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# Anxiolytic-*like* effect of succinic acid: A possible GABAergic intervention

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A B S T R A C T

In the modern era, physical and mental stress lead to anxiety at an alarming rate. Therefore, searching for new lead compounds that deal with anxiety has a practical demand. Our study aimed to evaluate succinic acid's (SUC) possibility of managing anxiety through *in vivo* and *in silico* studies. For this, SUC (5, 10, and 15) mg/kg were administered orally (p.o.) to the adult male *Swiss* mice, followed by several studies like open field, swing, hole cross, and light-dark residence tests. The GABAergic agonist diazepam (DZP-2 mg/kg) and an antagonist such as caffeine (CAF-10 mg/kg) were also studied in combination with the SUC group. In contrast to the DZP group, SUC significantly ($p < 0.0001$) increased the amount of time that animals spent in the light, suggesting that it may have relaxing impacts on mice. Additionally, *in silico* studies were carried out to understand the interaction between SUC and $GABA_A$ (α1, β2, and γ2) receptor subunits. Furthermore, pharmacokinetic and drug-like properties were examined by pkCSM and SwissADME online servers and found to be supportive of considering SUC as a potential drug candidate. Our experiment found that SUC-10 had an anxiolytic-like effect on the animals. SUC-10, when combined with DZP, produced more significant ($p < 0.0001$) anxiolytic effects than their individual groups, suggesting possible synergistic effects with this commonly used anxiolytic drug. SUC-10 also altered CAF-mediated effects in mice. An *in silico* study demonstrates that SUC has good interaction capacity with $GABA_A$ receptor subunits. Taken together, SUC-10 exerted anxiolytic-like effects in Swiss mice. It may act synergistically with DZP while acting antagonistically with CAF, possibly through GABAergic interaction pathways.

## 1. Introduction

Anxiety was identified by Greco-Roman philosophers and physicians as a distinct negative phenomenon and a separate medical condition (Crocq, 2022). Anxiety disorder is a mental condition that has an impact on a person's actions in the areas of thought, feeling, and behavior. The typical clinical signs include heart palpitations, dry mouth, tight muscles, headaches, perspiration, and emotional distress (Lin et al., 2021). Around 25% of people worldwide, across all age categories, suffer from anxiety disorders, which are the most common mental diseases and have significant societal and economic implications (Hacke et al., 2020; Stein, Scott, DeJonge, & Kessler, 2022). Most patients do not experience complete remission after receiving the standard pharmaceutical therapies for anxiolytic disorders since they frequently have negative side effects and have poor effectiveness in 40%–60% of patients (Souza et al., 2022). Therefore, it is crucial to create new treatment plans for anxiety disorders (see Scheme 1).

Gamma-aminobutyric acid (GABA), a suppressive neurotransmitter, is evidently decreased in the brain during anxiety (Meyerhoff et al., 2014). There is not much information on GABA toxicity and overdosing. According to the data, there have been no major adverse effects linked to GABA at doses up to 18 g per day for four days and 120 mg per day for 12

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weeks in longer investigations. A burning feeling in the throat and low blood pressure, however, may occur with large daily dosages of GABA (5–10 g). The maximum dosage that is commonly advised is 3 g per day, taken in doses of no more than 750 mg (Oketch-Rabah et al., 2021).

Benzodiazepines, or BZD (e.g., diazepam, lorazepam, and alprazolam), are used as anxiolytics, sedatives, hypnotics, muscle relaxants, and anticonvulsants (Hozack et al., 2022). BZD is among the drugs that are most frequently given globally because of their qualities and efficacy. As a result, they are also among the drugs that are most frequently misused (Kang et al., 2018). The combination of BZD and alcohol is particularly risky since it can significantly increase drowsiness, which raises the risk of home- or work-related injuries (Persona et al., 2015). Most of the time, when overdosed, they only exhibit mild to moderate indicators of toxicity (Bhuia et al., 2023), and they also have a very high therapeutic index (Zamani et al., 2022). However, these drugs have side effects, which include confusion, dizziness, drowsiness, unsteadiness, memory impairment, irritability, and aggression (Magro et al., 2016). Additionally, other medications used to treat neurological diseases included tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), and selective serotonin reuptake inhibitors (SSRIs). However, patients did not tolerate these medications well and they had negative side effects (Edinoff et al., 2021). On this occasion, complementary medicines based on naturally occurring bioactive substances might be a hopeful option to manage anxiety and anxiety disorders (Bhuia et al., 2023; Islam et al., 2022).

Diazepam (DZP), a common medication from the benzodiazepine class, specifically interacts with the $GABA_A$ receptor (Richter et al., 2012), which results in the inhibition of neurotransmission (Matsumoto et al., 2007) through a positive allosteric modulation. The $GABA_A$ receptor complex is made up of several different subunits, including the α, β, and γ subunits (Olsen, 2018). Among these subunits, α and γ serve as the main binding sites for benzodiazepines, including DZP (Sieghart et al., 2012). When DZP binds to the benzodiazepine binding site, the $GABA_A$ receptor undergoes conformational modifications, resulting in an increase in chloride ion channel opening and, thus, enhanced inhibitory neurotransmission (Jazvinscak Jembrek & Vlainic, 2015), which causes sedative, anxiolytic, muscle-relaxant, and anticonvulsant effects (Akkol et al., 2020).

Interaction of GABA with the $GABA_A$ receptor results in conformational changes in the receptor (Knoflach & Bertrand, 2021), which opens the $GABA_A$ channel, thereby causing Cl-influx (Sallard et al., 2021; Nawafleh et al., 2022). It causes the neuronal membrane to become hyperpolarized, which makes it more challenging for the neuron to produce an action potential and lowers its excitability (Pressey et al., 2023). It is evident that the $GABA_A$ receptor has ion channel selectivity, especially for $Cl^-$, which means that it largely permits the passage of Cl-. The unique subunit composition and structural characteristics of the $GABA_A$ receptor dictate this selectivity (Farrant & Kaila, 2007).

