Phytochemical Characterization of Hypericum perforatum Extract and Bioassay-Guided Isolation of Hypericin (Compound C1): Extraction Methodology, Structural Elucidation, and Monoaminergic Restorative Efficacy in a Reserpine-Induced Depression Model

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Abstract

Background: Major Depressive Disorder (MDD) is a debilitating global public health condition characterized by monoaminergic deficits, oxidative stress, and structural neurodegeneration. Botanical preparations of Hypericum perforatum (St. John's Wort) have demonstrated multi-target antidepressant potential, yet the precise phytochemical identity of their principal bioactive constituent remains a subject of ongoing pharmacological investigation.

Methods: In the present study, aerial parts of H. perforatum were subjected to a modified hydroalcoholic cold maceration protocol (70% ethanol, 72 h, 1:10 w/v). The lyophilized extract was standardized using HPLC for the primary pharmacopeial markers, hypericin and hyperforin. Bioassay-guided isolation was performed via extensive silica gel open-column chromatography (60–120 mesh, gradient elution), and the resultant pure fraction was subjected to comprehensive spectroscopic characterization (MS-qTOF, FT-IR, 1H-NMR, 13C-NMR). The monoaminergic efficacy of the isolated compound (Compound C1) and the crude extract was evaluated against reserpine-induced depression in adult male Wistar rats (n = 8/group) using HPLC-ECD quantification of serotonin (5-HT), norepinephrine (NE), and dopamine (DA) in discrete brain regions.

Results: The extraction yielded 18.5% (w/w) of a standardized dark-brown lyophilizate containing 0.38% hypericin and 4.6% hyperforin, surpassing pharmacopeial minima. Open-column chromatography of 20.0 g extract yielded 120 mg of a pure, dark-red crystalline compound. Spectroscopic analysis (m/z 505.09 [M+H]+; C30H16O8) unequivocally identified Compound C1 as Hypericin, a naphthodianthrone. In the reserpine-induced depression model, reserpine (2 mg/kg, i.p., three days) produced profound depletion of hippocampal 5-HT and NE, and striatal DA. Fourteen-day oral administration of Compound C1 (300 mg/kg) restored all three monoamines to levels statistically comparable to the reference standard imipramine (15 mg/kg, i.p.).

Conclusion: The present investigation provides a systematic phytochemical framework establishing Hypericin as the principal bioactive constituent of H. perforatum, and furnishes definitive neurochemical evidence for its robust monoaminergic restorative activity. These findings position standardized Hypericin (Compound C1) as a highly viable multi-target therapeutic candidate for major depressive disorder.

Keywords: Hypericum perforatum · St. John's Wort · Hypericin · Compound C1 · Bioassay-guided isolation · HPLC standardization · Monoamine restoration · Reserpine model · Major Depressive Disorder

1. Introduction

Major Depressive Disorder (MDD) is one of the most prevalent and disabling psychiatric conditions worldwide, affecting hundreds of millions of individuals and imposing substantial economic and societal burden[1–3]. The pathophysiology of MDD is multifactorial, encompassing deficiencies in the central monoaminergic systems—primarily serotonergic, noradrenergic, and dopaminergic—that regulate mood, cognition, reward processing, and stress responsivity[4–6]. Despite decades of pharmacological development, conventional antidepressants such as selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs) exhibit significant limitations, including high rates of treatment resistance (30–40% of patients), delayed therapeutic onset, and a broad spectrum of adverse effects[7–9].

Hypericum perforatum (St. John's Wort, SJW), a perennial flowering herb widely distributed across temperate regions, has been employed in traditional medicine for centuries as a remedy for depressive states and mood dysregulation[10–11]. Clinical and preclinical evidence has established its antidepressant efficacy, broadly attributed to the synergistic actions of its two principal marker compounds: hypericin, a naphthodianthrone pigment, and hyperforin, an acylphloroglucinol derivative[12–14]. However, the phytochemical characterization of isolated, pure bioactive constituents from standardized

SJW extracts, along with their individualized neurochemical mechanistic profiles in validated animal models, remains an important area requiring rigorous investigation[15].

