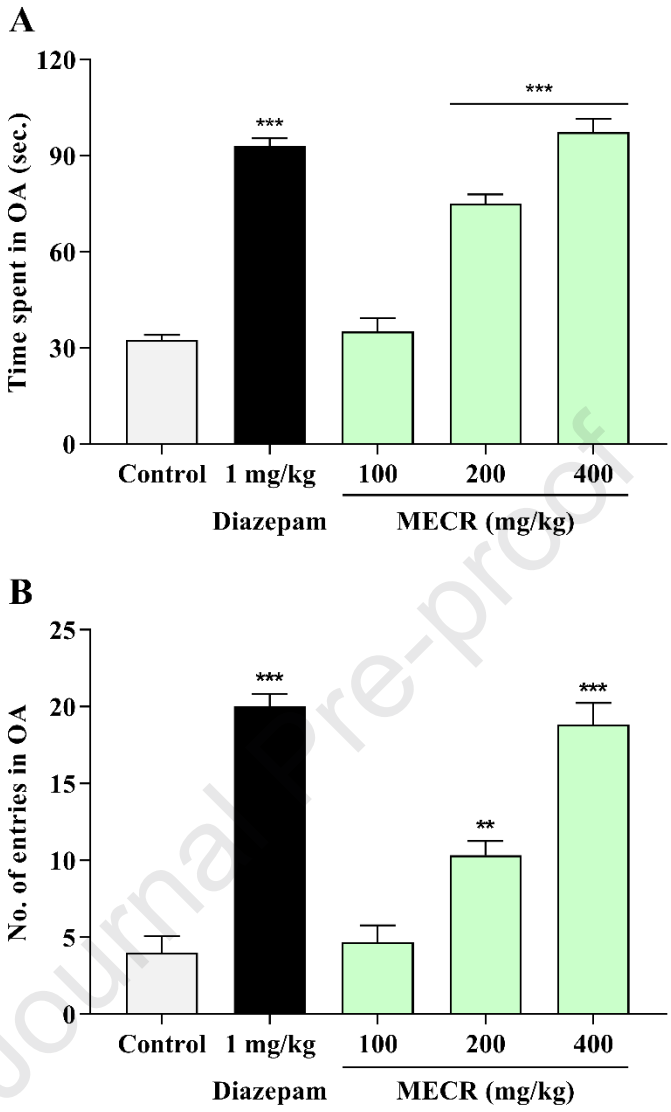
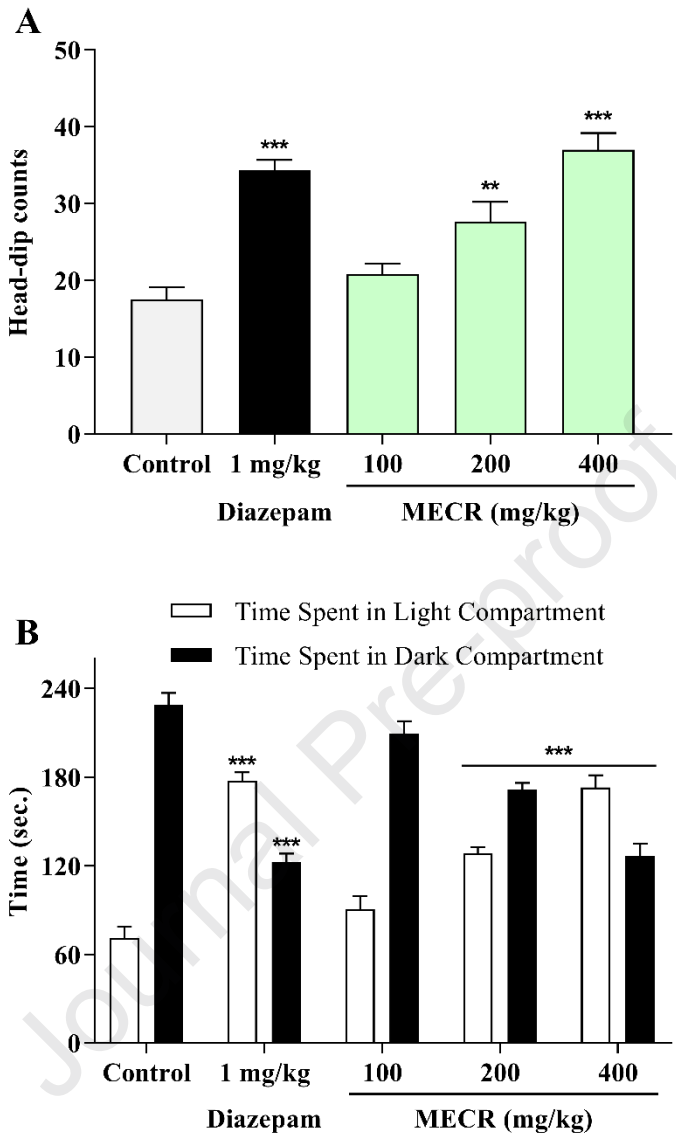