Succinic acid (SUC), which is also called butanedioic acid, 1,2-ethanedicarboxylic acid, and amber acid (Nghiem et al., 2017), can be found in nature either in its pure form or in a number of its derivatives. SUC is one of the naturally occurring acids that can be identified in foods including broccoli, rhubarb, sugar beets, fresh meat extracts, different cheeses, and sauerkraut (Featherstone, 2015). SUC is present in high concentrations in natural Baltic amber, up to 8% by weight (Latos-Brozio & Masek, 2021). Plants are a potential source of a wide range of chemicals (Zaman et al., 2022), which are the active components of many drugs used to treat a wide range of disorders (Islam et al., 2023; Jannah et al., 2023). Moreover, SUC has diverse bioactivities, including anti-bacterial (Feng et al., 2010), antioxidant (Zarubina et al., 2012), neuroprotective (Safonova et al., 2015), cardioprotective (Wang et al., 2020), and anti-thrombotic activity (Zhang et al., 2014). Some previous reports suggest that SUC has an anxiolytic effect in experimental animals (Chen et al., 2003; Pozdnyakov et al., 2021; Volchegorskii et al., 2015, 2016), and it may reduce seizures by interacting with the $GABA_A$ receptor (Zhang et al., 2020).

In the present investigation, we used open-field, hole-cross, swing, and light-dark box tests on Swiss albino mice to evaluate the possible anxiolytic impact of SUC. In order to determine if $GABA_A$ receptors could be involved in SUC's anxiolytic effects on experimental animals, we also paired it with and/or without $GABA_A$ receptor agonists or antagonists. The interaction between SUC and GABA receptors was also studied computationally to see whether it had anxiolytic-like effects in experimental animals.

## 2. Material and methods

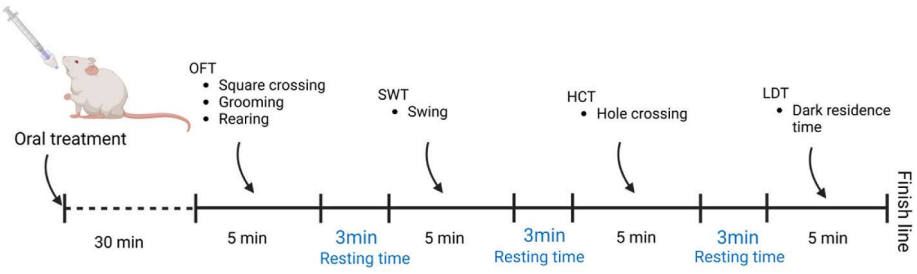
### 2.1. In vivo (animal) study

#### 2.1.1. Chemicals and reagents

Succinic acid (SUC) and 65% ethanol were purchased from Loba Chemie Pvt. Ltd., Mumbai, Maharashtra 400005, India. The sources of diazepam (DZP) and caffeine (CAF) were provided by Opsonin Pharma Ltd., Barishal, Bangladesh, and Incepta Pharmaceuticals Ltd., Dhaka, Bangladesh, respectively.

#### 2.1.2. Experimental animals

Male *Swiss* albino mice (weighing 24–30 g) were obtained from the animal house of Jahangirnagar University, Bangladesh, and were maintained at the Pharmacology Lab of Bangabandhu Sheikh Mujibur Rahman Science and Technology University (BSMRSTU), Gopalganj, for the present study. The animals were allowed free access to standard food and water, *ad libitum*. They were kept under controlled lighting (12-h dark/light cycle) at 27 ± 2 °C until the test commenced. The present experiment was performed from 9:00 a.m. to 3:00 p.m., and those animals were observed for 17 h to check their possible mortality after the study. Experimental design and procedures were approved by the Department of Pharmacy at the BSMRSTU (#bsmrstu16-11/22).




OFT: Open Field Test; HCT: Hole Cross Test; SWT: Swing Test; LDT: Light Dark Test

**Scheme 1.** Study outline for the anxiolytic test in *Swiss* albino mice.

#### 2.1.3. Study design

Animals used in experiments were fasted for 6 h prior to the test. The animals were then randomly divided into experimental and control groups with six animals each. The animals were separated into nine groups, which were designated as Gr.-I to Gr.-IX in brief. Table 1 lists the groups receiving treatment. Each group was given a negative control and a positive control, as well as several medications and medication combinations. Based on the weight of each mouse, the dosages of the sample substance and the control medications were adjusted.

SUC, DZP, and CAF were all regenerated in the vehicle (NC). The NC (10 ml/kg), DZP (2 mg/kg) (Rakhshandah et al., 2022), and CAF (10 mg/kg) (Basu Mallik et al., 2022) were administered to the first three groups (i.e., Gr.-I, Gr.-II, and Gr.-III) respectively. Then the next three groups (Gr.-IV to Gr.-VI) were treated with SUC at 15, 10, and 5 mg/kg, respectively (Chen et al., 2003). The animals were given treatments 30 min before the studies. DZP-2 was combined with CAF-10 and denoted as Gr.-VII and SUC-10 was denoted as Gr.-VIII. Furthermore, SUC-10 was combined with CAF-10 and marked as Gr.-IX to find out the combined activity of the test sample (SUC). All the treatments were given orally (Table 1).

#### 2.1.4. Open-field test

In this test, a wooded open-field portion with a pointed square floor ($30 \times 30 \times 30$ $cm^3$) was used, using the provided methodology, to evaluate the animals' curiosity activities over a 5-min period (Tan et al., 2011). Counts of squares crossed by each mouse's spontaneous movement activity, grooming routines, and observations of its environment (upright position) were documented. After each experiment, the floor of the apparatus was cleaned with 65% ethanol.

#### 2.1.5. Swing test

This study was conducted according to the method developed and described by Islam et al. (2014). After receiving the treatments under research, the test subjects were placed in the swing apparatus, and the number of swings generated by the regular movements of each subject within the device was tallied. The floor of the apparatus was cleaned with 65% ethyl alcohol following each test.

#### 2.1.6. Hole-cross test

The process was carried out exactly as described in Reference (de Almeida et al., 2012). In this experiment, we used a wooden barrier that was positioned in the center of a cage that measured $30 \times 20 \times 14$ $cm^3$. A 3-cm diameter hole was drilled in the lowest part of the dividing board of the cage. After a 3-min swing test, every mouse was promptly placed on one end of the hole-board gadget. The mice were visible, using the hole to freely move between rooms for 5 min. The floor of the apparatus was thoroughly cleaned after each test.

