Biological Evaluation of *Aegle marmelos* Fruit Extract and Isolated Aegeline in Alleviating Pain–Depression Dyad: In Silico Analysis of Aegeline on MAO-A and iNOS

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**ABSTRACT:** Pain and depression have been assessed to co-occur in up to 80% of patients, and this comorbidity is more debilitating and pricier for the patients as compared to either of these disorders alone. *Aegle marmelos* is a well-known medicinal plant with a broad spectrum of pharmacological activities. Aegeline is a relatively unexplored molecule present in *Aegle marmelos*. Therefore, the current investigation aims to explore the potential of *Aegle marmelos* fruit extract (AMFE) and isolated aegeline against the reserpine-induced pain–depression dyad. In the current investigation, aegeline was isolated from AMFE, followed by spectroscopic characterization, i.e., using NMR and mass analyses. AMFE (200 mg kg\(^{-1}\) p.o) and aegeline (10 mg kg\(^{-1}\) p.o.) were administered to reserpinized (0.5 mg kg\(^{-1}\) s.c.) mice, and clorgyline (3 mg kg\(^{-1}\) i.p.) was taken as the standard drug. AMFE and aegeline significantly alleviated the reserpine-induced reduction in a pain threshold and an increase in immobility as observed in behavioral tests of pain and depression, respectively. *In silico* molecular docking studies of aegeline showed a good binding interaction at the active sites of MAO-A and iNOS. The *in vivo* analysis showed that AMFE and aegeline treatment significantly decreased the monoamine oxidase-A (MAO-A) activity, serum interleukin-6 (IL-6) level, and lipid peroxidation, along with an increase in the reduced glutathione level in comparison to the reserpine-treated group. Immunofluorescence studies also showed that AMFE and aegeline abrogated the reserpine-induced increase in iNOS expression. Conclusively, the results delineate that AMFE and aegeline might exert a protective effect via downregulating the MAO-A hyperactivity, IL-6 level, oxidative and nitrosative stress.

**INTRODUCTION**

Pain and depression are turning out to be progressively common and leading causes of morbidity in the western world.\(^1\) They are closely related to each other in terms of worsening the genesis of each other.\(^2\) Clinically, it has been reported that chronic pain often induces depression, and in 85% of patients reporting chronic pain issues, they are also suffering from severe depression.\(^3\) Systematic analyses (1990–2017) for the global burden of the disease reported in 2017 revealed that more than 264 million people suffer from depression.\(^4\) On the other hand, in the case of pain, a report published by the International Association for the Study of Pain documented that the global burden of pain is enormous and mounting, accounting for one new chronic pain case per 10 medical issues.\(^5,6\) At the biochemical level, various neuronal substrates, such as neurotransmitters, cytokines, neurotrophins, etc., link these disorders with each other.\(^7\) The monoaminergic system has been implicated in depression and a specific type of painful condition, such as neuropathic pain.\(^8\) Moreover, monoamine oxidase (MAO) inhibitors are often implicated in a mental disorder, such as depression, and have also been

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reported to have an analgesic effect. However, classical MAO inhibitors have hepatotoxicity and are no longer in use for the treatment of depression nowadays.

Additionally, it has been reported that chronic pain can autonomously contribute to the pathogenesis of depression via increasing the level/expression of proinflammatory cytokines such as IL-6, TNF-α, etc. An increase in the serum IL-6 level corresponds directly to neuroinflammation and neuronal damage. Complexing of IL-6 with its receptor initiates a series of kinases viz. PI3K, MAPKs, and JAK/STAT signaling in upregulating pain perception. In addition to this, nitric oxide is also a critical inflammatory mediator responsible for triggering the immune response and inflammation. The inducible nitric oxide synthase (iNOS) enzyme is responsible for the production of nitric oxide. An extensive release of nitric oxide has been associated with triggering the production of proinflammatory cytokines, such as IL-6 and IL-8, leading to pain and depression. Aegle marmelos is one of the most exploited traditional medicinal plants of south Asian origin with a pharmacological profile ranging from anti-inflammatory, antifungal, antibacterial, antipyretic, antiproliferative, and hypoglycemic activities. Aegeline is one of the active natural components of Aegle marmelos fruits and leaves.

Reserpine-induced pain–depression dyad is a well-known and accepted animal model. Reserpine prevents the uptake and reduces the stored biogenic amines by blocking vesicular monoamine transporter-2 (VMAT-2). Studies have shown that reserpine administration reduces the pain threshold and induces a depression-like state in rodents. Using the reserpine-induced pain–depression model, the present study is designed to explore the potential of Aegle marmelos and isolated aegeline in mitigating the reserpine-induced behavioral and biochemical alterations.

Treatment for chronic pain and comorbid depression is complex and not well established. A number of hypothesis describing the interplay between pain and depression has been established. Whereas, estimating the extent of comorbidities between these two prime factors for establishing a treatment therapy is highly complex and ambiguous. The widely accepted hypothesis for their comorbidity states that depression precedes pain or vice versa or both may occur independent of each other. Meanwhile, others state that the risk linked to development of subsequent depression with new onset pain is higher in patients who have a history of depression. Clinical data reveals that many antidepressants are effective in mitigating pain but their efficacy is highly variable.

- Figure 1. HPTLC chromatograms of Aegle marmelos fruit extract (Track 1) and standard aegeline (Track 2) at 220 nm.
Tricyclic antidepressants (both secondary: desipramine, nortriptyline, and tertiary: amitriptyline, doxepin, imipramine), selective serotonin reuptake inhibitors (such as paroxetine and citalopram), and other agents with antidepressant effects, such as venlafaxine and bupropion, have shown analgesic action. Duloxetine is the only antidepressant agent approved by FDA for use in neuropathic pain. On the other hand, the potential of anti-inflammatory agents in abrogating depression have also been explored in various clinical studies. The results indicate a link between inflammation and depression (macrophage theory of depression). Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown antidepressant potential when tested against placebos. Selective COX-2 inhibitors (such as celecoxib) have potent anti-inflammatory activity; thus, their antidepressant potential is proportionally higher vs other NSAIDs. A limitation to their use is due to the cardiotoxic effect of these groups of molecules/agents.