The reserpine-induced depression model is a pharmacologically validated paradigm of high construct validity for MDD research[16]. Reserpine irreversibly blocks the vesicular monoamine transporter-2 (VMAT-2), preventing monoamine storage and inducing their precipitous enzymatic degradation by cytoplasmic monoamine oxidase (MAO)[17–18]. This results in a measurable, region-specific central monoamine collapse that recapitulates the core neurochemical pathology of MDD, making it ideally suited for the evaluation of monoaminergic mechanisms[19].

The present study was designed with two integrated objectives: (i) to establish a validated phytochemical workflow for the extraction, HPLC standardization, and bioassay-guided isolation of the primary bioactive constituent of *H. perforatum*, with comprehensive spectroscopic structural elucidation; and (ii) to assess the monoaminergic restorative capacity of the isolated compound and the crude extract via HPLC with electrochemical detection (HPLC-ECD) in a reserpine-induced depression model in Wistar rats[20].

2. Materials And Methods

2.1 Plant Material Procurement and Authentication

Fresh aerial parts of Hypericum perforatum were harvested during the peak flowering stage (June–July) from a certified herbal cultivation facility. The geographic coordinates and soil conditions of the collection site were fully documented. Rigorous morphological and taxonomic identification was conducted by a senior botanist at the Botanical Survey of India (02961).

The harvested plant material was thoroughly washed with distilled water to remove surface contaminants. The material was subsequently spread in thin layers on blotting paper and shade-dried in a well-ventilated, moisture-controlled environment (25–30°C) for 14 days, strictly avoiding direct sunlight to prevent photolytic degradation of photosensitive secondary metabolites. Following the confirmation of constant dry weight, the dried material was pulverized using an industrial mechanical grinder and passed through a No. 40 mesh sieve to achieve a uniform particle size that maximizes the surface area for extraction.

2.2 Hydroalcoholic Extraction

Extraction was performed utilizing a modified cold maceration technique. Precisely 500.0 g of the pulverized plant material was transferred into a 10 L amber-colored glass carboy and macerated with 5.0 L of 70% ethanol (v/v in distilled water), achieving a solid-to-solvent ratio of 1:10 (w/v). The maceration was conducted for 72 h at room temperature (25 ± 2°C) under continuous orbital shaking at 120 rpm to enhance mass transfer efficiency. Following maceration, the suspension was filtered through a muslin cloth and then vacuum-filtered through a Büchner funnel (Whatman No. 1 filter paper over a 1 cm Celite bed) to remove fine particulates.

The marc was subjected to two additional successive extractions under identical conditions, and all three filtrate pools were combined. The pooled filtrate was concentrated under reduced pressure using a rotary evaporator (Heidolph Laborota 4000), with the water bath strictly maintained at 40°C and the flask rotating at 100 rpm under a vacuum of 150 mbar to evaporate the ethanol without thermally degrading the phytoconstituents. The concentrated aqueous suspension was subsequently frozen at -80°C and lyophilized (freeze-dryer: -50°C, 0.01 mbar) for 48 h to yield a fine, hygroscopic, dark-brown powder.

2.3 HPLC Standardization of the Extract

The lyophilized extract was standardized for its primary bioactive markers—hypericin and hyperforin—by HPLC, conducted in accordance with United States Pharmacopeia (USP) monographs. The HPLC system was equipped with a C18 reverse-phase column (250 mm × 4.6 mm, 5 μm). For hypericin quantification, the mobile phase consisted of methanol, ethyl acetate, and 0.1 M sodium dihydrogen phosphate buffer (pH 2.5), with detection at 590 nm. For hyperforin, gradient elution of acetonitrile and phosphoric acid-acidified water was utilized, with detection at 275 nm.

2.4 Bioassay-Guided Isolation by Open-Column Chromatography

Silica gel (60–120 mesh, Merck) was activated at 110°C for 2 h prior to use. A glass chromatography column (100 cm × 5 cm internal diameter) was uniformly packed with 500 g of activated silica gel via the wet slurry method in petroleum ether (40–60°C). Precisely 20.0 g of the lyophilized SJW extract, pre-adsorbed onto 40.0 g of silica gel, was loaded as a uniform dry layer over the packed bed. A 2 cm layer of acid-washed sea sand was placed over the loaded sample to prevent bed disruption.

