**In vitro** antioxidant and **in vivo** antidepressant activity of green synthesized azomethine derivatives of cinnamaldehyde

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Abstract:

OBJECTIVES: In this study, three (CS-1 to CS-3) azomethine derivatives of cinnamaldehyde were green synthesized, characterized, and their antioxidant and antidepressant activities were explored.

MATERIALS AND METHODS: The antioxidant effect of these compounds was initially performed in vitro using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay methods before subjecting them to in vivo experiments. Compounds showing potent antioxidant activity (CS-1 and CS-2) were investigated further for their antidepressant activity using the forced swim test (FST) and tail suspension test (TST). Ascorbic acid (AA) and fluoxetine (20 mg/kg, p.o) were used as reference drugs for comparison in the antioxidant and antidepressant experiments, respectively.

RESULTS: It was observed that CS-2 and CS-3 exhibited highest DPPH (half maximal inhibitory concentration [IC₅₀]: 16.22 and 25.18 µg/mL) and ABTS (IC₅₀: 17.2 and 28.86 µg/mL) radical scavenging activity, respectively, compared to AA (IC₅₀: 15.73 and 16.79 µg/mL) and therefore, both CS-2 and CS-3 were tested for their antidepressant effect using FST and TST as experimental models. Pretreatment of CS-2 and CS-3 (20 mg/kg) for 10 days considerably decreased the immobility time in both the FST and TST models.

CONCLUSION: The antioxidant and antidepressant effect of CS-2 and CS-3 may be attributed to the presence of azomethine linkage in the molecule.

Keywords: 1, 1-diphenyl-2-picrylhydrazyl and 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonate), antidepressant, antioxidant, azomethine, cinnamaldehyde, forced swim test, green synthesis, Schiff base, tail suspension test

Introduction

The green synthesis and structure of Schiff bases (also called as azomethines) have attracted much attention in the field of pharmaceutical research. Schiff bases are obtained by condensing primary amines with carbonyl compounds.¹⁻³ A considerable amount of research has been done by Hugo (Ugo) Schiff⁴⁻⁵ in the course of time to produce a huge variety of Schiff bases using different chemical procedures. The general structural feature of Schiff bases is the azomethine group with a general formula RHC = N‑R₁, where R and R₁ represent alkyl, aryl, cyclo alkyl, or heterocyclic groups. Several studies⁶⁻⁹ revealed that the incidence of a lone pair of electrons in a sp² hybridized orbital of the nitrogen atom of the azomethine group is of considerable chemical and biological importance.
Recent literature revealed the use of cinnamaldehyde as an antimicrobial,
and flavoring agent for chewing gums. Similarly, Schiff bases have been reported for antibacterial, antifungal, antiproliferative, and antipyretic properties. During the entire preceding history of medicine, fewer drugs had known the focus of action, and then fewer had been submitted to synthetic investigations. It is an evident fact that azomethines with aryl substituents are more stable and readily synthesized, whereas those containing alkyl substituents are relatively unstable. Azomethines of aliphatic aldehydes are usually readily polymerizable and unstable while those with conjugated aromatic aldehydes are more stable.

In an attempt to identify a potent and safer antidepressant agent, this study avoided the usage of hazardous chemicals and focused in green synthesis of novel azomethines of cinnamaldehyde (CS-1, CS-2, and CS-3). Both cinnamaldehyde and their azomethine derivatives were reported for their antioxidant and antidepressant effects. Hence, the synthesized cinnamaldehyde azomethines were subjected to in vitro antioxidant and in vivo antidepressant studies.

Materials and Methods

Drugs and chemicals
1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid (AA), and fluoxetine hydrochloride (FLX) were obtained from Sigma-Aldrich (USA). All other chemicals used are of analytical grade were purchased commercially from local vendors and were utilized without further purification.

Instrumentation
The melting points of the compounds were determined using an electric melting point apparatus (Tosniwal Pvt. Ltd., India) and the values were uncorrected. Thin layer chromatography (TLC) technique on silica gel-GF254 coated plates (Merck, Germany) is used for purity checking. Spots of TLC were recognized by iodine chamber. The ultraviolet (UV)-visible spectra of all the compounds were obtained at 200–400 nm using a double beam Shimadzu UV 1800 spectrophotometer at a concentration of 50 µg dissolved in methanol. The infrared (IR) spectra of synthesized compounds were recorded on a Thermo Nicolet Nexus 670-FTIR using KBr disc method with minimum forty scans. 1H-NMR spectra were obtained on Bruker UX-NMR instrument at 300 MHz, using tetramethylsilane as an internal standard and CDCl3, as a solvent, and chemical shift values were expressed in δ ppm. 13C NMR spectra were recorded on Bruker DPX at 300 Hz. The mass spectra were recorded on MASPEC (MSW/9629), and values were expressed as %relative abundance. Elemental analyses for synthesized compounds were carried over Perkin Elmer 240 CHN analyzer.