**RESULTS AND DISCUSSION**

**Extraction, Isolation, and Characterization of Aegeline.** AMFE was prepared by using the Soxhlet extraction method. The preliminary TLC screening of extract with known standards yielded a spot similar to that for aegeline. Furthermore, high-performance thin-layer chromatography (HPTLC) analysis of the extract confirmed the presence of aegeline; the chromatogram corresponding to the standard aegeline has a retardation factor (Rf) of 0.24 at 250 nm (Figure 1). After that, AMFE was subjected to column chromatography, and the fraction eluted at hexane: ethyl acetate (6.5:3.5), yielding a single spot similar to the known standard, was collected. NMR and mass spectrometry analyses of an isolated molecule were performed.

**NMR and Mass Analyses.** \[^1H]_{\text{NMR}} = -2.5 (0.4, \text{CH}_2\text{OH}); \[^1H]_{\text{NMR}} (500 \text{MHz}, \text{CDCl}_3, 25 ^\circ \text{C}, \text{TMS } \delta) : 3.15 (d, J = 3.26 \text{ Hz, 1H, CH of CH}_2), 3.43-3.48 (m, 1H, CH), 3.81 (s, 3H, OCH}_3), 3.83-3.84 (m, 1H, CH of CH}_2), 4.87-4.88 (m, 1H, OH), 6.02 (br, 1H, NH), 6.39 (d, J = 15.62 Hz, 1H, ArCH), 6.90 (d, J = 8.39, 2H, ArH), 7.32 (d, J = 8.39, 2H, ArH), 7.36-7.37 (m, 3H, ArH), 7.49-7.50 (m, 2H, ArH), 7.65 (d, J = 6.03 Hz, 1H, ArCH).\[^13C]_{\text{NMR}} (125 \text{MHz, CDCl}_3 \delta): 47.6 (-, ve, CH2), 55.3 (+ve, OCH3), 73.4 (+ve, CH), 114.0 (+ve, ArCH), 120.0 (+ve, ArCH), 127.1 (+ve, ArCH), 127.8 (+ve, ArCH), 128.8 (+ve, ArCH), 129.8 (+ve, ArCH), 133.8 (ArC), 134.6 (ArC), 141.7 (+ve, ArCH), 159.3 (ArC), 167.0 (C=O); HRMS (microTOF-QII, MS, ESI): [M + H]+ calcd for C18H19O3N 298.1437; found, 298.1435. HRMS (microTOF-QII, MS, ESI): [M + K]+ calcd for C18H19O3N 336.0996; found, 336.0938 (Figure 2).

**In Silico Molecular Docking Studies.** Following the docking procedure, the compound (Figure 3) was docked at the catalytic binding pocket of enzymes MAO-A (PDB ID 2Z5Y), MAO-B (PDB ID 2V5Z), and iNOS (PDB ID 3E7G). Aegeline is successfully docked in the catalytic pocket of MAO-A and MAO-B with a binding energy of −10.06 kcal/mol and −10.09 kcal/mol, respectively. Aegeline in the catalytic pocket of MAO-A interacts with Tyr407 through an H bond (2.37 Å), while in the active site of MAO-B, aegeline shows H-bonding interaction with Gln264 (2.03 Å) and pi–pi stacking interactions with Tyr398 and Trp119 with its two aromatic rings. Aegeline was also docked at the active site of iNOS (PDB ID 3E7G). The best pose formed a hydrogen bond with Leu264 (1.93 Å), displaying binding energy of −31.54 kcal/
Based on these observations, it was indicated that aegeline had good binding interaction with enzymes MAO-A and iNOS (Figures 4 and 5).

**Behavioral Studies.** Pain and depression have been assessed to co-occur in up to 80% of patients. This comorbidity is more debilitating and pricier for the patients than either of these disorders alone.\(^3^5\) Clinically, it has been reported that pain perception in patients with depression is much higher than the cutoff for normal individuals.\(^3^6\) Reserpine-induced pain–depression dyad is a well-established and widely accepted model of these comorbid disorders.\(^2^3\) In the present study, reserpine administration induced pain and a depression-like state. A significant reduction in the paw withdrawal threshold was observed in PAM and eVF tests on days 4 and 6, delineating the algesic effect of reserpine (Figure 6a,b). Moreover, in the forced swim test, reserpine significantly...

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**Figure 4.** (a) Aegeline docked in the catalytic pocket of enzyme monoamine oxidase A [PDB ID 2Z5Y] showing hydrogen bonding (pink dotted lines) with Y407 [2.37 Å]. (b) Ligand interaction diagram of aegeline docked in the catalytic pocket of monoamine oxidase A [PDB ID 2Z5Y]. (c) Aegeline docked in the catalytic pocket of enzyme monoamine oxidase B [PDB ID 2V5Z] showing hydrogen bonding (pink dotted lines) with Q206 [2.03 Å] and pi–pi stacking interactions (green lines) with Y398 and W119 residues. (d) Ligand interaction diagram of aegeline docked in the catalytic pocket of monoamine oxidase B [PDB ID 2V5Z].