The column was eluted using a stepwise gradient of increasing polarity: 100% petroleum ether → petroleum ether:ethyl acetate (90:10, 80:20, 70:30, 50:50 v/v) → 100% ethyl acetate → ethyl acetate:methanol (90:10, 80:20 v/v). Fractions of 50 mL were collected via an automated fraction collector at a constant flow rate of 2.0 mL/min (250 total fractions). All fractions were monitored by analytical TLC on Silica gel 60 F254 aluminum plates (Merck), developed in appropriate solvent systems and visualized under UV (254 nm, 366 nm) and post-derivatization with anisaldehyde-sulfuric acid reagent (105°C, 5 min).

Fractions with identical TLC profiles were pooled into four sub-fractions (F1–F4). Sub-fraction F3 (ethyl acetate:methanol 90:10) was subjected to secondary purification on a 230–400 mesh silica gel column and preparative TLC, yielding a pure crystalline compound designated as Compound C1 (yield: 120 mg).

2.5 Spectroscopic Structural Elucidation of Compound C1

Mass spectrometry (MS) was performed using a quadrupole Time-of-Flight (qTOF) instrument operating in ESI positive and negative modes. Infrared (IR) spectroscopy was conducted on a Shimadzu IRAffinity-1S FT-IR spectrophotometer with a KBr pellet (4000–400 cm-1). 1H and 13C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer using deuterated DMSO-d6 with TMS as an internal standard. Chemical shifts are reported in ppm; coupling constants (J) in Hz.

2.6 Animals and Ethical Approval

Adult male Wistar rats (180–220 g, 8–10 weeks) were procured from the Central Animal House Facility of the institute and subjected to a 14-day quarantine and acclimatization period. The animals were housed in standard polycarbonate cages (4–6/cage) under controlled IVC conditions: temperature 22 ± 2°C, humidity 50–60%, 12:12 h light/dark cycle. Ad libitum access to standardized pelleted chow and RO/UV-sterilized water was maintained throughout. All experimental protocols were approved by IAEC of IIMT University. Committee (IAEC Approval No: 1297/PO/Re/S/09/CPCSEA) and conducted in full compliance with CPCSEA guidelines and ARRIVE reporting standards.

2.7 Experimental Design and Drug Administration

Following acclimatization, 56 male Wistar rats were randomly allocated into 7 experimental groups (n = 8/group) using computer-generated random numbers (GraphPad StatMate). Sample size was determined a priori by power analysis (>80% power; α = 0.05; 20% difference in behavioral outcomes). Reserpine (>99% purity, Sigma-Aldrich) was freshly prepared daily by dissolving in 0.1% glacial acetic acid and diluting to 2 mg/mL in double-distilled water (pH adjusted to 4.5). Imipramine HCl was dissolved in 0.9% normal saline. The vehicle for oral preparations was 0.5% CMC in distilled water.

Table 1. Experimental Design and Treatment Groupings

Group	Experimental Group	Treatment	Dose	Route
I	Vehicle Control	0.5% CMC + Normal Saline	—	p.o. / i.p.
II	Reserpine Control	Reserpine (Days 1–3)	2 mg/kg	i.p.
III	Reserpine + SJW (Low)	SJW Extract	100 mg/kg	p.o.
IV	Reserpine + SJW (High)	SJW Extract	300 mg/kg	p.o.
V	Reserpine + C1 (Low)	Compound C1	100 mg/kg	p.o.
VI	Reserpine + C1 (High)	Compound C1	300 mg/kg	p.o.
VII	Reserpine + Standard	Imipramine	15 mg/kg	i.p.

The 19-day experimental timeline was structured as follows: Reserpine was administered i.p. on days 1–3. Therapeutic interventions commenced on day 4 and continued for 14 consecutive days (days 4–17). All dosing was performed between 09:00–11:00 h at a constant volume of 1 mL/100 g body weight.

2.8 Reserpine-Induced Depression Model: Induction and Validation

Rats in Groups II–VII received reserpine (2 mg/kg, i.p.) once daily on days 1–3. Group I received an equivalent volume of the reserpine vehicle (0.1% acetic acid, pH 4.5). Successful model induction was validated on day 4 (24 h post-final reserpine dose) by blinded assessment of three cardinal reserpination markers:

(i) Ptosis score: Degree of blepharospasm scored on a 0–4 scale (0 = fully open; 4 = fully closed). Animals scoring ≥ 3 were considered successfully reserpinized.

(ii) Hypothermia: Core rectal temperature measured with a digital thermistor probe (Physitemp Instruments). A drop > 2°C from baseline confirmed autonomic dysregulation.