#### 2.1.7. Light-dark test

The experimental apparatus was made of wood, divided into two compartments (the light chamber and the dark chamber), and joined by a little door (Subhan et al., 2008). The lightbox ($27 \times 18 \times 29$ $cm^3$) is brighter than the dark box (black portion: 271,829 $cm^3$) and is illuminated by ambient light. Immediately following a 3-min hole-cross test, each mouse was placed on one end of the light-dark equipment. Each animal's time (in seconds) in the dark and light was counted over the course of 5 min using a stopwatch. The equipment's floor was thoroughly cleaned after each test.

#### 2.1.8. Statistical analysis

The results are arranged as mean ± S.E.M. (standard error of the mean) or percentile values. An analysis of variance (ANOVA) was followed by *t*-Student–Newman–Keuls's posthoc test using the statistical software GraphPad Prism v9.5 (Bappi et al., 2023), (GraphPad Software LLC, accessed on November 18, 2022), and we compared the experimental groups with the vehicle (control) group. At 95% confidence intervals, P values ($p < 0.05$) were regarded as significant (Habib et al., 2017), and p values ($p < 0.0001$) were regarded as most significant.

### 2.2. In silico studies

#### 2.2.1. GABA homology model and macromolecule

The SWISS-MODEL online server (https://swissmodel.expasy.org/interactive) was used to generate the homology of gamma-aminobutyric acid (GABA) subunits (Karim et al., 2021). Prior to creating the actual models, the sequence of $GABA_A$ (α1, β2, and γ2) was received through the UniProt protein database (UniProt accession : P14867, P47870, and P18507) (UniProt Consortium, 2015), and the NCBI BLAST (Kishk et al., 2020) tools was used to conduct a BLAST analysis to select the template. PROCHECK was employed for tasks like the Homology Model's verification (Park et al., 2022). The interaction pathwayof GABA was studied using the computational approaches of SUC, CAF, and DZP molecules.

*2.2.1.1. Ligand preparation.* The Swiss-PDB Viewer software program v4.1.0 (accessed on July 11, 2021) was utilized to minimize the energy consumption of the crystal structure before docking (Rana et al., 2021). Moreover, the chemical formulae of succinic acid (PubChem ID: 1110), caffeine (PubChem ID: 2519), and diazepam (PubChem ID: 3016) have all been provided (PubChem ID: 2519). The internal energies of each ligand were all optimized using the Chem3D Pro21.0 (PerkinElmer Informatics, Inc., accessed on July 7, 2022) software (Wang et al., 2022).

*2.2.1.2. Docking protocol.* A computerized drug design method in drug discovery is docking study simulation. Through the examination and placement of molecules at specific binding sites, the PyRx v0.8 (The Scripps research institute, accessed on January 2, 2023) virtual analysis approach is used to evaluate the pharmacodynamic characteristics of an active medication (ligand) (Abdullahi & Adeniji, 2020). The results of docking indicate the degree of binding to a target molecule's catalytic site. Pymol v1.7.4.5 Edu and BIOVIA Discovery Studio v21.1.0 (Dassault Systemes, accessed on April 5, 2022) are used to investigate the properties of the ligands in the first target protein grids for these active binding areas of the target protein (Bappi et al., 2023).

#### 2.2.2. ADME and toxicity prediction

To examine the physicochemical characteristics of effective candidates, such as aqueous solubility, lipid affinity, and pharmacokinetics, the SwissADME online platform (http://www.swissadme.ch/) was employed for analysis (Daina et al., 2017). The pkCSM online application was used to evaluate the pharmacokinetic activities, and the

**Table 1**
Study groups, compositions, and treatments.

| Treatment Groups | Description | Dose (p.o.) |
|---|---|---|
| Gr.-I: NC | Vehicle (0.05% Tween 80 + 0.9% NaCl solution) | 10 ml/kg |
| Gr.-II: DZP | Standard 1: Diazepam [agonist] | 2 mg/kg |
| Gr.-III: CAF | Standard 2: Caffeine [antagonist] | 10 mg/kg |
| Gr.-IV: SUC-15 | Test sample: Succinic acid (upper dose) | 15 mg/kg |
| Gr.-V: SUC-10 | Test sample: Succinic acid (middle dose) | 10 mg/kg |
| Gr.-VI: SUC-5 | Test sample: Succinic acid (lower dose) | 5 mg/kg |
| Gr.- VII: (Gr.- II + Gr.- III) | Diazepam + Caffeine | 2 mg/kg + 10 mg/kg |
| Gr.- VIII: (Gr.-V + Gr.-II) | Succinic acid + Diazepam | 10 mg/kg + 2 mg/kg |
| Gr.- IX: (Gr.-V + Gr.- III) | Succinic acid + Caffeine | 10 mg/kg + 10 mg/kg |
| (p.o.: Per oral; n = 6) | | |

publicly available ProTox-II server (https://tox-new.charite.de/protox_II/) was used to test the toxicity qualities of succinic acid (Ahmed & Alkali, 2019).

## 3. Results

### 3.1. In vivo studies

#### 3.1.1. Open-field test

Experimental animals from the negative control group (NC) exerted the most field crossing and grooming compared to SUC groups and DZP. DZP and SUC-10 considerably ($p < 0.0001$) reduced the number of field crossings and grooming values. In comparison to NC, SUC groups improved rearing, but DZP had a lower value. All of the experimental parameters were decreased by SUC-10 and DZP in a substantial manner. After CAF pretreatment, most field crossings, rearing, and grooming were observed. The number of animals reared and groomed for (CAF + DZP) and (CAF + SUC-10) significantly decreased, although the number of field crosses was nearly comparable to CAF. Surprisingly, the DZP + SUC-10 group exhibited the most significant anxiolytic effect in the square cross (38.4 ± 01.63) and rearing (0.00 ± 0.00) among the other groups, but the SUC-10 group showed a considerably lower value (0.80 ± 0.58) in the grooming test. Fig. 1 shows the number of square crosses, grooming, and rearing that were noticed in the various treatment groups.

#### 3.1.2. Swing test

In this test, pre-treated animals with the NC (29.20 ± 1.46) caused the most swings. In comparison to the NC group, both DZP and SUC at all doses significantly ($p < 0.0001$) decreased the number of swing parameters in the animals. Test animals that received CAF as a pretreatment had more swings (40.00 ± 1.70). The number of swings in test animals was considerably decreased by both DZP (18.40 ± 1.20) and SUC-10 (14.80 ± 1.28). When compared to the NC, DZP, and CAF groups, it was discovered that (CAF + DZP) (7.00 ± 1.78) and (CAF + SUC-10) (15.60 ± 1.88) significantly reduced the number of swings. SUC-5 provided the lowest number of swings (12.20 ± 1.39) among the three concentrations. However, the lowest number of swing values was observed in the DZP + SUC-10 group (2.80 ± 0.66). Fig. 2(A) shows the number of swings that were noticed in the various treatment groups.