**Figure 5.** (a) Aegeline docked in the catalytic pocket of enzyme iNOS [PDB ID 3E7G] showing hydrogen bonding (pink dotted lines) with Y347 [1.93 Å]. (b) Ligand interaction diagram of aegeline docked in the catalytic pocket of iNOS [PDB ID 3E7G].
increased the immobility time on both days of tests (Figure 6c). These outcomes corroborate with the existing literature evidence.23,37 AMFE and aegeline pretreatment significantly increased the paw withdrawal threshold in both PAM and eVF, along with a reduction in immobility time in the FST on both days of tests. The behavioral effects observed with AMFE and aegeline treatment were analogous with the standard clorgyline.

Serum IL-6 Estimation. Preclinical and clinical studies have demonstrated that among all the proinflammatory cytokines, IL-6 plays a unique role in the pathophysiology of depression and its somatic consequences.12 A report by Rizzo et al. showed that intracerebroventricular treatment of recombinant IL-6 to mice elicits depression-like behaviors in mice.38 Furthermore, it has been demonstrated that patients with increased depressive symptoms and higher IL-6 levels experience more pain.36 In the present study, reserpine treatment significantly increased serum IL-6 compared to the normal control group. Pretreatment with AMFE and aegeline significantly reduced the level of IL-6 as compared to reserpinized animals. Although clorgyline treatment reduced the IL-6 level, the change was insignificant vs the reserpine group (Figure 7).

Immunohistochemical Analysis of iNOS. iNOS is an important isoform of the nitric oxide synthase family responsible for the production of nitric oxide.39 However, increased activation of iNOS results in nitric oxide’s abrupt production, which results in neurotoxic insult leading to oxidative insult, neuroinflammation, and pain.40−42 Furthermore, it has been well reported in the literature that inhibition of iNOS elicits an antinociceptive activity.43,44 iNOS also plays a pathological role in the progression of depressive symptoms, and its inhibition alleviates depression.45,46 In the current study, reserpine administration significantly increased the iNOS expression in the spine and brain tissue samples.47 AMFE and aegeline administration reversed the reserpine-induced increased expression of iNOS in the spine and the brain (Figure 8).

Brain MAO-A and Oxidative Stress Analysis. MAO-A is an important enzyme responsible for the oxidative deamination of biogenic amines.48 The pathological role of MAO-A in depression has been well established.49 Furthermore, it has been documented that monoamine depletion is directly linked with the dysfunction of the descending pain inhibitory system, which amplifies pain response.50,51 Various research groups have also shown the analgesic potential of MAO-A inhibitors, such as moclobemide and clorgyline.52−54 In the current study, reserpine treatment significantly increased the brain MAO-A activity (Figure 9). This finding was consistent with previously published scientific reports.55−57 AMFE and aegeline pretreatment significantly abrogated the MAO-A activity potentiating effect of reserpine. Furthermore, it has been demonstrated that metabolic end products of oxidative deamination of biogenic amines, such as hydrogen peroxide, results in enhanced production of reactive oxygen species (ROS), which leads to an increased oxidative insult and promotion of inflammatory cytokines.58−60 ROS are a set of biologically unstable entities known for their detrimental effect on cellular lipids, proteins, DNA, etc. High oxidative stress results in lipid peroxidation and inactivation of certain enzymes.51,52,39 The reduction in antioxidant levels, such as GSH, is associated with enhanced...
production of reactive oxygen species. In the present investigation, reserpine treatment significantly increased oxidative stress as evidenced by the increased brain TBARS level and the reduced brain GSH level. These alterations were abrogated considerably by AMFE and aegeline treatment (Table 1).

**CONCLUSION**

The above findings state that AMFE and isolated aegeline displayed an antinociceptive and antidepressant activities in reserpinized animals. The biochemical analysis showed that the protective effect of AMFE and aegeline against reserpine-induced pain–depression might be through the down-regulation of MAO-A hyperactivity, IL-6, and the iNOS level (Figure 10).

**Limitations.** The current study is a single-dose study. Further investigations involving multiple dose levels can be worth it. Moreover, the monoamine oxidase-A assay done in this study was carried out in the whole brain. Estimations of this in different MAO-A neuroanatomical-rich regions might also be worthy.

**Future Possible Treatment.** Since pain and depression are often comorbid, their pathophysiology is highly intercoiled. Chronic inflammation may further cause a shift in monoamine metabolism by activating certain cytotoxic pathways mediated via the proinflammatory cytokines. NA and 5-HT via modulation of specific receptor subtypes in the descending tracts are known to show nociception or antinociception. Anomalies in the descending tracts tend to alter pain perception or response. The off-label use of antidepressants for treating chronic pain is on the rise in the current clinical...
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MATERIALS AND METHODS

Table 1. Effect of Various Treatments on Oxidative Stress Parameters Employed in the Study

| biochemical parameter | groups | control (0.1% CMC)  | reserpine (0.5 mg/kg) | clorgyline (3 mg/kg) | AMFE (200 mg/kg) | aegeline (10 mg/kg) |
|------------------------|--------|--------------------|------------------|----------------|----------------|--------------------|
| TBARS (nM/mg)          | mean ± SD | 0.177 ± 0.020       | 0.311 ± 0.044*   | 0.158 ± 0.010*   | 0.166 ± 0.016*   | 0.173 ± 0.011*     |
| reduced GSH (μg/mg)    | mean ± SD | 0.573 ± 0.084       | 0.137 ± 0.035*   | 0.374 ± 0.033*   | 0.458 ± 0.031*   | 0.419 ± 0.023*     |

*Values are represented as mean ± SD where *p < 0.05 vs control and #p < 0.05 vs reserpine for both TBARS and reduced GSH.