Figure 1. Effects of MECR (100, 200, 400 mg/kg, p.o.) and diazepam (1 mg/kg, p.o.) on the (**A**) time spent in the open arms (in seconds) and (**B**) number of entries in the open arms in the EPM test. Values are presented as means ± SEM ($n = 6$). The data sets were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered significant as compared to the control. MECR, methanol extract of *C. resiniferum* leaves.

219 *3.2.2 Effects of MECR in the hole-board test (HBT)*

220 The effects of MECR on the head-dip counts in the HBT are illustrated in Figure 2A. MECR (at
221 doses of 400 and 200 mg/kg) significantly and dose-dependently increased the number of head-
222 dips by 111.43% (37 ± 2.19; $P < 0.001$) and 58.01% (27.67 ± 2.56; $P < 0.01$), respectively
223 compared to the control group. The extract at a dose of 400 mg/kg showed a head-dip count and
224 % head-dips increase superior to that of the positive control diazepam at 1 mg/kg (34.33 ± 1.33;
225 96.19%). No significant effect was recorded at the dose of 100 mg/kg.

226



227

228 **Figure 2.** Effects of MECR (100, 200, 400 mg/kg, p.o.) and diazepam (1 mg/kg, p.o.) (**A**) on the
229 head-dip counts in the HBT and (**B**) the time spent in the light and the dark compartments in the
230 LDB test. Values are presented as means ± SEM (n = 6) along with the mean % increase in head-
231 dip counts. The data sets were analyzed by one-way ANOVA followed by Dunnett's multiple
232 comparisons test. $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered significant as compared to the
233 control. MECR, methanol extract of *C. resiniferum* leaves.

234

235

3.2.3 Effects of MECR in the light-dark box (LDB) test

The effects of MECR on the time spent in the light and the dark compartments in the LDB test are illustrated in Figure 2B. When compared to the control, MECR at 400 and 200 mg/kg significantly ($P < 0.001$) increased the time spent in the light box (173.05 ± 8.11 and 128.27 ± 4.34 s, respectively) and significantly ($P < 0.001$) decreased the time spent in the dark box (126.95 ± 8.11 and 191.73 ± 4.34 s, respectively). The group of animals treated with 100 mg/kg did not manifest a significant increase/decrease ($P > 0.05$) in the time spent in the light /dark box. In the standard drug diazepam-treated group (1 mg/kg), the time spent in the light and dark box were 177.57 ± 5.64 and 122.43 ± 5.64 s, respectively ($P < 0.001$ vs. control group). The values for the time spent in the light/dark box observed for MECR (400 mg/kg) were comparable to those obtained after administration of the standard drug diazepam.

3.3. Evaluation of the antidepressant activity

3.3.1 Effects of MECR in the tail suspension test (TST) and the forced swim test (FST)

The effects of MECR on the duration of immobility in the TST and FST are illustrated in Figure 3A and B, respectively. In both tests, MECR (100,200,400 mg/kg) dose-dependently reduced the duration of immobility compared to untreated animals (control) and this was significant ($P < 0.001$ vs control) for all doses, except for the dose of 100 mg/kg in the FST model. The standard drug imipramine (1 mg/kg) showed a significant reduction in the duration of immobility ($P < 0.001$ vs control), and the effect of MECR at the highest dose of 400 mg/kg was comparable to that of imipramine in both tests.