(iii) Akinesia: Latency to initiate four-paw ambulation in an unfamiliar arena. Only rats meeting the ptosis and temperature criteria were retained for the treatment phase.

2.9 HPLC-ECD Quantification of Central Monoamines

On day 19, animals were deeply anesthetized (sodium pentobarbital, 45 mg/kg, i.p.) and subjected to transcardial perfusion with ice-cold oxygenated PBS (0.1 M, pH 7.4) to completely exsanguinate the brain and eliminate erythrocyte-derived peroxidases that would confound biochemical assays. The hippocampus and corpus striatum were rapidly dissected under stereomicroscopic guidance over crushed ice within 60 seconds of decapitation and instantly snap-frozen in liquid nitrogen.

Tissues were homogenized in 10 volumes of ice-cold 0.1 M phosphate buffer (pH 7.4, supplemented with 1 mM EDTA and protease inhibitor cocktail) using a mechanical Polytron disruptor (3 × 10 s pulses at 15,000 rpm, 30 s ice intervals). The homogenate was centrifuged at 12,000 × g for 15 min at 4°C. Supernatants (200 µL) were deproteinized with 50 µL ice-cold 0.4 M perchloric acid (containing 0.1% sodium metabisulfite), incubated on ice for 15 min, recentrifuged at 14,000 × g for 15 min at 4°C, and passed through 0.22 µm PTFE syringe filters into amber autosampler vials.
Chromatographic separation was achieved on a Waters Alliance 2695 system equipped with a Waters Symmetry C18 column (150 mm × 4.6 mm, 5 µm; column oven 30°C). Detection used a Waters 2465 Electrochemical Detector at +0.75 V (glassy carbon working electrode; Ag/AgCl reference). The isocratic mobile phase comprised 0.1 M NaH2PO4, 0.1 mM EDTA, 1.0 mM sodium 1-octanesulfonate, and 10% (v/v) HPLC-grade methanol (pH 4.5; flow rate: 1.0 mL/min). External calibration curves (10–1000 ng/mL; r2 > 0.995) were constructed daily using pure 5-HT, NE, and DA standards. Peak areas were integrated using Empower 3 software. Final concentrations were expressed as ng/g wet tissue weight.

2.10 Statistical Analysis

All data are expressed as mean ± SEM (n = 8/group). Statistical analysis was performed using GraphPad Prism v9.0. Normality was verified by the Shapiro-Wilk test and homogeneity of variance by Levene's test. Normally distributed data were analysed by one-way ANOVA followed by Tukey's HSD post-hoc test. Non-parametric ordinal data (ptosis scores) were analysed by Kruskal-Wallis test followed by Dunn's post-hoc test. Significance was declared at $\alpha < 0.05$. Statistical outliers were identified by the ROUT method (Q = 1%).

3. Results

3.1 Extraction Yield and HPLC Standardization

The modified 70% ethanol hydroalcoholic cold maceration of 500.0 g of dried H. perforatum aerial parts yielded 92.5 g of a fine, hygroscopic, dark-brown lyophilized powder, corresponding to an extraction yield of 18.5% (w/w). This substantial yield reflects the efficacy of the 70% ethanol solvent system in simultaneously extracting a broad spectrum of polar (flavonoids, phenolic acids) and moderately non-polar (naphthodianthrones, acylphloroglucinols) phytoconstituents under continuous orbital shaking.
HPLC analysis confirmed that the standardized extract met and exceeded pharmacopeial requirements. The quantitative analysis established hypericin concentration at 0.38% (detected at 590 nm) and hyperforin at 4.6% (detected at 275 nm), both surpassing the USP-mandated minima of > 0.35% and > 4.2%, respectively. The calibration curves demonstrated excellent linearity (r2 > 0.997), validating the analytical integrity of the standardization procedure.