Figure 10. Mechanistic diagram of the protective effect of aegeline in pain–depression dyad.

approaches. Recently published data have not only proved their effectiveness but also have unlocked fresher mechanistic insights. Tricyclic antidepressants and other atypical antidepressants have demonstrated effectiveness in treating chronic pain associated with inflammatory bowel disease. Serotonin and norepinephrine reuptake inhibitors (SNRIs), such as duloxetine, are known for their modulatory effect on the recovery of the noradrenergic descending inhibitory system in the spinal regions, thus effectively treating neuropathic pain. The above-stated evidence strongly advocates the use of agents modulating neurotransmitter metabolism for treating pain. Although, the only limiting factor in their use is the adverse events, such as insomnia, sexual dysfunction, hypotension, tachycardia, nausea, blurred vision, constipation, etc., associated with their use. Furthermore, nonsteroidal anti-inflammatory drugs (NSAIDs) have shown antidepressant potential when tested against placebos. The role of proinflammatory cytokines, primarily the interleukins (IL)-1β, IL-6, IL-18; TNF-α; and interferon-γ, has been well-published in the scientific literature. Agents such as Celecoxib, known for their selective cox-2 inhibitory action, have demonstrated antidepressant activity proportionately higher than other class agents.

These literature evidence advocate the future use and development of agents who primarily target proinflammatory cytokines and prevent the depletion of biogenic amines.

■ MATERIALS AND METHODS

Extract Preparation. Aegle marmelos fruits were collected from the botanical garden of Guru Nanak Dev University, Amritsar, Punjab. Authentication of the plant material was done by Dr. AS Sooden from the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab (ref no. 855 Bot. and Env. Sci., H/V-123). Collected fruits were thoroughly washed to remove any dust or foreign particles. The fruit was cut open; the pulp was removed and deseeded. Pulp was air-dried, and a coarse powdered material was obtained. The dried and powdered plant matter was held in a hermetic container at 4 °C till future use. Powdered fruit extract was subjected to soxhlation with 75% methanol at 70 °C for 120 min. The crude extract thus obtained was dried under reduced pressure and subjected to lyophilization. Until further use, the resultant product was refrigerated at 4 °C.

HPTLC Analysis of the Extract. HPTLC was performed with a CAMAG instrumental setup (Switzerland) consisting of Linomat-V, an automatic sample applicator (100 μL syringe), CAMAG TLC scanner III for detection, and a winCATS planner chromatography manager for result recording and interpretation. Extract (15 μL) and standard were applied as bands (8 mm from the base; band length of 6 mm) on TLC sheets (precoated silica gel 60 F254) of dimensions 30 mm × 100 mm (Mercck, Germany) with an application rate of 150 nL/s. Chromatogram development was executed using a twin trough glass chamber (in an ascending mode) presaturated for 10 min. During the test procedure, the standard room temperature and relative humidity were maintained (25 ± 2 °C; 55 ± 5%). Hexane and ethyl acetate were taken as the mobile phase solvents in a ratio of 6:4 (v/v). After chromatogram development, the plates were dried suitably to remove the solvent and subjected to densitometric scanning using the TLC scanner (with winCATS); in a reflectance absorbance mode, TLC scan was run between 240 to 260 nm. Densitometric analysis was performed in a reflectance mode at 250 nm. Slit dimensions were fixed at 4.00 × 30 mm, scanning speed was limited to 20 mm/s, and data resolution was set to 100 μm/step (second-order optical filter). The chromatographic peaks were observed and recorded for further analysis.

Isolation of Aegeline. The methanolic extract of Aegle marmelos was subjected to column chromatography. Dried extract (20 g) was packed into a glass column and eluted with the mobile phase (n-hexane:ethyl acetate, ratio pattern from 100:0 to 100:1) in a gradient manner. The column’s flow rate was maintained at 20 drops per minute, and the fraction volume was fixed at 50 mL for optimal isolation and separation of components. Different fractions were collected, concentrated, and pooled based on chromatographic similarities (TLC). Fractions obtained at the hexane to ethyl acetate ratio of 65:35 (single spot at the same RF compared to standard) were pooled. The pooled fraction was subjected to mass spectroscopy and NMR analysis for the characterization of the obtained molecule.

Characterization of Aegeline by NMR and MASS Analyses. 1H and 13C NMR spectra were logged with JEOL 400 MHz, Bruker 500 Hz, and 125 Hz NMR spectrophotometers. Deuterated chloroform was used as the base solvent. Chemical shifts were recorded (as particle per million)
In Silico Molecular Docking Studies. Isolated aegeline was docked at the active binding sites of enzyme monoamine oxidase and inductive nitric oxide synthase to ascertain its potential action.71,72

Molecular Docking. Schrödinger software (release 2014-4, Maestro, version 10.0) was used for molecular docking following three major steps:

Step 1: Protein Preparation. For protein preparation, X-ray crystal structures of MAO-A (PDB code 2Z5Y), MAO-B (PDB code 2VSZ), and iNOS (PDB code 3E7G) were taken from Protein Data Bank (PDB). By using Protein Preparation Wizard (PrepWiz) of Schrödinger, these structures were then processed. In this preprocessing step, hydrogens were added, and bond orders were assigned following the deletion of excess water molecules. The heteroatoms were then ionized by Epik at biological pH to consider the protein permeability and drug solubility. The H bonds were optimized to reduce the steric clashes by histidine, aspartate, glutamate, and hydroxyl-containing amino acids. Lastly, the OPLS 2005 force field was used to minimize the complete protein structure to the least possible energy state.

Step 2: Ligand Preparation. Ligand preparation is done using the Lig prep tool of Schrödinger, which converts 1D/2D structures of a ligand to 3D. It is required to perform because molecules generally lack 3D coordinates, stereochemistry, ionization, and tautomers. Before docking, the ligand should have 3D coordinates along with all the above properties. Therefore, the lowest energy state of the ligand was needed to be prepared. Finally, this ligand molecule was minimized with the help of the OPLS 2005 force field.