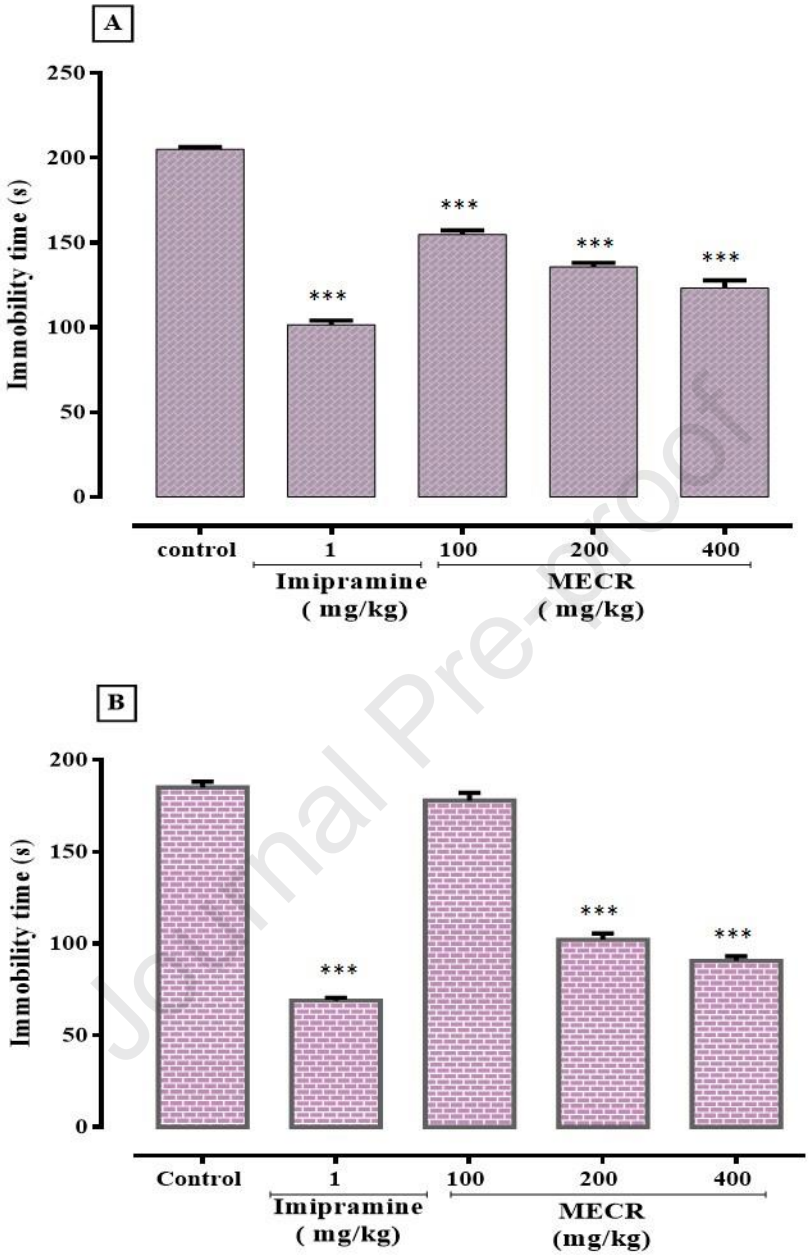


Figure 3. Effects of MECR (100, 200, 400 mg/kg, p.o.) and imipramine (1 mg/kg, i.p.) on the duration of immobility (**A**) in the TST and (**B**) in the FST. Values are presented as means ± SEM (n = 6). The data sets were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. ***$P < 0.001$ was considered significant as compared to the control. MECR, methanol extract of *C. resiniferum* leaves.

265 *3.4. Qualitative phytochemical analysis*

266 Preliminary phytochemical profiling of MECR revealed the presence of alkaloids, carbohydrates,
267 proteins, phenols, tannins, and flavonoids (Table 1).

268

269 **Table 1.** Preliminary phytochemical screening of MECR

Phytoconstituents	Test performed	Observations
Alkaloids	Mayer's test	+
	Wagner's test	+
Carbohydrates	Benedict's test	+
	Molisch's test	+
Proteins	Biuret test	+
Glycosides	Borntrager's test	-
Phenols	Ferric Chloride test	+
Tannins	Gelatin test	+
Flavonoids	Alkaline Reagent test	+
Terpenoids	Salkowski test	-

270 +/- sign indicates presence/absence of the phytoconstituent.

271

272 *3.5. Determination of TPC, TFC, antioxidant activity*

273 The total phenolic and flavonoid content of MECR were determined as 90.94 ± 0.75 mg GAE/g
274 and 51.54 ± 0.78 mg QE/g of dried extract, respectively (Table 2).