Synthesis of azomethine derivatives of cinnamaldehyde
An equimolar concentration of cinnamaldehyde (0.01M) and substituted aromatic amines (0.01M) were dissolved in 5 ml of ethanol at room temperature until completely dissolved and then they were triturated for the mentioned time to give cinnamaldehyde azomethines [Figure 1]. The crystalline product was collected after filtration, washed with water, and then recrystallized using aqueous ethanol. A base catalyst triethylamine was added during trituration. The completion of the reaction was confirmed by Rf with TLC technique, using the appropriate solvent system, benzene:pyridine:ammonia (8:2:1), and the spots were recognized with the help of iodine chamber. The formed crystalline powder was dried in oil pump, further dried in a desiccator. The UV-visible, IR, NMR, and mass spectra supported the structures of all synthesized compounds.

In vitro antioxidant assay
1,1-diphenyl-2-picrylhydrazyl assay
The stable free radical-scavenging potential was obtained by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. Preparation of DPPH solution was done...
using Molyneux[30] and Blois[31] method with minor modification. The samples used to dissolve the test compounds were 95% v/v ethanol. In brief, 0.5 mL of test compounds (0-blank control, 10, 25, 50, 100, 250, 500, and 1000 µg/mL) was added to 0.5 mL of DPPH (2 µM in 95% v/v ethanol) and incubated the mixture at room temperature for 30 min. The absorbance was measured at a wavelength of 517 nm, and the percentage scavenging of free radicals by the test compounds was calculated from the following equation using Microsoft Excel software (Version 2010, Microsoft Corp., Seattle, WA, USA.) The standard used for comparison was AA.[32]

\[
\% \text{ Inhibition} = \left(1 - \frac{\text{absorbance sample}}{\text{absorbance control}}\right) \times 100 \quad (1)
\]

The half maximal inhibitory concentration (IC₅₀) was calculated using constructing a nonlinear regression graph between %inhibition versus concentration, using GraphPad Prism software (version 6, GraphPad Software, Inc., CA 92037, USA).

2,2'‑azinobis(3‑ethylbenzothiazoline‑6‑sulfonic acid) assay
ABTS was dissolved in distilled water at the concentration of 7 × 10⁻³ M and 2.45 × 10⁻³ M of potassium persulfate was added. This reaction mixture was left overnight (12–16 h) in the dark, at room temperature and used for the assay.[33] Various concentrations of test compounds were prepared similar to that of DPPH assay and were incubated with the ABTS solution for 30 min. The absorbance was measured at 734 nm,[34] and the %scavenging of free radicals by the test compounds was determined using the formula described in equation (1) and the IC₅₀ was calculated. AA was selected as standard drug.

Antidepressant activity
Animals
Twenty‑four male Sprague‑Dawley rats (standard deviation: 150–200 g) and 24 male Swiss Albino mice (18–25 g) were selected and housed in Central Animal House, AIMST University, Malaysia. The animals were separately housed in groups (6/cage) and were maintained at laboratory standard conditions (room temperature: 22°C ± 2°C, relative humidity: 50%–70%, photoperiod: 12 h light/dark) with free access to pellet rodent feed (Gold Coin, Port Klang, Malaysia) and reverse osmosis treated water ad libitum. All animals were acclimatized to laboratory standard conditions for a week before the start of the experiment. The experimental protocol was approved by AIMST University Human and Animal Ethics Committee (Ref. No: AUHAEC 13/FOP/SP/2015).

Study design and treatment
Based on the in vitro antioxidant results, CS‑1 was excluded from animal studies, and CS‑2 and CS‑3 were selected for in vivo study. At the end of acclimatization period, rats and mice were randomly divided into four groups (n = 6/group) for forced swim test (FST) and tail suspension test (TST), respectively. Control group animals were administered with vehicle (10 mL/kg), positive control group animals were treated with FLX (20 mg/kg), and test group animals were treated with CS‑2 and CS‑3 (20 mg/kg).