Step 3: Molecular Docking. For docking, the grids were generated by using the grid-based energy descriptor, which had a default set of options with a van der Waals radius of 1.0 Å and the grid size is 20 Å. This grid was then used to calculate the prepared ligand’s interaction with the receptor using the XP ligand docking in Glide. We considered only poses having low energy conformations and good H-bond geometries.

In Vivo Studies. Animals. Male Swiss albino mice (30–40 g) were acquired from the authorized breeders (NIPER, Mohali, Punjab). The lab animals were contained in the central animal facility present within the university (GNDU). The fresh batch of animals was quarantined and acclimatized with the new environment for not less than 2 weeks. The animals were provided unrestricted access to food (Aashirwad Industries Ltd., Chandigarh.) and portable water. The animal study protocol was presented before the Ethics Committee (IAEC) and was duly approved (226/CPCSEA/2018/39). All the ethical guidelines for conducting animal experiments were duly followed as laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

The Experimental Strategy. Mice were randomly distributed into five groups (N = 5) as follows: Group 1 is obliged as the normal control and was injected with 0.1% carboxymethylcellulose; group 2 is the disease control and was administered with reserpine (0.5 mg kg\(^{-1}\) s.c.); group 3, standard treatment was administered using clorgyline (3 mg kg\(^{-1}\) i.p.) 30 min preliminary to the reserpine shot; group 4 was treated with the extract (200 mg kg\(^{-1}\) p.o.); and group 5 was administered with aegeline (10 mg kg\(^{-1}\) p.o.) respectively 30 min before reserpine administration. Behavioral studies were carried out on the second, fourth, and sixth day. At the end of the six day observations, test animals were euthanized, and brain tissue was collected for further evaluation.

Biochemical Analysis. Thio-Barbituric Acid Reactive Substances (TBARS) Estimation. The degree of lipid oxidized in the test sample was determined by determining the amount of TBARS.80,81 TBA-TCA-HCl trimix (0.6 mL) was added to the cytosolic fraction. This was followed by keeping the reaction mixture heated for 15 min over a water bath. After cooling the reaction mixture to standard lab conditions, it was centrifuged for 10 min (3000 g). The upper layer was separated and stored.

Reduced Glutathione Estimation (GSH). This assay is a direct measure of oxidative potential in terms of its ability to measure the reducing capacity of glutathione in the test sample by directly measuring the level of reduced glutathione.82 Disodium hydrogen phosphate (2 mL) was added to the homogenate. Freshly prepared DTNB was added to the resultant mixture. The chromophore thus obtained was measured spectrophotometrically at 535 nm.

Monoamine Oxidase Activity. The animals were euthanized as per the protocol, and the brain sample was isolated. The sample (10 mg) was weighed and homogenized in 100 μL of an extracting assay buffer. This was followed by centrifugation of the homogenate at 2000 rpm (10 min). The resultant upper layer thus obtained was pooled and subjected to MAO-A activity assay using the commercially available ELISA kit (Sigma-Aldrich).
Serum IL-6 Estimation. On the sixth day, the animals were anesthetized, and blood samples were withdrawn via the cardiac puncture route. The blood samples were centrifuged (5000 rpm) for 10 min at 10 °C to obtain the serum. A commercially available ELISA kit for IL-6 (Krishgen Biotech, Mumbai Ltd.) was used to estimate the level of IL-6, and the results are expressed in picogram per milliliter (pg/ml).

Immunofluorescence Studies. At the end of the six day procedures, the animals were anesthetized suitably and preperfused with a 4% paraformaldehyde phosphate buffer solution. L4 and L5 of the spinal cord was removed post-preperfusion and stored for 4 h in the 4% paraformaldehyde phosphate buffer solution. This was followed by immersion of the removed section in sucrose solution (30% w/v) at 4 °C overnight. 10 μm-thick sections were randomly cut using a cryomicrotome (Leica Biosystems). These sections were sequentially incubated in a mouse anti-iNOS antibody (1:100) followed by intermittent washing using the buffer. In the next step, the sections were subjected to treatment with fluorescent Alexa fluor and a secondary goat antimouse antibody for 2 h, followed by washing with buffer solution. In the terminal stage of the procedure, the sections were stained with DAPI and fixed on a glass slide for viewing. The slide imaging and microphotographs were taken using a confocal microscope (Olympus, FV 1200; at CIL, Central University of Batinda).

Statistical Analysis. Results were expressed as mean ± SEM. Intergroup variations were determined by one-way analysis of variance (ANOVA), followed by Tukey’s multiple comparisons post-hoc analysis by Prism 7.0 software (Graph Pad Prism Inc. La Jolla California U.S.A.).

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Author Contributions
A.P.S. performed aforementioned experiments and wrote the manuscript. L.S. assisted in pharmacological studies and statistical analysis. P.S. assisted in the in silico analysis and spectroscopic characterization. R.B. conceptualized the idea, and supervised the work and funding acquisition.

Notes
The authors declare no competing financial interest.

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REFERENCES

(1) Rayner, L.; Hotopf, M.; Petkova, H.; Matcham, F.; Simpson, A.; McCracken, L. M. Depression in patients with chronic pain attending a specialised pain treatment centre: prevalence and impact on health care costs. Pain. 2016, 157, 1472–1479.
(2) Ihsak, W. W.; Wen, R. Y.; Nagdechi, L.; Vanle, B.; Deng, J.; Knoop, M.; Dascal, J.; Marcia, L.; Gohar, Y.; Eskander, L.; Yadegar, J.; Hanna, S.; Sadek, A.; Aguilar-Hernandez, L.; Danovitch, I.; Louy, C. Pain and depression: a systematic review. Harv. Rev. Psychiatry 2018, 26, 352–363.
(3) Bair, M. J.; Robinson, R. L.; Katon, W.; Kroenke, K. Depression and pain comorbidity: a literature review. Arch. Intern. Med. 2003, 163, 2433–2445.