275

276

277

278

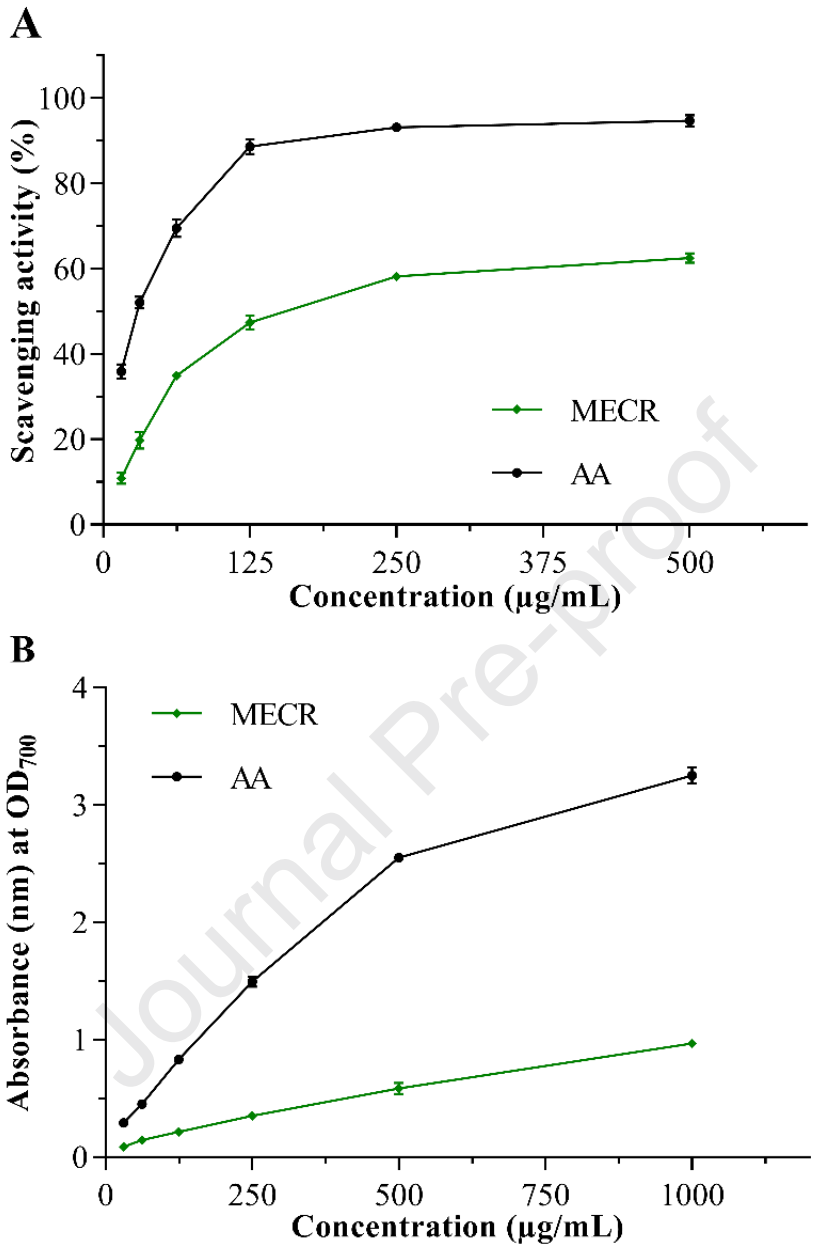
279

Table 2. Total phenolic content (TPC), total flavonoid content (TPC), and radical scavenging activity of MECR

	TPC (GAE in mg /g of dry extract)	TFC (mg QE/g of dry extract)	DPPH Assay IC_{50} (µg/mL)
MECR	90.94 ± 0.75	51.54 ± 0.78	177.82 ± 2.77
AA	-	-	25.62 ± 0.68

Values are expressed as mean ± SEM (n = 3). MECR, methanol extract of *C. resiniferum* leaves; AA, ascorbic acid; GAE, gallic acid equivalent; QE, quercetin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; -: not assessed.

In the DPPH assay, MECR showed concentration-dependent radical scavenging activity, with the maximum percentage (62.44%) recorded at the highest concentration (500 µg/mL). The IC_{50} value obtained for MECR (177.82 ± 2.77 µg/mL) was higher than that of the standard ascorbic acid (IC_{50} value of 25.62 ± 0.68 µg/mL) (Figure 4A). In the ferric reducing antioxidant power (FRAP) assay, MECR demonstrated concentration-dependent reducing capability compared to ascorbic acid. At 1000 µg/mL, the absorbance of MECR and ascorbic acid were 0.969 and 3.251, respectively (Figure 4B).