#### 3.1.3. Hole-cross test

In this experiment, NC (19.40 ± 2.06) increased the number of hole-cross in test animals compared to DZP (11.20 ± 1.15) and SUC-10 (12.80 ± 2.17). (SUC-10+DZP) significantly ($p < 0.0001$) decreased the number of hole-crosses (9.80 ± 1.71) in comparison to NC and SUC-10 but was nearly equivalent to DZP. The most valuable hole-crossing was provided by CAF (27.00 ± 1.94). However, CAF combined with SUC-10 and DZP showed a lower number of hole-crosses. Fig. 2(B) shows the number of hole-crosses that were noticed in the various treatment groups.

#### 3.1.4. Light–dark test

In this investigation, SUC at all doses provided the highest amount of time spent in the lightbox compared to NC, DZP, and CAF. When compared to the other two concentrations (15 mg/kg and 5 mg/kg), the SUC-10 pre-treatment group spent the most time in the lightbox (120.6 ± 2.58). DZP (61.40 ± 2.33) increased the light residence time significantly ($p < 0.0001$) compared to NC (52.00 ± 1.30) and CAF (42.60 ± 1.72). SUC-10 combined with CAF or DZP lowers the light residence time in the lightbox. (CAF + DZP) increased light residence time (77.80 ± 2.69) compared to NC, DZP, and CAF. Fig. 2(C) shows the light residence time that was noticed in the various treatment groups.

### 3.2. In silico study

#### 3.2.1. GABA homology model

With the development of homology modeling into a potent structural biology technique, the time between empirically verified protein structures and known protein sequences has been greatly shortened (Hamedu h et al., 2020). The Swiss model was utilized to generate the best homology model for GABAA from the UniProt sequence of amino acids, which was reported to the NCBI Blast Programs. In Fig. 3, a 3D homology model of GABA receptors is shown. As illustrated in Fig. 4, the GABA homology models were optimized using the Swiss-PDB View software tool (version 4.1.0) and validated using the Ramachandran plot produced by PROCHECK (Bhuia et al., 2023).

The Ramachandran plot would be a straightforward method for observing the distribution of torsion angles in protein complexes. Also, it provides an overview of the torsion angle values that are allowed and forbidden, which is crucial for determining the validity of proteins' three-dimensional structures. The phi-psi torsion orientations of each residue in the structure are shown on the Ramachandran map (except those at the chain termini). (Fig. 4). According to Ramachandran plot statistics, residues in the most favored areas are around 93.31%, 94.68%, and 93.52% for GABA α1, β2, and γ2.

*3.2.1.1. Interaction of SUC with GABA receptor.* SUC binds to $GABA_A$ receptor subunits α1, β2, and γ2 with affinities of −5.0, −4.5, and −4.8 kcal/mol, respectively (Table 2). SUC was able to connect to the GABA α1 subunit through five conventional hydrogen bonds (ASN378, LEU379, ALA380, THR376, and ASN378) and one carbon-hydrogen (PRO377) bond. SUC also attaches to the GABA β2 subunit through five conventional hydrogen bonds (ASN378, LEU379, ALA380, THR376,

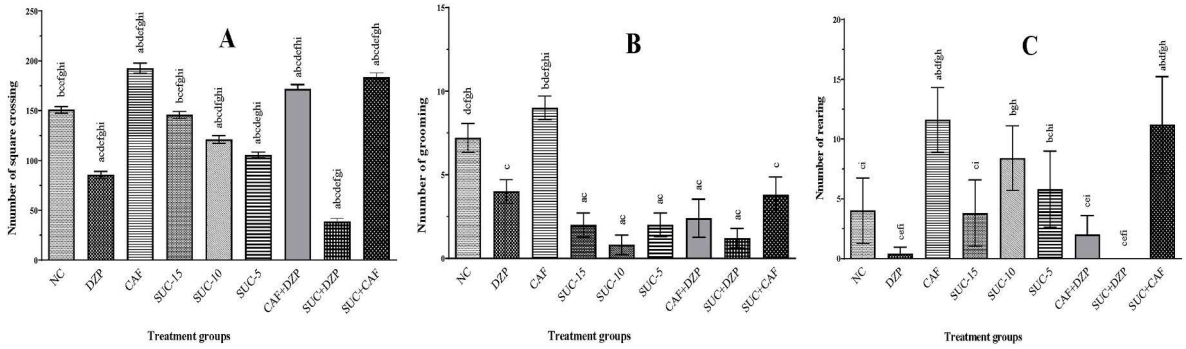



**Fig. 1.** [(A) Number of square crossings observed in test samples, controls, and combinations. {$^{ci}p<0.05$, $^{gi}p<0.001$, $^{abcdefghi}p<0.0001$}; (B) Number of grooming observed in test samples, controls, and combinations. {$agp<0.01$, $abcdfip<0.001$, $acdefghp<0.0001$}; (C) Number of rearing observed in test samples, controls, and combinations. {$^{bcfhi}p<0.05$, $^{acdegi}p<0.01$, $^{bcdeh}p<0.001$, $^{bcghi}p<0.0001$}; compared to the [a]NC (vehicle); [b]DZP (positive control); [c]CAF (positive control); [d]SUC-15 (test sample); [e]SUC-10; [f]SUC-5; [g]CAF + DZP; [h]SUC + DZP; [i]SUC + CAF; Values are mean ± S.E.M. (n = 6) (one-way ANOVA and t-Student-Neuman-Keuls post hoc test with multiple comparisons)].