(4) James, S. L.; Abate, D.; Abate, K. H.; Abay, S. M.; Abbafati, C.; Abbasi, N.; Abbastabar, H.; Abd-Allah, F.; Abdela, J.; Abdelalim, A.; Abdollahpour, I.; et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 2018, 392, 1789–1858.
(5) Gaskin, D. J.; Richard, P. The economic costs of pain in the United States. J. Headache Pain 2012, 13, 71–724.
(6) Enright, A.; Goucke, R. The Global Burden of Pain. Anesth. Analg. 2016, 123, 529–530.
(7) Maletic, V.; Raison, C. L. Neurobiology of Depression, Fibromyalgia and Neuropathic Pain. Front. Biosci. 2009, 14, 5291–5338.
(8) Bravo, L.; Llorca-Torralba, M.; Berrocoso, E.; Micó, J. A. Monoamines as Drug Targets in Chronic Pain: Focusing on Neuropathic Pain. Front. Neurosci. 2019, 13, 1268.
(9) Youdim, M. B. H.; Bakhle, Y. S. Monoamine Oxidase: Isoforms and Inhibitors in Parkinson’s Disease and Depressive Illness. Br. J. Pharmacol. 2006, 147, 5287–5296.
(10) Sheng, J.; Liu, S.; Wang, Y.; Cui, R.; Zhang, X. The Link between Depression and Chronic Pain: Neural Mechanisms in the Brain. Brain. 2017, 2017, 1–10.
(11) Zhang, J. M.; An, J. Cytokines, Inflammation, and Pain. Int. Anesthesiol. Clin. 2007, 45, 27–37.
(12) Ting, E. Y. C.; Yang, A. C.; Tsai, S. J. Role of Interleukin-6 in Depressive Disorder. Int. J. Mol. Sci. 2020, 21, 2194.
(13) Rothaug, M.; Becker-Pauly, C.; Rose-John, S. The Role of Interleukin-6 Signaling in Nervous Tissue. Biochim. Biophys. Acta, Mol. Cell Res. 2016, 1863, 1218–1227.
(14) Zhou, Y.-Q.; Liu, Z.; Liu, Z.-H.; Chen, S.-P.; Li, M.; Shahveranov, A.; Ye, D.-W.; Tian, Y.-K. Interleukin-6: An Emerging Regulator of Pathological Pain. 2016, 13 (1), 141, DOI: 10.1186/s12974-016-0607-6.
(15) Hodes, G. E.; Ménard, C.; Russo, S. J. Integrating Interleukin-6 into Depression Diagnosis and Treatment. Neurobiol. Stress 2016, 4, 15–22.
(16) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. Free Radicals and Antioxidants in Normal Physiological Functions and Human Disease. Int. J. Biochem. Cell Biol. 2007, 39, 44–84.
(17) Villarete, L. H.; Remick, D. G. Nitric oxide regulation of interleukin-8 gene expression. Shock 1997, 9, 64–69.
(18) Demirel, I.; Vumma, R.; Mohlin, C.; Svensson, L.; Šäve, S.; Persson, K. Nitric Oxide Activates IL-6 Production and Expression in Human Renal Epithelial Cells. Am. J. Nephrol. 2012, 36, 524–530.
(19) Sarkar, S.; Sonkar, R.; Bhatia, G.; Tadigoppula, N. Synthesis of New N-Acryl-1-Amino-2-Phenylethanol and N-Acyl-1-Amino-3-Aryloxypropanol and Evaluation of Their Antihyperlipidemic, LDL-
Oxidation and Antioxidant Activity. *Eur. J. Med. Chem.* 2014, 80, 135–144.

(20) Manandhar, B.; Paudel, K. R.; Sharma, B.; Karki, R. Phytochemical Profile and Pharmacological Activity of Aegle Marmelos Linn. *J. Integr.Med.* 2018, 16, 153–163.

(21) Nugroho, A. E.; Ryanto, S.; Sukari, M. A.; Maeyama, K. Effects of Aegelina, a Main Alkaloid of Aegle Marmelos Correa Leaves, on the Histamine Release from Mast Cells. *Pak. J. Pharm. Sci.* 2011, 24, 359–367.

(22) Derf, A.A.; Sharma, A.; Bharate, S. B.; Chaudhuri, B. Aegelina, a Natural Product from the Plant Aegle Marmelos, Mimics the Yeast SNARE Protein Sec22p in Suppressing α-Synuclein and Box Toxicity in Yeast. *Bioorg. Med. Chem. Lett.* 2019, 29, 454–460.

(23) Sousa, F. S. S.; Birmann, P. T.; Baldinotti, R.; Fronza, M. G.; Balaguez, R.; Alves, D.; Brünig, C. A.; Savegnago, L. α-(Phenylselenyl) Acetophenone Mitigates Reserpine-Induced Pain—depression Dysad: Behavioral, Biochemical and Molecular Docking Evidences. *Brain Res. Bull.* 2018, 142, 129–137.

(24) Santos, J. R.; Cunha, J. A. S.; Dierschnabel, A. L.; Campelo, C. L. C.; Leão, A. H. F. F.; Silva, A. F.; Engelberth, R. C. G. J.; Izidio, G. S.; Cavalcante, J. S.; Abílio, V. C.; Ribeiro, A. M.; Silva, R. H. Cognitive, Motor and Tyrosine Hydroxylase Temporal Impairment in a Model of Parkinsonism Induced by Reserpine. *Behav. Brain Res.* 2013, 253, 68–77.