295

296 **Figure 4.** Antioxidant activity of MECR in the DPPH free radical scavenging and ferric reducing
297 antioxidant power (FRAP) assay. (**A**) % DPPH free radical scavenging activity of MECR and
298 ascorbic acid at different concentrations. (**B**) Ferric reducing power capacity of MECR and
299 ascorbic acid at different concentrations. Values are presented as means ± SEM (n = 3). MECR,
300 methanol extract of *C. resiniferum* leaves; AA, ascorbic acid.

301

4. Discussion

Anxiety and depressive disorders are common mental disorders that are often experienced simultaneously and that can severely impair the quality of life of sufferers.[14] In this study, the effects of MECR on anxiety-related behavior were assessed in mice using the EPM, HBT, and LDB tests; three behavioral models widely used to investigate the anxiolytic potential of drugs, including plant-based ones. [36,37] The EPM test is one of the most commonly used animal models for testing anxiety-related behavior. It is based on the fact that elevated and open sections of the maze trigger fear and anxiety in rodents which in turn tend to avoid spending time in these places and prefer safer (closed arm) sections. Treatment with an anxiolytic agent encourages exploratory behaviour and increases the time spent and the number of entries in the open arms of the maze.[38] In the present study, MECR (200 and 400 mg/kg) as well as diazepam (1 mg/kg) showed significant anxiolytic activity by increasing both the time spent and the number of entries of treated animals in the open arms. In the HBD test, the degree of anxiety in animals is assessed by observing head-dipping behavior, with anxiolytic drugs triggering an increase in the head-dip counts.[23] In this study, MECR (at doses of 400 and 200 mg/kg) significantly increased the number of head-dips compared to the control group, and at 400 mg/kg showed a head-dip count and % head-dips increase superior to that of the standard drug diazepam. The anxiolytic effect of MECR was further investigated using the LDB test. The LDB apparatus comprises of a dark (safe) compartment and a bright (aversive) compartment. The LDB test relies on the inherent aversion of rodents to bright areas, with anxiolytic drugs increasing the time spent by animals in the light compartment rather than the dark one. [24] MECR at 400 and 200 mg/kg significantly increased the time spent in the light box and significantly decreased the time spent in the dark box. The effects observed for MECR at 400 mg/kg were comparable to those obtained after administration of diazepam. MECR

at the dose of 400 mg/kg only showed significant anxiolytic activity compared to untreated animals in all three tests.

The antidepressant activity of MECR was evaluated in mice using the well-established TST and FST behavioral models. When animals are placed under stressful conditions (i.e. inescapable positions), they tend to remain immobile for a long duration. This state of immobility is a reflection of an inability to adjust to the stressful situation, despair or loss of hope to be able to escape. Such behavior closely resembles what is observed in depression. [39,40] Treatment with an antidepressant leads to a decrease in the duration of immobility. In the present study, MECR significantly reduced the duration of immobility compared to untreated animals for all doses, except for the dose of 100 mg/kg in the FST model. The effect of MECR at 400 mg/kg was comparable to that of the standard drug imipramine in both tests.

Several mechanisms have been proposed to explain the pathogenesis of anxiety and depression. The latter has been linked to a dysregulation of the neurotransmitter systems (mainly serotonin and norepinephrine) in the CNS, with antidepressants like selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs) inhibiting the re-uptake of these neurotransmitters, and monoamine oxidase inhibitors (MAOIs) inhibiting their degradation. [41-44] It has also been linked with excessive activation of the hypothalamic-pituitary-adrenal axis which stimulates neurons to discharge the stress-related neuropeptide corticotropin-releasing factor.[45] Other, more recent, studies have highlighted the role of oxidative stress/damage in depression as well as anxiety disorders.[19,20] Natural products such as polyphenols and flavonoids are well-known for their free radical scavenging/antioxidant activity.[46,47] The DPPH and the FRAP assays are two colorimetric in vitro tests that are commonly employed to measure the free-radical scavenging activity and the reducing power of antioxidant drugs, respectively.[48] In the present

investigation, MECR showed high radical scavenging activity in the DPPH assay as well as some ferric reducing antioxidant power. This may be attributable to the high total phenolic and total flavonoid contents of MECR.