Forced swim test
The effect of CS‑2, CS‑3, and FLX treatment on depressive‑like behavior of rats was measured in the FST.[35,36] The entire FST was performed after 2 h of treatment on the 9th and 10th day. On the 9th day, rats were exposed to a 15 min FST (pretest) and followed by a 6 min FST (test) on the next (10th) day. Rats were individually allowed to perform FST inside a plastic cylinder (height 35 cm and diameter 25.5 cm; containing 25 cm of water maintained at 25°C). The rats were removed from the water and then placed in the warm enclosure before accommodating into the respective cages. Cylinders were cleaned thoroughly and filled with fresh water after performing FST with each rat. In total, 6 min test period, the immobility was recorded only for the last 4 min. A time‑sampling technique was used, whereby the predominant behavior in each 5 s interval of the 4 min test swim was scored, the rat’s behavior was judged as one of the following three behaviors as described as follows:[37,38]

• Immobility behavior: This includes the floating behavior of rat with no additional activity other than that required to keep its head above the water
• Climbing behavior: This behavior includes the upward‑directed vigorous thrashing movements with the forepaws, along the side of the swim chamber
• Swimming behavior: This behavior includes the movement of rat on the water surface throughout the swim chamber, with its horizontal position

Tail suspension test
The TST was performed as per the methods earlier described[39,40] with minor modifications. All the mice of four different groups received their respective treatments for 10 days and 2 h after the last treatment (10th day) the mice were suspended upside down on a metal rod which was placed at the height of 60 cm from the ground level using an adhesive tape placed approximately 1 cm from the tip of the tail. Within 6 min period, the total duration of immobility was rated with each mice used only once.

Statistical analysis
The means of total immobility time in seconds for FST, TST, and behavioral scores (immobility, swimming, and
climbing counts) in FST were analyzed using one-way analysis of variance further followed by Tukey’s multiple comparison test. *P < 0.05* was measured statistically significant. All statistical analyses were performed using GraphPad Prism statistical software (Version 6, GraphPad Software, Inc., CA 92037, USA).

## Results

### Chemistry

The synthetic experiment offered three cinnamaldehyde azomethines (CS-1, CS-2, and CS-3). The structures of all synthesized compounds were established by UV-visible, IR, NMR, and mass spectral data. The spectral data for the newly synthesized compounds are as follows:

N-(3-phenylallylidene) benzamine (CS-1): Yield: 70%; mp: 98–100°C; Anal. Calcd. for C_{13}H_{16}N: C, 86.92; H, 6.32; N, 6.76%. Found C, 86.90; H, 6.28; N, 6.67%; IR (KBr, cm\(^{-1}\)): 3085 (=C-H stretching of aromatic ring), 3039 (=C-H stretching of alkenyl group), 1668 (=C=N stretching azomethine group), 1540–1600 (C=C-stretching of aromatic ring), 1320 (C-N, stretching of azomethine group); \(^1\)H-NMR (500.1 MHz, CDCl\(_3\), \(\delta / ppm\)): 5.76 (1H, t, \(J = 9.5\) Hz, H-2'), 6.52 (1H, d, \(J = 12\) Hz, H-3'), 7.23–7.60 (10H, m, phenyl), 8.24 (1H, d, \(J = 7.8\) Hz, H-1'); MS (m/z, (relative abundance, %)): 207 (M+), 189, 174, 160, 146, 130, 102, 77, 53, 51 (BP, 100); UV-Vis (MeOH) (\(\lambda_{max}/\text{nm}\)): 353.

4-(3-Phenylallylideneamino) phenol (CS-2): Yield: 80%; mp: 120–130°C; Anal. Calcd. for C_{13}H_{16}NO: C, 80.68; H, 5.87; N, 5.28%. Found C, 80.56; H, 5.78; N, 5.32%; IR (KBr, cm\(^{-1}\)): 3640 (Broad, O-H Str), 3055 (=C-H stretching of alkenyl group), 3039 (=C-H stretching of aromatic ring), 1668 (C=C-stretching of azomethine group), 1540–1600 (C=C-stretching of aromatic ring), 1320 (C-N, stretching of azomethine group); \(^1\)H-NMR (500.1 MHz, CDCl\(_3\), \(\delta / ppm\)): 5.4 (1H, s, -OH, D\(_2\)O exchangeable), 5.72 (1H, t, \(J = 9.5\) Hz, H-2'), 6.59 (1H, d, \(J = 12\) Hz, H-3'), 7.4–7.8 (10H, m, phenyl), 8.29 (1H, d, \(J = 7.8\) Hz, H-1'); MS (m/z, (relative abundance, %)): 223 (M+, 18.5), 146, 130, 102, 93, 77, 53, 51 (BP, 100); UV-Vis (MeOH) (\(\lambda_{max}/\text{nm}\)): 302.