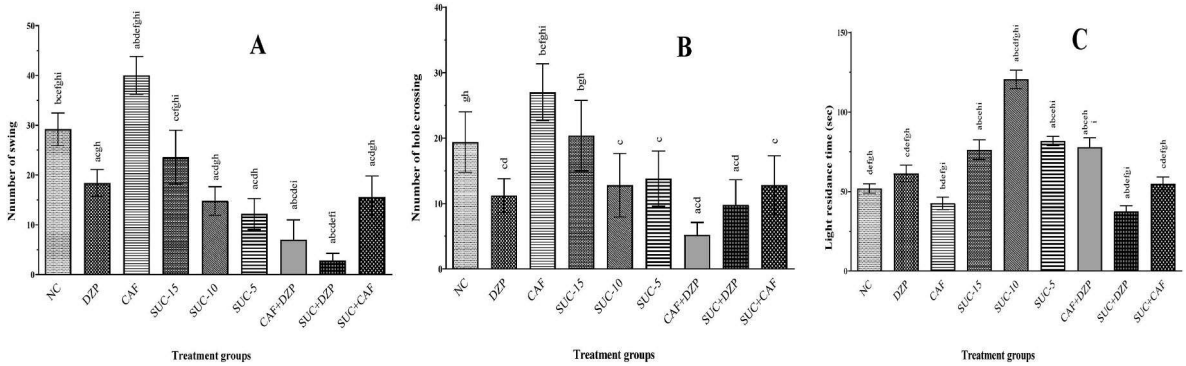



**Fig. 2.** [(A)Number of swings observed in test samples, controls, and combinations. {$^{degi}p<0.05$, $^{fh}p<0.01$, $^{abcdefgh}p<0.001$, $^{abcdefghi}p<0.0001$}; (B) Number of hole crossing observed in test samples, controls, and combinations. {$^{ahbd}p<0.05$, $^{dh}p<0.01$, $^{acefgi}p<0.001$, $^{bcdgh}p<0.0001$}; (C) Light residence time (sec) observed in test samples, controls, and combinations. {$^{ci}p<0.01$, $^{abdh}p<0.001$, $^{abcdefghi}p<0.0001$} $^{a}$compared to the NC (vehicle); $^{b}$compared to the DZP (positive control); $^{c}$compared to the CAF (positive control); $^{d}$compared to the SUC-15(test sample); $^{e}$compared to the SUC-10(test sample); $^{f}$compared to the SUC-5(test sample); $^{g}$compared to the CAF + DZP; $^{h}$compared to the SUC + DZP; $^{i}$compared to the SUC + CAF; Values are mean ± S.E.M. (n = 6) (one-way ANOVA and t-Student-Neuman-Keuls post hoc test with multiple comparisons)].

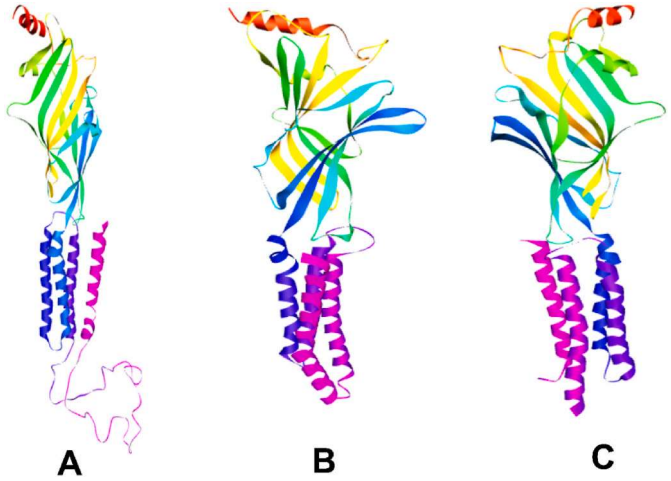



**Fig. 3.** The Homology modeling of $GABA_A$ receptors (A) α1, (B) β2, & (C) γ2 through the *Swiss* model.

and ASN378) and one carbon-hydrogen (PRO377). Furthermore, SUC binds to the GABA γ2 subunit through two conventional hydrogen bonds (TYR235, GLN200). Fig. 5 (A, B, and C) depicts the three-dimensional (3D) structures of SUC non-bond interactions with $GABA_A$ receptor subunits.

*3.2.1.2. Interaction of CAF with GABA receptor.* CAF binds to GABA receptor subunits α1, β2, and γ2 with affinities of −5.5, −5.2, and −5.1 kcal/mol, respectively (Table 2). CAF was able to connect to the GABA α1 subunit through one conventional hydrogen bond (ARG424), two carbon-hydrogen (TRP273, GLU277) bonds, and three pi-alkyls (TRP344, TRP344, VAL416). CAF also attaches to the GABA β2 subunit through two conventional hydrogens (TYR97, TYR157) and three carbon-hydrogen bonds (GLU155, TYR157, TYR157), one pi-sigma (TYR205), three pi-pi stacked (TYR157, PHE200, TYR205), and three pi-alkyl (TYR97, TYR157, PHE200) hydrophobic bonds. Furthermore, CAF binds to the GABA γ2 subunit through two conventional hydrogens (GLN200, ARG232), one carbon-hydrogen (TYR199) bond, one pi-pi stacked (TYR235), one alkyl (ARG231), and two pi-alkyl (TYR235, PHE236) hydrophobic bonds.

**Table 2**
Best binding affinity values and non-bond interactions of succinic acid (SUC), caffeine (CAF), and diazepam (DZP) with $GABA_A$ (α, β, and γ) receptors.

| Ligands | Protein (Receptor) | Binding affinity (Kcal/mol) | Number of Hydrogen Bond | Number of Hydrophobic Bond | Number of Others Bond |
|---|---|---|---|---|---|
| SUC | GABA- α1 | −5.0 | 6 | – | – |
| | GABA- β2 | −4.5 | 4 | 1 | – |
| | GABA- γ2 | −4.8 | 2 | – | – |
| CAF | GABA- α1 | −5.5 | 3 | 3 | – |
| | GABA- β2 | −5.2 | 5 | 7 | – |
| | GABA- γ2 | −5.1 | 3 | 4 | – |
| DZP | GABA- α1 | −6.7 | 1 | 7 | 1 |
| | GABA- β2 | −6.3 | 2 | 2 | 2 |
| | GABA- γ2 | −7.0 | 5 | 2 | – |