Qualitative phytochemical analysis showed that MECR contained a range of structurally-diverse secondary metabolites, including alkaloids, tannins, phenols, and flavonoids. Previous studies have demonstrated that alkaloids, flavonoids, and phenols had anxiolytic activity owing to their high affinity for the benzodiazepine (BZD)-binding site of $GABA_A$ receptors.[49] Gamma-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in the central nervous system (CNS). The binding of BZDs to $GABA_A$ receptors increases the opening of the linked chloride channel, leading to neuronal membrane hyperpolarization and anxiolytic activity.[50] Other studies reported that plants rich in total phenolics and tannins could exert beneficial effects in anxiety and depression via upregulating the expression of $GABA_A$ and $5\text{-}HT_{1A}$ receptors, serotonin, norepinephrine, dopamine, brain-derived neurotrophic factor, cAMP response element-binding protein, and reducing serum cortisol levels in animals.[51,52]

5. Conclusion

The above results revealed the lack of acute oral toxicity (up to a dose of 4000 mg/kg) and significant anxiolytic and antidepressant activity (at a dose of 400 mg/kg) of the methanol extract of *C. resiniferum* leaves in mice. They also showed that this extract was rich in phenolic compounds, including flavonoids, and possessed a high free radical scavenging effect *in vitro*. This suggests that *C. resiniferum* leaves may represent an alternative treatment for anxiety and depressive disorders at a human equivalent dose (HED) of 32.5 mg/kg, and one that would be safe (up to HED 325 mg/kg).[53] Further investigations are warranted to link the anxiolytic/antidepressant

activity of MECR to the presence of individual bioactive phytoconstituent(s). The antioxidant phenolics/flavonoids, or other phytoconstituents with a separate mechanism of action, in MECR may serve as templates for the development of new treatments for depression and/or anxiety in the future.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

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

References

1. World Flora Online. 2021. http://www.worldfloraonline.org. Accessed on: 01 Dec 2021
2. Nahar S, Rahmatullah M. Plants, animals, birds, insects, minerals–all are medicines to a folk medicinal practitioner in Nilphamari district, Bangladesh. *World J Pharm Pharm Sci.* 2016;5(4):2422−2439.
3. Mogana R, Wiart C. *Canarium* L. : A Phytochemical and Pharmacological Review. *J Pharm Res.* 2011;4(8):2482–2489.

4. Kao Y, Yeh Y, Chiang A, Hsieh S. Study on Mechanisms Underlying the Preventive Effects of *Canarium album* L. ethanol Extract on Modulation of Hyperglycemia and Hypercholesterolemia in Diabetic Rats. *J Food Nutr Res.* 2018;6:261–270.
5. Kadir N, Azlan A, Abas F, Ismail IS. Effect of Defatted Dabai Pulp Extract in Urine Metabolomics of Hypercholesterolemic Rats. *Nutrients.* 2020;12(11):3511.
6. Quan, NV, Xuan TD, Tran HD, Thuy N, Trang LT, Huong CT, Andriana Y, Tuyen PT. Antioxidant, α-Amylase and α-Glucosidase Inhibitory Activities and Potential Constituents of *Canarium tramdenum* Bark. *Molecules* 2019;24(3):605.
7. Wu J, Fang X, Yuan Y, Dong Y, Liang Y, Xie Q, Ban J, Chen Y, Lv Z. UPLC/Q-TOF-MS profiling of phenolics from *Canarium pimela* leaves and its vasorelaxant and antioxidant activities. *Rev Bras Farmacogn.* 2017;27(6):716–723.
8. Chen F, Yang L, Zhai L, Huang Y, Chen F, Duan W, Yang J. Methyl brevifolincarboxylate, a novel influenza virus PB2 inhibitor from *Canarium Album* (Lour.) Raeusch. *Chem Biol Drug Des. 2020;*96*:*1280–1291.
9. Yeh YT, Lu TJ, Lian GT, et al. Chinese olive (*Canarium album* L.) fruit regulates glucose utilization by activating AMP-activated protein kinase. *FASEB J.* 2020;34(6):7866–7884.
10. Venkatachalapathi A, Abdul Kaffoor H, Paulsamy S. Antipyretic activity of methanolic leaf extract of *Canarium strictum* Roxb. *J Ayurvedic Herb Med*. 2017;3(2):60–62.
11. Choi E, Kim MY, Cho JY. Anti-inflammatory activities of *Canarium subulatum* Guillaumin methanol extract operate by targeting Src and Syk in the NF-κB pathway. *J Ethnopharmacol*. 2019;238:111848.