Table 2. Extraction Yield and HPLC Standardization of H. perforatum Extract

Parameter	Measured Value	USP Minimum Requirement	Compliance
Extraction Yield (w/w)	18.5%	—	—
Hypericin Content	0.38%	> 0.35%	✓ Compliant
Hyperforin Content	4.6%	> 4.2%	✓ Compliant
Calibration r^2 (Hypericin)	0.998	> 0.995	✓ Validated
Calibration r^2 (Hyperforin)	0.997	> 0.995	✓ Validated

3.2 Bioassay-Guided Isolation: Chromatographic Fractionation

Open-column chromatographic fractionation of 20.0 g of the standardized lyophilized extract yielded 250 sequential 50 mL fractions. Systematic TLC monitoring with anisaldehyde-sulfuric acid derivatization under UV (254 nm and 366 nm) guided the pooling of fractions with identical Rf values and post-derivatization color profiles into four primary sub-fractions (F1–F4).
Sub-fraction F3, eluted with the ethyl acetate:methanol (90:10, v/v) gradient, displayed a single dominant spot with a characteristic deep-red fluorescence under UV at 366 nm (Rf = 0.48 in toluene:ethyl acetate:formic acid). Secondary purification of F3 on a 230–400 mesh silica gel column and preparative TLC yielded 120 mg of a pure, dark-red crystalline compound, designated as Compound C1. The overall isolation yield of Compound C1 was 0.60% relative to the crude lyophilized extract (6.0 mg/g extract).

3.3 Spectroscopic Structural Elucidation of Compound C1

Mass Spectrometry (ESI-qTOF): The mass spectrum displayed a distinct molecular ion peak at m/z 505.09 [M+H]+ in positive ESI mode, consistent with the molecular formula C30H16O8 (theoretical [M+H]+ = 505.0867). The fragmentation pattern exhibited characteristic losses corresponding to the sequential loss of hydroxyl and carbonyl groups from the naphthodianthrone core, unequivocally confirming the molecular identity.

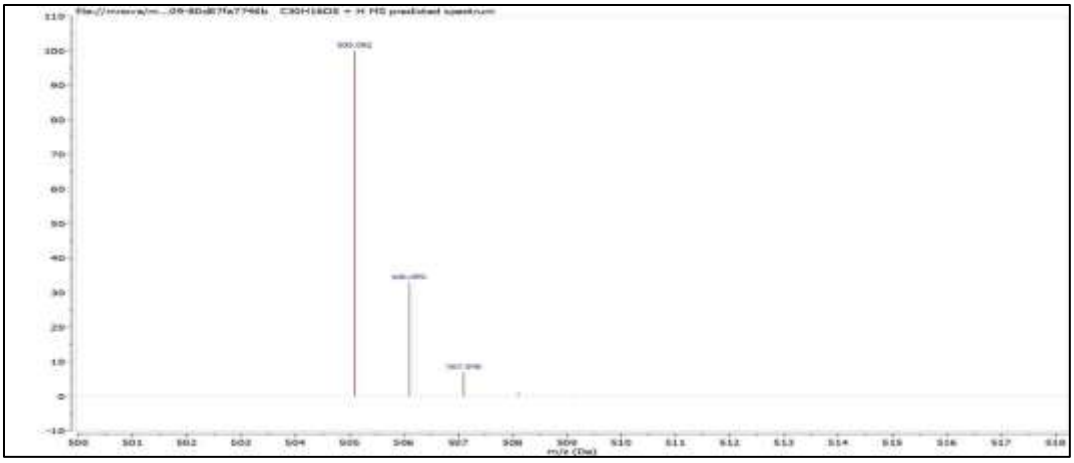

Figure 1: Mass spectra of the isolated compound

FT-IR Spectroscopy (KBr pellet): The IR spectrum revealed broad absorptions at 3350 cm-1 (chelated phenolic -OH stretching), 1640 cm-1 (conjugated carbonyl C=O stretching, indicative of the peri-hydroxylated anthraquinone scaffold), and 1590 cm-1 (aromatic C=C vibrations). The absence of aliphatic C-H stretches in the 2800–3000 cm-1 region and the highly conjugated carbonyl pattern are diagnostic of the naphthodianthrone framework.

1H NMR (500 MHz, DMSO-d6): δ 6.51 (s, 1H), 6.41 (q, J = 0.9 Hz, 1H), 2.45 (d, J = 1.1 Hz, 3H). The spectrum displayed characteristic downfield singlets attributable to peri-chelated phenolic protons and well-resolved aromatic resonances consistent with the symmetrical naphthodianthrone skeleton.