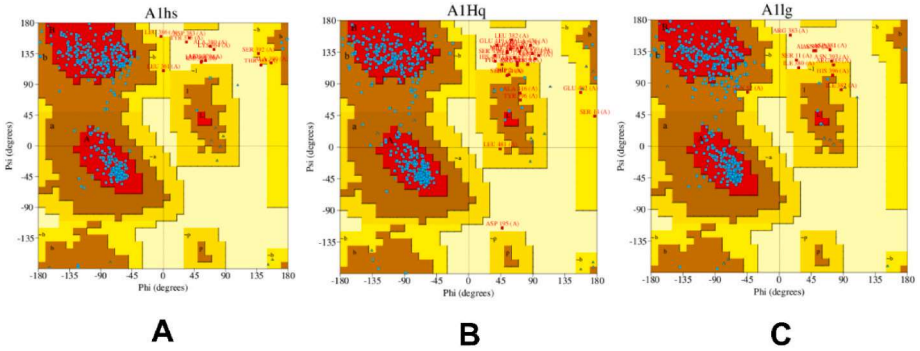



**Fig. 4.** The Swiss model was used to optimize the predictions of $GABA_A$ receptors (A) α1, (B) β2, & (C) γ2.

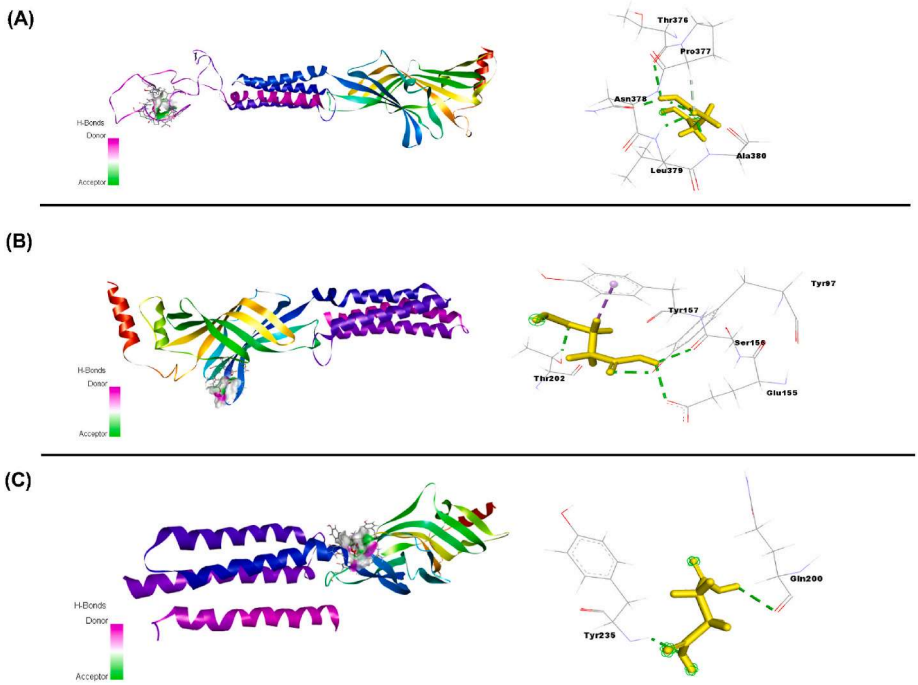



**Fig. 5.** The three-dimensional (3D) structures of molecular docking contacts between GABAA receptors. Here, (A) SUC and GABA- α1, (B) SUC and GABA- β2, (C) SUC and GABA- γ2.

*3.2.1.3. Interaction of DZP with GABA receptor.* Table 2 shows that DZP has binding affinities of −6.7, −6.3, and −7.0 kcal/mol for the α1, β2, and γ2 subunits of the GABA receptor, respectively. DZP's connection to the GABA α2 subunit involved a carbon-hydrogen bond (ASN414), two pi-pi stacked bonds (TRP344, TRP344), one pi-pi T-shaped (TRP344), four pi-alkyls (TRP344, TRP344, TRP344, ALA343), hydrophobic bonds, and one attractive charge (GLU277) electrostatic bond. DZP also attaches to the GABA β2 subunit through conventional hydrogen (ASN85) and two carbon-hydrogen bonds (ARG114, LEU83), two amide-pi stacked (ASN113, ARG144), one alkyl (MET115), and two pi-alkyls (ARG129, MET115) hydrophobic bonds. Furthermore, DZP binds to the GABA γ2 subunit through two conventional hydrogen bonds (GLY234, TYR235) and three carbon-hydrogen bonds (TYR199, GLN200, ARG232), one pi-pi stacked (TYR235), and one pi-alkyl (TYR199) hydrophobic bonds.

*3.2.2. Pharmacokinetic and drug-likeness properties*

One such area is the development of drugs, where computational techniques from biology and chemistry are crucial. The pre-clinical procedure needed to generate and use the target molecule as a therapeutic is called computer-aided drug design. A number of *in silico* techniques, including *Swiss*-ADME, pkCSM, and the ProTox II server, assist in assessing a molecule's ADMET properties. The pharmacokinetic properties of a chemical describe the characteristics of its ADME (absorption, distribution, metabolism, and excretion). The pkCSM and ProTox II servers were used to forecast the ADMET parameters of SUC (Fig. 6). Utilized toxicity parameters include toxicity class, Ames toxicity, hepatotoxicity, oral rat acute toxicity ($LD_{50}$), mutagenicity, carcinogenicity, and many others.

The human intestine has a 71.748% chance of absorbing SUC, according to the astoundingly high levels of human intestinal absorption (HIA) statistics. If a chemical's log Papp value is greater than 0.90 cm/s, it is considered to have high-level Caco-2 permeability, according to the pkCSM website, whereas SUC has low Caco-2 permeability (0.603 cm/s). When evaluating a compound's ability to cause mutations in bacteria, the AMES toxicity test is frequently utilized. Due to the fact that SUC has no hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, or skin sensitization, it is safe to assume that taking significant doses of it won't be harmful (Table 3).

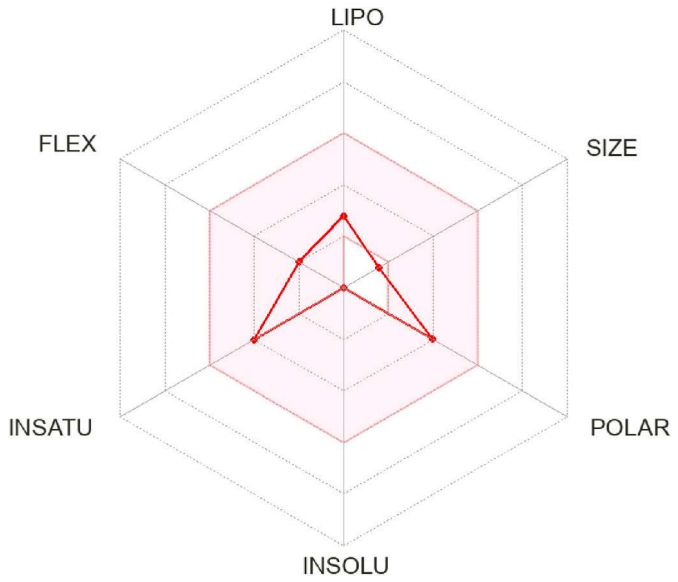



**Fig. 6.** Bioavailability radar related to physicochemical properties of Succinic acid. Lipophilicity (LIPO): −0.7 < XLOGP3 < +5.0, Polarity(POLAR): 20 $Å^2$ < TPSA <130 $Å^2$, Insolubility(INSOLU): −6 < log S (ESOL) < 0, Insaturation (INSATU) 0.25 < Fraction Csp3 < 1, Flexibility(FLEX): 0 < Num, rotatable bonds <9.