4-(3-Phenylallylideneamino) benzoic acid (CS-3): Yield: 86%; mp: 130–140°C; Anal. Calcd. for C_{15}H_{16}NO: C, 76.42; H, 5.29 N, 5.52%. Found C, 76.31; H, 5.32N, 4.88%; IR (KBr cm\(^{-1}\)): 3052 (=C-H stretching of aromatic ring), 3036 (=C-H stretching of alkenyl group), 2926, 2872 (C-H stretching of methyl group), 1668 (C=C-stretching azomethine group), 1540–1600 (C=C-stretching of aromatic ring), 1320 (C-N, stretching of azomethine group); \(^1\)H-NMR (500.1 MHz, CDCl\(_3\), \(\delta / ppm\)): 5.85 (1H, t, \(J = 9.5\) Hz, H-2'), 6.76 (1H, d, \(J = 12\) Hz, H-3'), 7.64–7.92 (10H, m, phenyl), 8.41 (1H, d, \(J = 7.8\) Hz, H-1'), 11.00 (1H, s, COOH); MS (m/z, (relative abundance, %)): 251 (M+, 32.6), 174, 130, 121, 102, 77, 53, 51 (BP, 100); UV-Vis (MeOH) (\(\lambda_{max}/\text{nm}\)): 307.

### Antioxidant activity

#### 1,1-diphenyl-2-picrylhydrazyl method

The antioxidant potency of cinnamaldehyde azomethines evaluated using DPPH [Figure 2] and ABTS method [Figure 3].

The IC\(_{50}\) (µg/mL) values obtained with DPPH assay [AA (15.73) > CS-3 (16.22) > CS-2 (25.18) > CS-1 (35.47)] and ABTS assay [AA (16.79) > CS-3 (17.2) > CS-2 (28.86) > CS-1 (50.97)] are presented in the decreasing order. Among the cinnamaldehyde azomethines except CS-1, all other compounds showed the high (CS-3) to moderate (CS-2) antioxidant potency. Hence, CS-1 was excluded from our in vivo experiment.

### Antidepressant activity

#### Effect of CS-2, CS-3, and fluoxetine treatment on immobility time in forced swim test and tail suspension test

The effects of CS-2, CS-3, and FLX treatment on the mean immobility time in FST and TST are shown in Table 1. On by visual observation, in 15 min FST (pretest), rats were very active, vigorously swimming, climbing the wall, or diving down. At the end of 2–3 min, the above-mentioned activities subsides, in which rat made only those movements which are necessary to keep its head above the water indicating the characteristic behavior called as immobility. At 24 h later, on the test date when rats subjected to 6 min test session, there is increased immobility time.

Similarly, in TST, initially, by making vigorous movements, the mice tried to escape but became immobile after a while. The animals were exposed to this kind of...
unavoidable and inescapable stress to imitate behavioral despair which in turn could reflect depressive disorders in humans. The mean total duration of immobility time (seconds) for CS-2 \( (P < 0.05) \), CS-3 \( (P < 0.01) \), and FLX \( (P < 0.01) \) treated mice were found to be significant when compared to the control group.

**Effect of CS-2, CS-3, and fluoxetine treatment on different behavioral responses in rats**

The effect of CS-2, CS-3, and FLX on mean total numbers of counts for different behavioral responses such as swimming, climbing, and immobility is shown in Table 2. The results obtained with the behavioral responses are in accordance with that of FST. CS-2, CS-3, and FLX treatment affected the mean counts of immobility, swimming, and climbing behavior significantly compared to control animals.