(25) Buhidma, Y.; Rukavina, K.; Chaudhuri, K. R.; Duty, S. Potential of Animal Models for Advancing the Understanding and Treatment of Pain in Parkinson’s Disease. *npj Parkinson’s Dis.* 2020, 6, 1–7.

(26) Kleiber, B.; Jain, S.; Trivedi, M. H. Depression and Pain: Implications for Symptomatic Presentation and Pharmacological Behav. *Brain Res.* 2013, 153, 686–692.

(27) Nekovarova, T.; Yamamotova, A.; Vales, K.; Stuchlik, A.; Fricova, J.; Rokyta, R. Common Mechanisms of Pain and Depression: Are Antidepressants Also Analgesics? *Front. Behav. Neurosci.* 2014, 8, 99.

(28) Gallagher, R. M. Management of Neuropathic Pain: translating mechanistic advances and evidence-based research into clinical practice. *Clin. J. Pain* 2006, 22, S2–S8.

(29) Wolfe, G. I.; Trivedi, J. R. Painful Peripheral Neuropathy and Its Nonsurgical Treatment. *Curr. Neuropharmacol.* 2013, 11, 756–781.

(30) Muntean, D. M.; Boia, E. S. Monoamine Oxidase-Related Vascular Neurotoxic Catecholamine Metabolite in Nociceptors Contributes to the nociceptive Effect of Moclobemide Is Mediated by Noradrenergic Pathways. *Exp. Physiol.* 2012, 97, 2164.

(31) Thase, M. E. The Role of Monoamine Oxidase Inhibitors in Depression Treatment Guidelines. *J. Clin. Psychiatry* 2012, 73, 10–16.

(32) Wood, P. B. Role of Central Dopamine in Pain and Analgesia. *Expert Rev. Neurother.* 2008, 8, 781–797.

(33) Yam, M.; Loh, Y.; Tan, C.; Khadijah Adam, S.; Abdul Manan, N.; Basir, R. General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain Regulation. *Int. J. Mol. Sci.* 2018, 19, 2164.

(34) Schreiber, S.; Getslev, V.; Weizman, A.; Pick, C. G. The Anti-nociceptive Effect of Moclobemide Is Mediated by Noradrenergic Pathways. *Neurosci. Lett.* 1997, 237, 544.

(35) Dina, O. A.; Khasar, S. G.; Alessandri-Haber, N.; Bogen, O.; Chen, X.; Green, P. G.; Reichling, D. B.; Messing, R. O.; Levine, J. D. Neurotoxic Catecholamine Metabolite in Noceptrors Contributes to Painful Peripheral Neuropathy. *Eur. J. Neurosci.* 2008, 28, 1180–1190.

(36) Villarinho, J. G.; Pinheiro, K. M.; Iovu, C. M.; Oliveira, S.; Machado, P.; Martins, M. A. P.; Bonacorsco, H. G.; Zanatta, N.; Fachinetti, R.; Ferreira, J. The Anti-nociceptive Effect of Reversible Monoamine Oxidase-A Inhibitors in a Mouse Neuropathic Pain Model. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* 2013, 44, 136–142.

(37) Zayek, E.; Zoka, A.; Bogatko, K.; Wyska, E.; Wokos, K.; Świąder, K.; Doboszewski, U.; Właz, A.; Wróbel, A.; Właz, P.; Sereńko, A. Antidepressant-Like Activity of Typical Antidepressant
Drugs in the Forced Swim Test and Tail Suspension Test in Mice Is Augmented by DMPX, an Adenosine A<sub>2A</sub> Receptor Antagonist. Neurotoxic. Res. 2019, 35, 344–352.

(56) Tabari, S. A.; Esfahani, M. L.; Hosseini, S. M.; Rahimi, A. Neurobehavioral Toxicity of Triclosan in Mice. Food Chem. Toxicol. 2019, 130, 154–160.

(57) Meyer, J. H.; Ginovart, N.; Boovariwala, A.; Sagrati, S.; Hussey, D.; Garcia, A.; Young, T.; Fraschak-Rieder, N.; Wilson, A. A.; Houle, S. Elevated Monoamine Oxidase A Levels in the Brain: An Explanation for the Monoamine Imbalance of Major Depression. Arch. Gen. Psychiatry 2006, 63, 1209–1216.

(58) Ahmad, A.; Rasheed, N.; Banu, N.; Palit, G. Alterations in Monoamine Levels and Oxidative Systems in Frontal Cortex, Striatum, and Hippocampus of the Rat Brain during Chronic Unpredictable Stress. Stress 2010, 13, 356–365.

(59) Patel, P. R. Norepinephrine Reduces Reactive Oxygen Species (ROS) and DNA Damage in Ovarian Surface Epithelial Cells. J. Bioanal. Biomed. 2015, 07, 75.

(60) Cartwright, C.; Gibson, K.; Read, J.; Cowan, O.; Dehar, T. Long-term anti-depressant use: patient perspectives of benefits and adverse effects. Patient prefer.ence and adherence. 2016, Volume 10, 1401.

(61) Golecki, P.; Szemraj, J.; Bienkiewicz, M.; Florzkoew, A.; Galecka, E. Lipid Peroxidation and Antioxidant Protection in Patients during Acute Depressive Episodes and in Remission after Fluoxetine Treatment. Pharmacol. Rep. 2009, 61, 436–447.

(62) Maria Michel, T.; Pulschen, D.; Thome, J. The Role of Oxidative Stress in Depressive Disorders. Curr. Pharm. Des. 2012, 18, 5890–5899.