## 4. Discussion

Uncontrollable stress, dread, and nervousness are symptoms of the common mental health condition known as anxiety (Mariani et al., 2020). Numerous neurotransmitters, receptors, and parts of the brain interact intricately in the neurobiology of anxiety (Olivier & Olivier,

**Table 3**
Pharmacokinetic and drug-likeness properties of succinic acid (SUC).

| Properties | Factors | SUC |
|---|---|---|
| Physicochemical Properties | Formula | $C_4H_6O_4$ |
| | Molecular weight (g/mol) | 118.09 |
| | Num. heavy atoms | 8 |
| | Num. H-bond donors | 2 |
| | Num. of aroma-heavy atoms | 0 |
| | Num. H-bond acceptors | 4 |
| | Fraction Csp3 | 0.50 |
| | TPSA ($Å^2$) | 74.60 |
| | Num. rotatable bonds | 3 |
| | Molar Refractivity | 24.89 |
| Lipophilicity | Log Po/w (iLOGP) | 0.32 |
| | Log Po/w (SILICOS-IT) | −0.63 |
| | Log Po/w (XLOGP3) | −0.59 |
| Water Solubility | Log S (ESOL) | Very soluble |
| Pharmacokinetics | GI absorption | High |
| | Log $K_p$ (skin permeation) (cm/s) | −7.44 |
| Druglikeness | Lipinski | Yes; 0 violation |
| | Bioavailability Score | 0.85 |
| Medicinal Chemistry | Synthetic accessibility | 1.29 |
| Absorption | Caco2 permeability (Log Papp in $10^{-6}$ cm/s) | 0.603 |
| | Intestinal absorption (% Absorbed) | 71.748 |
| | BBB permeant | No |
| Distribution | $VD_{ss}$ (log L/kg) | −1.013 |
| | Fraction unbound (Fu) | 0.638 |
| | CNS permeability (log PS) | −3.06 |
| Metabolism | CYP2D6 substrate | No |
| | CYP3A4 substrate | |
| | CYP1A2 inhibitor | |
| | CYP2C19 inhibitor | |
| Excretion | Total Clearance (log ml/min/kg) | 0.722 |
| | Renal OCT2 substrate | No |
| Toxicity **test** | Hepatotoxicity | Inactive |
| | Carcinogenicity | |
| | Immunotoxicity | |
| | Mutagenicity | |
| | Cytotoxicity | |
| | Skin Sensitization | |
| | AMES toxicity | |
| | Oral Rat Acute Toxicity (mol/kg) | 1.618 |
| | Human Max. tolerated dose (log mg/kg/day) | 0.641 |
| | Minnow toxicity (log mM) | 2.829 |

2020). GABA serves as the central nervous system's main inhibitory neurotransmitter. It helps to balance the activity of excitatory neurotransmitters like glutamate by lowering neural excitability (Bi et al., 2020). GABA binds to $GABA_A$ receptors, which are ligand-gated chloride channels present in the brain, to provide its inhibitory effects (Knoflach et al., 2016). Although GABA and $GABA_A$ receptors are important in the control of anxiety, anxiety disorders are complicated illnesses involving several neurotransmitters, different parts of the brain, and hereditary factors (Bystritsky et al., 2013). There is no one "cause" of anxiety, so treatment usually entails a mix of therapies, such as cognitive-behavioural therapy (CBT), lifestyle adjustments, and, occasionally, medication (DeMartini et al., 2019). However, the network of neurotransmitter systems implicated in anxiety is far larger than the $GABA_A$ receptor system, which is just one component of it (Chellappa & Aeschbach, 2022). Furthermore, serotonin, norepinephrine, and dopamine are other neurotransmitters that are important in the control of anxiety (Bhuia et al., 2023).

The principal route for inhibitory signalling in the central nervous system (CNS) is referred to as GABAergic signalling which includes the $GABA_A$ receptor as a crucial part and is a particular class of neurotransmitter receptors that are triggered by GABA (Lusche et al., 2011). Chloride ion (Cl-) transportation and the $GABA_A$ receptor have a strong connection. A Cl-channel is opened when GABA binds to the $GABA_A$ receptor, enabling Cl-ions to enter the cell. The neuron is inhibited by the inflow of Cl-through the $GABA_A$ receptor. It causes the cell membrane to become hyperpolarized, making it more challenging for the neuron to produce an action potential and convey signals (Luo and Balle, 2022). The balance between excitatory (e.g., glutamate) and inhibitory (e.g., GABA) signals in the brain, which is essential for healthy brain function, must be maintained through this inhibition (Nelson & Valakh, 2015). The balance between excitation and inhibition is maintained by this process, which is also crucial for controlling neuronal activity. This process also affects other aspects of brain function, such as sedation, anxiety, epilepsy, and neural growth (Carver & Reddy, 2013).

DZP is commonly used in combination with other commonly accessible over-the-counter medications that also have psychoactive properties, such as CAF, to reduce the efficacy of DZP in animals (Teoh et al., 2019; Verma et al., 2023). The brain's GABA-succinate shunt and Krebs cycle produce SUC, a dicarboxylic acid. It can exert an antianxiety effect by interacting with the $GABA_A$ receptor (Chen et al., 2003). The findings of our study also demonstrate that SUC-10 exerted a calming effect on experimental animals, it decreased the number of hole-crossings, grooming, rearing, and swings while increasing the dark residence time. In combination, SUC-10 also significantly ($p < 0.0001$) improved the anxiolytic effect of DZP. In contrast, SUC-10 altered the test parameters observed in CAF when combined with it.