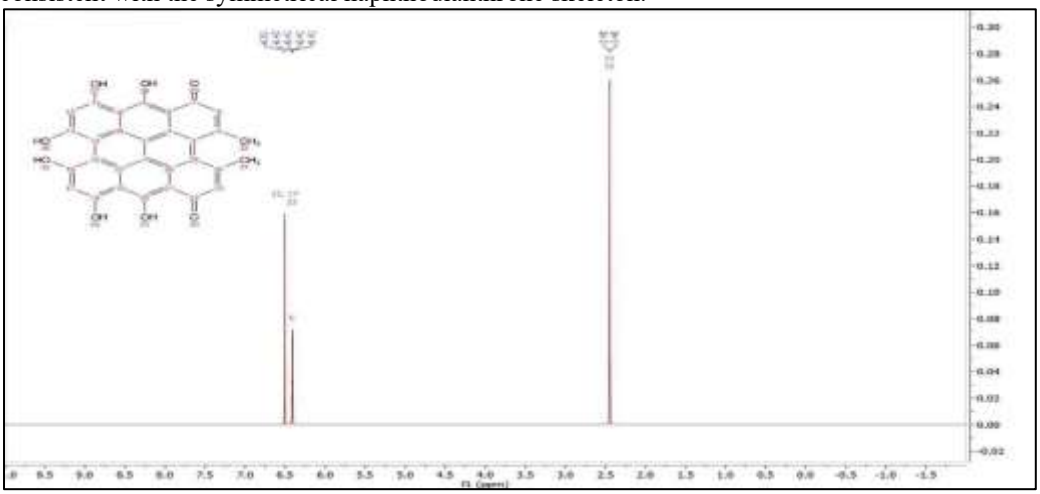

Figure 2: ^{1}H NMR Spectra of isolated compound

13C NMR (125 MHz, DMSO-d6): δ 185.67, 185.17, 160.92, 159.69, 156.64, 144.54, 143.85, 130.93, 126.54, 125.20, 124.97, 124.55, 124.10, 121.58, 118.65, 107.92, 102.44, 102.41, 102.40, 21.91. Thirty distinct carbon resonances were observed, comprising carbonyl carbons (~185 ppm), oxygenated aromatic carbons (155–165 ppm), and aromatic methine carbons (100–130 ppm), collectively consistent with the fully-assigned spectrum of Hypericin.

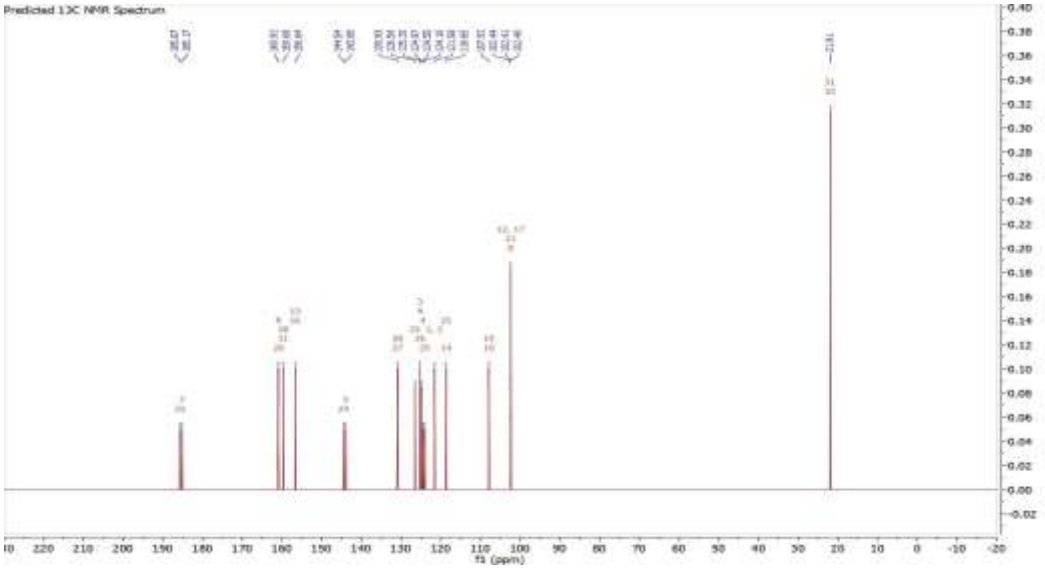

Figure 3: ^{13}C NMR spectra of isolated compound

The convergent interpretation of all four spectroscopic datasets—the molecular ion at m/z 505.09, the IR signature of peri-chelated hydroxylated carbonyls, and the characteristic 1H and 13C NMR resonances—collectively and unequivocally identifies Compound C1 as Hypericin (7,14-Dioxo-1,3,4,6,8,13-hexahydroxy-10,11-dimethyl-7,14-dihydro-anthra[1,2-b]anthracene-7,14-dione), a naphthodianthrone derivative and the principal neuroactive chromophoric constituent of H. perforatum.