12. Basri DF, Alamin ZA, Chan KM. Assessment of cytotoxicity and genotoxicity of stem bark extracts from *Canarium odontophyllum* Miq. (dabai) against HCT 116 human colorectal cancer cell line. *BMC Complement Altern Med*. 2016;16:36.
13. Cajuday LA, Membreve DMS, Serrano JE. Evaluation of the antioxidant and anticancer activities of *Canarium ovatum* (Burseraceae) pulp extracts. *Int J Biosci.* 2017;11(3):247–256.
14. World Health Organization. Depression and other common mental disorders: global health estimates (No. WHO/MSD/MER/2017.2). 2017. World Health Organization, Geneva, Switzerland.
15. Wang SM, Han C, Bahk WM, Lee SJ, Patkar AA, Masand PS, Pae CU. Addressing the side effects of contemporary antidepressant drugs: A comprehensive review. *Chonnam Med J.* 2018;54:101–112.
16. Edinoff AN, Nix CA, Hollier J, Sagrera CE, Delacroix BM, Abubakar T, Cornett EM, Kaye AM, Kaye AD. Benzodiazepines: Uses, Dangers, and Clinical Considerations. *Neurol. Int.* 2021;13(4):594–607.
17. Sarris J, Panossian A, Schweitzer I, Stough C, Scholey, A. Herbal medicine for depression, anxiety and insomnia: A review of psychopharmacology and clinical evidence. *Eur Neuropsychopharmacol.* 2011;21(12):841–860.
18. García-Ríos RI, Mora-Perez A, Ramos-Molina AR, Soria-Fregozo C. Neuropharmacology of Secondary Metabolites from Plants with Anxiolytic and Antidepressant Properties. In: Rodriguez-Landa JF, Cueto-Escobedo J, eds. Behavioral Pharmacology-From Basic to Clinical Research. London, UK: IntechOpen Ltd; 2020:685–471.

19. Fedoce A, Ferreira F, Bota RG, Bonet-Costa V, Sun PY, Davies K. The role of oxidative stress in anxiety disorder: cause or consequence?. *Free Radic Res.* 2018;52(7):737–750.
20. Bhatt S, Nagappa AN, Patil CR. Role of oxidative stress in depression. *Drug Discov* Today. 2020;25(7):1270–1276.
21. Sonavane GS, Sarveiya VP, Kasture VS, Kasture SB. Anxiogenic activity of *Myristica fragrans* seeds. *Pharmacol Biochem Behav.* 2002;71(1-2):239–244.
22. Jain NN, Ohal CC, Shroff SK, Bhutada RH, Somani RS, Kasture VS, Kasture SB. *Clitoria ternatea* and the CNS. *Pharmacol Biochem Behav.* 2003;75(3):529–536.
23. Takeda H, Tsuji M, Matsumiya T. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. *Eur J Pharmacol.* 1998;350(1):21–29.
24. Bourin M, Hascoët M. The mouse light/dark box test. *Eur J Pharmacol.* 2003;463(1-3):55–65.
25. Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacol.* 1985;85(3):367–370.
26. Porsolt RD, Anton G, Blavet N, Jalfre M. Behavioural despair in rats: A new model sensitive to antidepressant treatments. *Eur J Pharmacol.* 1978;47(4):379–391.
27. Bajaj J, Dwivedi J, Sahu R, et al. Antidepressant activity of *Spathodea campanulata* in mice and predictive affinity of spatheosides towards type A monoamine oxidase. *Cell Mol Biol (Noisy-le-grand)*. 2021;67(1):1-8.
28. Shilpi JA, Taufiq-Ur-Rahman M, Uddin SJ, Alam MS, Sadhu SK, Seidel V. Preliminary pharmacological screening of *Bixa orellana* L. leaves. *J Ethnopharmacol.* 2006;108(2):264–271.

29. Kifayatullah M, Mustafa MS, Sengupta P, Sarker MMR, Das A, Das SK. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of *Pericampylus glaucus* (Lam.) Merr. in BALB/c mice. *J. Acut. Dis.* 2015;4(4):309–315.

30. Pellow S, File SE. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol Biochem Behav.* 1986;24(3):525–529.

31. Tiwari P, Kaur M, Kaur H. Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia.* 2011;1:98–106.

32. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Meth Enzymol.* 1999;299: 152–178.

33. Skerget M, Kotnik P, Hadolin M, Hraš AR, Simonič M, Knez Ž. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem.* 2005;89(2):191–198.

34. Braca A, De Tommassi N, Di Bari L, Pizza C, Politi M, Morelli I. Antioxidant principles from *Bauhinia t arapotensis. J Nat Prod.* 2001;64(7):892–895.