### Discussion

**Chemistry**

Equimolar concentration of cinnamaldehyde and substituted aromatic amines in the presence of a basic catalyst; triethylamine resulted in the formation of azomethines of cinnamaldehyde moiety. The \( \lambda_{\max} \) for the newly synthesized azomethines was found to be in a range, i.e., from 300 to 440 nm. The IR stretch at around 1650–1680 cm\(^{-1}\) showed the C = N bond formation. The formation of azomethines was shown by the presence of triplet between 5.7 and 5.8 ppm, in proton NMR spectra. The aliphatic and aromatic protons were detected within the expected regions. The novel compounds were further confirmed by their characteristic mass fragment spectra. This part confirmed the green synthesis of a series of three Schiff bases of cinnamaldehyde.

Oxidative stress is found to exist in both peripheral as well central systems of patients suffering from major depression.\(^{[41]}\) Antioxidants maintain redox homeostasis by maintaining the level of reactive oxygen species (ROS); thus, the excess of ROS is counteracted by antioxidants escaping the oxidation of cellular components and consequently their damage. Thereby in the present study, first, we proved CS-2 and CS-3 have a significant antioxidant activity. Later, because cinnamaldehyde has earlier reported to have central nervous system activity and antioxidants play a significant role in ameliorating depression, we evaluated cinnamaldehyde base CS-2 and CS-3 for their antidepressant activity. The animal models were used for screening substances for antidepressant activity as some of the underlying biochemical mechanisms were common to clinical depression and experimentally produced depression in animals.\(^{[42]}\)

In the present study, CS-2 and CS-3 produced a significant antidepressant effect in FST and TST. These models of depression are extensively used to screen new antidepressant drugs. The FST is the most widely used tool for assessing antidepressant effect preclinically mainly due to its ability to detect a broad spectrum of antidepressant agents.\(^{[39]}\) It is evident from our study that CS-2 and CS-3 significantly affected the immobility and swimming behaviors and only CS-3 affected the climbing behavior in FST. The total immobility time in seconds was found to be significant in both FST and TST when compared with the control group. Therefore, one of the possible antidepressant mechanisms of CS-2 and CS-3 may be attributed due to its antioxidant potential. Moreover, our findings for CS-3 (climbing behavior) are in accordance with the study of Detke et al.\(^{[37]}\) that

### Table 1: Effect of CS-2, CS-3 and fluoxetine hydrochloride treatment on immobility time (s) in animals after forced swim test and tail suspension test

| Groups   | Immobility time (s) |
|----------|----------------------|
|          | FST                  | TST                  |
| Control  | 160.8±4.167          | 141.7±4.216          |
| FLX      | 115.8±5.231**        | 98.33±4.014**        |
| CS-2     | 133.3±2.789*         | 115.00±4.082*        |
| CS-3     | 122.5±7.159**        | 103.30±5.578**       |

**P<0.01 and *P<0.05 when compared with control, values are represented as mean±SEM (n=6). FST=Forced swim test, TST=Tail suspension test, SEM=Standard error of mean, FLX=Fluoxetine hydrochloride, CS=Cinnamaldehyde azomethines**

### Table 2: Effect of CS-2, CS-3 and fluoxetine hydrochloride treatment on different behavioral responses in rats after forced swim test

| Groups   | Behavioral responses |
|----------|----------------------|
|          | Immobility count     | Swimming count       | Climbing count      |
| Control  | 32.17±0.833          | 6.33±1.926           | 2.16±0.542          |
| FLX      | 23.17±1.046**        | 37.17±3.978**        | 9.33±0.802*         |
| CS-2     | 26.67±0.557*         | 25.00±1.633*         | 6.50±1.586          |
| CS-3     | 24.50±1.432**        | 41.67±1.856**        | 8.16±0.980*         |

**P<0.01 and *P<0.05 when compared with control, values are represented as mean±SEM (n=6). FLX=Fluoxetine, SEM=Standard error of mean, CS=Cinnamaldehyde azomethines**

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Figure 3: The percentage 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of cinnamaldehyde azomethines.
showed the climbing behavior was specifically affected by norepinephrine reuptake inhibitors. It has been argued that TST is less stressful than FST and has a greater pharmacological understanding. The results obtained from TST are also in agreement with the proved FST findings.

**Conclusion**

Azomethines of cinnamaldehyde (CS-1 to CS-3) were successfully synthesized using greener technique, and CS-3 appeared to be an important class of antioxidant and antidepressant agents.

**Acknowledgments**

The authors are thankful to AIMST University, Malaysia, for providing the fund from University grant and for the facilities to carry out the research. Authors are also thankful to Faculty of Pharmacy, UiTM, Puncak Alam Campus, Malaysia, for providing the analytical data.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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