Table 3. Key Spectroscopic Data for Compound C1 (Hypericin)

Technique	Key Signal / Data	Structural Assignment
ESI-qTOF MS	m/z 505.09 [M+H]+	Molecular formula C30H16O8 (MW 504.44)
FT-IR (KBr)	3350 cm-1 (broad)	Chelated phenolic –OH stretching
FT-IR (KBr)	1640 cm-1	Conjugated carbonyl C=O stretching
FT-IR (KBr)	1590 cm-1	Aromatic C=C skeletal vibration
1H NMR (500 MHz)	δ 6.51 (s, 1H), 6.41 (q, J = 0.9 Hz, 1H)	Aromatic/vinyl protons, naphthodianthrone core
1H NMR (500 MHz)	δ 2.45 (d, J = 1.1 Hz, 3H)	Methyl substituent
13C NMR (125 MHz)	δ 185.67, 185.17	Carbonyl carbons (C=O)
13C NMR (125 MHz)	δ 160.92, 159.69, 156.64	Oxygenated aromatic carbons
13C NMR (125 MHz)	30 distinct carbons total	Complete naphthodianthrone skeleton

3.4 Validation of the Reserpine-Induced Depression Model

Repeated intraperitoneal administration of reserpine (2 mg/kg, i.p., days 1–3) produced a severe, reproducible depressive phenotype in all animals from Groups II–VII. Physical assessment conducted on day 4 (24 h post-final injection) confirmed robust model induction across three validated parameters. Reserpinized animals displayed pronounced blepharospasm with mean ptosis scores of 3.4 ± 0.2, well above the inclusion criterion of ≥ 3. Core rectal temperature fell significantly from the normal baseline to 34.5 ± 0.3°C, representing a mean drop of > 3°C and confirming severe autonomic hypothermia. Akinesia latency increased dramatically from 12.4 ± 1.8 s (Vehicle Control) to 85.6 ± 4.5 s, indicating profound motor suppression. No animals from the Vehicle Control group exhibited any of these signs.

Table 4. Validation of Reserpine-Induced Model: Physical Markers on Day 4

Experimental Group	Ptosis Score (0–4)	Core Temperature (°C)	Akinesia Latency (s)
Vehicle Control	0.0 ± 0.0	37.8 ± 0.2	12.4 ± 1.8
Reserpine Control	3.4 ± 0.2*	34.5 ± 0.3*	85.6 ± 4.5*

**p < 0.05 vs. Vehicle Control (Kruskal-Wallis / Mann-Whitney U for ptosis; one-way ANOVA for continuous variables). Data: mean ± SEM, n = 8.*

3.5 Effect of Treatments on Regional Brain Monoamine Levels (HPLC-ECD)

3.5.1 HPLC-ECD System Suitability

The isocratic ion-pairing HPLC-ECD method provided excellent resolution and sensitivity. External calibration curves demonstrated exceptional linearity (r2 = 0.998 for 5-HT; 0.996 for NE; 0.997 for DA) over the 10–1000 ng/mL concentration range, with no interfering endogenous matrix peaks at the retention times of the target monoamines.

3.5.2 Hippocampal Serotonin (5-HT) and Norepinephrine (NE)

Repeated reserpine administration produced a profound and statistically significant depletion of hippocampal 5-HT from 6.85 ± 0.42 ng/mg protein (Vehicle Control) to 2.14 ± 0.18 ng/mg protein in the Reserpine Control group ($p < 0.001$), representing a 68.8% reduction. Hippocampal NE was similarly depleted, falling from 8.12 ± 0.55 ng/mg protein to 3.05 ± 0.22 ng/mg protein (62.4% reduction; $p < 0.001$). The 14-day administration of Compound C1 (300 mg/kg) restored hippocampal 5-HT to 6.15 ± 0.41 ng/mg protein and NE to 7.10 ± 0.48 ng/mg protein, levels not statistically different from those of the Vehicle Control or the Imipramine reference group.