35. Ojwang RA, Muge EK, Mbatia BN, Mwanza B, Ogoyi DO. Comparative Analysis of Phytochemical Composition and Antioxidant Activities of Methanolic Extracts of Leaves, Roots and Bark of Jackfruit (*Artocapus heterophyllus*) from Selected Regions in Kenya and Uganda. *J Adv Biol Biotechnol.* 2017;16:1–13.

36. Ortiz-Mendoza N, Zavala-Ocampo LM, Martínez-Gordillo MJ, González-Trujano ME, Peña F, Rodríguez I, Chávez J, Dorazco-González A, Aguirre-Hernández E.

Antinociceptive and anxiolytic-like effects of a neo-clerodane diterpene from *Salvia semiatrata* aerial parts. *Pharm Biol.* 2020;58(1):620–629.

37. Zhou X, Hassan W, Bakht S, Hussain K, Ahmed, H. Abutilon indicum Exhibits Anxiolytic and Antidepressant Effects in Mice Models. *Doklady. Biochem Biophys.* 2021;500(1):341–346.
38. Walf A, Frye C. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc.* 2007;2:322–328.
39. Can A, Dao DT, Terrillion CE, Piantadosi SC, Bhat S, Gould TD. The tail suspension test. *J Vis Exp.* 2012;59:e3769.
40. Yankelevitch-Yahav R, Franko M, Huly A, Doron R. The forced swim test as a model of depressive-like behavior. *J Vis Exp.* 2015;97:52587.
41. Ressler KJ, Nemeroff CB. Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. *Depress Anxiety.* 2000;12 Suppl 1:2–19.
42. Sansone RA, Sansone LA. Serotonin norepinephrine reuptake inhibitors: a pharmacological comparison. *Innov. Clin Neurosci.* 2014;11(3-4):37–42.
43. Clevenger SS, Malhotra D, Dang J, Vanle B, IsHak WW. The role of selective serotonin reuptake inhibitors in preventing relapse of major depressive disorder. *Ther Adv Psychopharmacol.* 2018;8(1):49–58.
44. Naoi M, Maruyama W, Shamoto-Nagai M. Type A monoamine oxidase and serotonin are coordinately involved in depressive disorders: from neurotransmitter imbalance to impaired neurogenesis. *J Neural Transm.* 2018;125:53–66.

503 45. Jiang Y, Peng T, Gaur U, Silva M, Little P, Chen Z, Qiu W, Zhang Y, Zheng W. Role of
504 Corticotropin Releasing Factor in the Neuroimmune Mechanisms of Depression:
505 Examination of Current Pharmaceutical and Herbal Therapies. *Front Cell Neurosci.*
506 2019;13: 290.
507 46. Ozgen M, Scheerens JC, Reese RN, Miller RA. Total phenolic, anthocyanin contents and
508 antioxidant capacity of selected elderberry (*Sambucus canadensis* L.)
509 accessions. *Pharmacogn Mag.* 2010;6(23):198–203.
510 47. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction
511 Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules.*
512 2016;21(7):901.
513 48. Gulcin İ. Antioxidants and antioxidant methods: an updated overview. *Arch Toxicol.*
514 2020; 94(3):651–715.
515 49. Fedotova J, Kubatka P, Büsselberg D, Shleikin AG, Caprnda M, Dragasek J, Rodrigo L,
516 Pohanka M, Gasparova I, Nosal V, Opatrilova R, Qaradakhi T, Zulli A, Kruzliak P.
517 Therapeutical strategies for anxiety and anxiety-like disorders using plant-derived natural
518 compounds and plant extracts. *Biomed Pharmacother.* 2017;95:437–446.
519 50. Haefely W. Benzodiazepine interactions with GABA receptors. *Neurosci Lett.*
520 1984;47(3):201–206.
521 51. Chandrasekhar Y, Phani Kumar G, Navya K, Ramya EM, Anilakumar KR. Tannins from
522 *Terminalia chebula* fruits attenuates GABA antagonist-induced anxiety-like behaviour
523 via modulation of neurotransmitters. *J Pharm Pharmacol.* 2018;70(12):1662–1674.
524 52. Chandrasekhar Y, Ramya EM, Navya K, Phani Kumar G, Anilakumar KR.
525 Antidepressant like effects of hydrolysable tannins of *Terminalia catappa* leaf extract via

modulation of hippocampal plasticity and regulation of monoamine neurotransmitters subjected to chronic mild stress (CMS). *Biomed Pharmacother.* 2017;86:414–425.

53. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm.* 2016;7(2):27–31.

Declaration of competing interest

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