Anxiety is produced in the light-dark box test by the tension between the desire to explore and the need to flee a well-lit new environment (Jakaria et al., 2019). Our study suggests that DZP and SUC-10 have anxiety-reducing effects, which was confirmed by the fact that they increased the amount of time spent by the animals in the light portion. SUC (5, 10, and 15 mg/kg) increased the light residence time, but when combined with DZP in the DZP + SUC-10 group, it significantly reduced the light residence time, indicating that it may have relaxing effects on mice.

At present, *in silico* studies open a new potential window for drug discovery and development. To date, many neuropharmacological studies have been performed *in silico* (Mastinu et al., 2023). In our *in silico* experiment, SUC, DZP, and CAF were separately bound to the $GABA_A$ receptor subunits α1, β2, and γ2, and computational techniques were used to investigate the molecular docking method. Our findings reveal that DZP exhibits the best binding affinities of −6.7, −6.3, and −7.0 kcal/mol and a number of H-Bonds (1,2,5 respectively) with the α1, β2, and γ2 subunits of the GABA receptor. SUC also demonstrates a good interaction with $GABA_A$ receptor subunits α1, β2, and γ2 with affinities of −5.0, −4.5, and −4.8 kcal/mol and a number of H-Bonds (6,4, 2 respectively). Additionally, the antagonist drug CAF binds to GABA receptor subunits α1, β2, and γ2 with affinities of −5.5, −5.2, and −5.1 kcal/mol and a number of H-Bonds (3,5,3 respectively). The development of a complex between biomacromolecules and their target ligands depends on hydrogen-bond interactions, which are also crucial in defining the selectivity of ligand binding (Takaya et al., 2021). In addition to stimulating ligand binding, hydrogen bonding also makes ligands more pharmacologically interesting (Pyrkov et al., 2010). According to our research, SUC exhibited a higher number of hydrogen bonds than either conventional or antagonist medications. Our *in silico* studies also suggest that SUC has acceptable physicochemical, pharmacokinetic, and drug-like qualities.

We found a good correlation among the *in vivo* test results. For instance, SUC and its combination showed a similar reduction in the number of square crossings, hole crossings, swings, and light residence times in comparison to the control group animals. Moreover, a correlation analysis between *in vivo* bioactivities and *in silico* methods has been carried out to offer evidence for the projected pharmacological activities of products (Uddin et al., 2021). In this study, animal models with SUC showed anxiolytic effects. Using data from our *in vivo* and *in silico* research, Fig. 7 shows the possible anxiolytic mechanism of SUC.

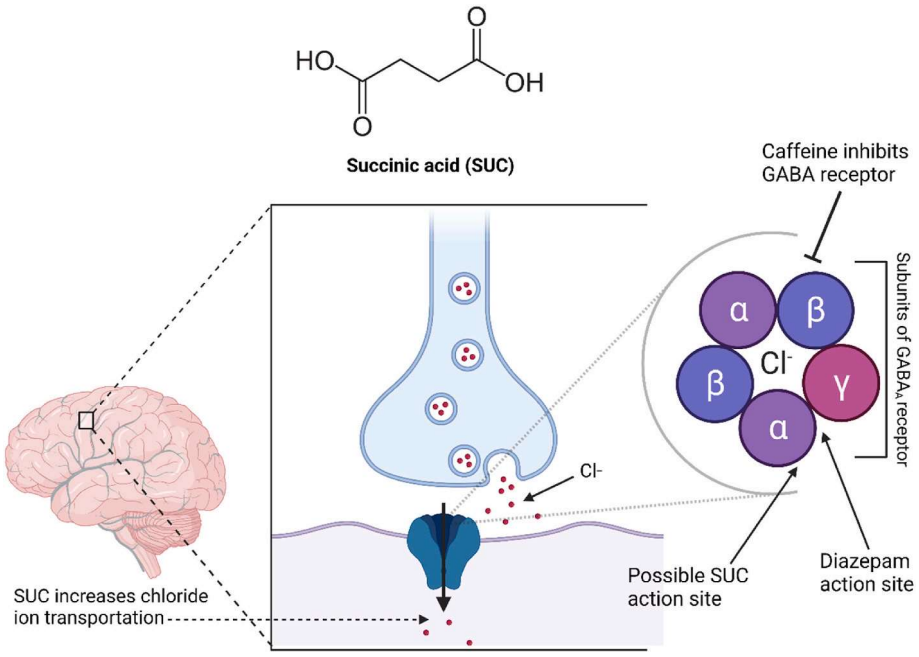



**Fig. 7.** The possible anxiolytic mechanism of succinic acid.

## 5. Conclusion

Psychological symptoms of mental diseases can affect many facets of a person's life. The most common stress-related mood disorders that lead to harm and early mortality include anxiety. Numerous conventional medications with neuroprotective properties are available to treat these diseases. Our *in vivo* study demonstrates that SUC-10 has an anxiolytic-*like* effect in experimental animals. Combining SUC-10 with DZP also considerably enhanced ($p < 0.0001$) its anxiolytic effects. Conversely, when paired with CAF, SUC-10 changed the test parameters shown in CAF. Furthermore, *in silico* molecular docking studies were carried out, and they suggest that SUC exhibits interactions with $GABA_A$ receptor subunits, particularly interactions with $GABA_A$ (α1, β2, and γ2). As SUC possesses good drug-like properties and shows potential anxiolytic activity, further studies are needed to elucidate the exact molecular mechanism of action behind this effect.

## Author contributions

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Shanita Zaman Smrity - CONCEPTUALIZATION.
Mehedi Hasan Bappi - METHODOLOGY.
Hossam Kamli - METHODOLOGY.
Tawhida Islam - METHODOLOGY.
Abdullah Al Shamsh Prottay - METHODOLOGY.
Md. Showkoth Akbor - INVESTIGATION.
Md. Abdul Latif - INVESTIGATION.
Shoriful Islam - SOFTWARE.
Kushal Bhakta - SOFTWARE.
Manik Chandra Shill - SUPERVISION.
Francisco Claudeni Pereira de Sousa – FIRST DRAFT OF THE MANUSCRIPT.
Gilberto de Luna – FIRST DRAFT OF THE MANUSCRIPT.
Henrique Douglas Melo Coutinho – PROJECT ADMINISTRATION.
Muhammad Torequl Islam - SUPERVISION.

## 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.

## Data availability

Data will be made available on request.

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