(63) Armstrong, J. S.; Steinauer, K. K.; Hornburg, B.; Irish, J. M.; Lecane, P.; Birrell, G. W.; Peehl, D. M.; Knox, S. J. Role of Glutathione Depletion and Reactive Oxygen Species Generation in Apoptotic Signaling in a Human B Lymphoma Cell Line. Cell Death Differ. 2002, 9, 252–263.

(64) Van den Ameele, S.; Fuchs, D.; Coppens, V.; de Boer, P.; Timmers, M.; Sabbe, B.; Malfait, A. M. Markers of Inflammation and Monoamine Metabolism Indicate Accelerated Aning in Bipolar Disorder. Front. Mol. Psychiatry 2018, 9, 250.

(65) Marks, D.; Shah, M.; Patkar, A.; Masand, P.; Park, G.-Y.; Pae, C.-U. Serotonin-Norepinephrine Reuptake Inhibitors for Pain Control: Premise and Promise. Curr. Neuropharmacol. 2009, 7, 331–336.

(66) Bannister, K.; Dickenson, A. H. What Do Monoamines Do in Pain Modulation? Curr. Opin. Support Palliat. Care 2016, 10, 143–148.

(67) Urrits, I.; Peck, J.; Orhurhu, M. S.; Wolf, J.; Patel, R.; Orhurhu, V.; Kaye, A. D.; Viswanath, O. Off-Label Antidepressant Use for Treatment of Management of Chronic Pain: Evolving Understanding and Comprehensive Review. Curr. Pain. Headache Rep. 2019, 23, 66.

(68) Ikandar, H. N.; Cassell, B.; Kanuri, N.; Gyawali, C. P.; Gutierrez, A.; Dassopoulos, T.; Ciorba, M. A.; Sayuk, G. S. Tricyclic Antidepressants for Management of Residual Symptoms in Inflammatory Bowel Disease. J. Clin. Gastroenterol. 2014, 48, 423–429.

(69) Köhler, O.; Benros, M. E.; Nordentoft, M.; Farkouh, M. E.; Iyengar, R. L.; Mors, O.; Krogh, J. Effect of Anti-Inflammatory Treatment on Depression, Depressive Symptoms, and Adverse Effects. JAMA Psychiatry 2014, 71, 1381.

(70) Fischer, R.; Maier, O. Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. Oxid. Med. Cell. Longevity 2015, 2015, 610813.

(71) Abid, S. M. A.; Aslam, S.; Zaih, S.; Bakht, S. M.; Ahmad, M.; Ahar, M. M.; Gardiner, J. M.; Iqbal, J. Pyrazolobenzothiazine-Based Carbothioamides as New Structural Leads for the Inhibition of Monoamine Oxidases: Design, Synthesis, in Vitro Bioevaluation and Molecular Docking Studies. Medchemcomm 2017, 8, 452–464.

(72) Wei, J.; Cheng, Y.; Guo, W. H.; Wang, D. C.; Zhang, Q.; Li, D.; Rong, J.; Gao, J. M. Molecular Diversity and Potential Anti-Neuroinflammatory Activities of Cyathane Diterpenoids from the Basidiomycete Cyathus Africanus. Sci. Rep. 2017, 7, 8883.

(73) Greenwood, J. R.; Calkins, D.; Sullivan, A. P.; Shelley, J. C. Towards the Comprehensive, Rapid, and Accurate Prediction of the Favorable Tautomeric States of Drug-like Molecules in Aqueous Solution. J. Comput-Aided Mol. Des. 2010, 24, 591–604.

(74) Shelley, J. C.; Cholleti, A.; Fye, L. L.; Greenwood, J. R.; Timlin, M. R.; Uchimaya, M. Epik: A Software Program for pKa Prediction and Protonation State Generation for Drug-like Molecules. J. Comput-Aided Mol. Des. 2007, 21, 681–691.

(75) Shivakumar, D.; Williams, J.; Wu, Y.; Damm, W.; Shelley, J.; Sherman, W. Prediction of Absolute Solvation Free Energies Using Molecular Dynamics Free Energy Perturbation and the Opls Force Field. J. Chem. Theory Comput. 2010, 6, 1509–1519.

(76) Jorgensen, W. L.; Tirado-Rives, J. The OPLS Potential Functions for Proteins. Energy Minimizations for Crystals of Cyclic Peptides and Crambin. J. Am. Chem. Soc. 1988, 110, 1657–1666.

(77) Jorgensen, W. L.; Maxwell, D. S.; Tirado-Rives, J. Development and Testing of the OPLS All-Atom Force Field on Conformational Ennergetics and Properties of Organic Liquids. J. Am. Chem. Soc. 1996, 118, 11225–11236.

(78) Ko, H.-G.; Oh, S.-B.; Zhuo, M.; Kaang, B.-K. Reduced Acute Nociception and Chronic Pain in Shank2−/−Mice. Mol. Pain 2016, 12, 174480619664705.

(79) Miller, R. E.; Ishihara, S.; Bhattacharyya, B.; Delaney, A.; Menichella, D. M.; Miller, R. J.; Malfait, A. M. Chemogenetic Inhibition of Pain Neurons in a Mouse Model of Osteoarthritis. Arthritis Rheumatol. 2017, 69, 1429–1439.

(80) Oikawa, H.; Ohishi, N.; Yagi, K. Assay for Lipid Peroxides in Animal Tissues by Thiobarbituric Acid Reaction. Anal. Biochem. 1979, 95, 351–358.

(81) Wills, E. D. Mechanisms of Lipid Peroxide Formation in Tissues Role of Metals and Haematin Proteins in the Catalysis of the Oxidation of Unsaturated Fatty Acids. Biochim. Biophys. Acta, Lipids Lipid Metab. 1965, 98, 238–251.

(82) Ellman, G. L. Tissue Sulphydryl Groups. Arch. Biochem. Biophys. 1959, 82, 70–77.