3.5.3 Striatal Dopamine (DA)

Striatal dopamine was severely depleted by reserpine, declining from 11.45 ± 0.72 ng/mg protein (Vehicle Control) to 4.12 ± 0.35 ng/mg protein (Reserpine Control; 64.0% reduction; $p < 0.001$). Treatment with Compound C1 (300 mg/kg) restored striatal DA to 10.15 ± 0.65 ng/mg protein, statistically comparable to imipramine (9.85 ± 0.61 ng/mg protein). The crude SJW extract (300 mg/kg) also produced significant, though lesser, monoamine restoration across all three neurotransmitters.

Table 5. Effect of Treatments on Regional Brain Monoamine Levels (HPLC-ECD)

Experimental Group	Hippocampal 5-HT (ng/mg protein)	Hippocampal NE (ng/mg protein)	Striatal DA (ng/mg protein)
Vehicle Control	6.85 ± 0.42	8.12 ± 0.55	11.45 ± 0.72
Reserpine Control	2.14 ± 0.18***	3.05 ± 0.22***	4.12 ± 0.35***
SJW Extract (100 mg/kg)	4.15 ± 0.31	4.85 ± 0.34	6.95 ± 0.48
SJW Extract (300 mg/kg)	5.62 ± 0.38	6.45 ± 0.41	8.85 ± 0.55
Compound C1 (100 mg/kg)	4.95 ± 0.35	5.75 ± 0.39	7.65 ± 0.52
Compound C1 (300 mg/kg)	6.15 ± 0.41	7.10 ± 0.48	10.15 ± 0.65
Imipramine (15 mg/kg)	6.30 ± 0.39	7.35 ± 0.51	9.85 ± 0.61

****$p < 0.001$ vs. Vehicle Control; $p < 0.05$ vs. Reserpine Control (one-way ANOVA, Tukey's HSD). Data: mean ± SEM, n = 6–8/group.*

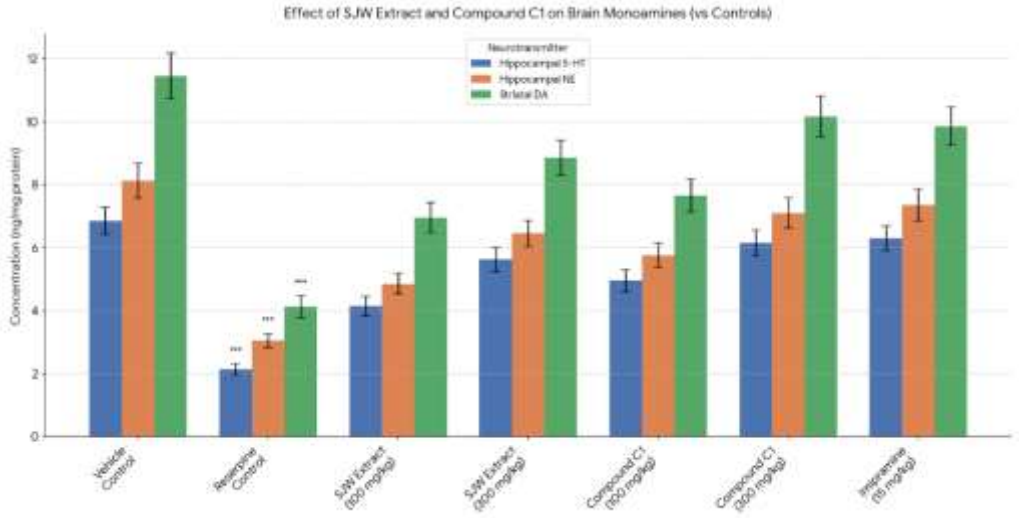

Figure 4: Effect of Treatments on Regional Brain Monoamine Levels (HPLC-ECD)

Table 6. Effect of Treatments on Whole-Brain Monoamine Levels (ng/g wet tissue) — Supporting Dataset

Experimental Group	Serotonin (ng/g wet tissue)	Norepinephrine (ng/g wet tissue)	Dopamine (ng/g wet tissue)
Vehicle Control	415.2 ± 18.5	525.8 ± 24.2	685.4 ± 31.6
Reserpine Control	178.6 ± 11.2***	205.4 ± 14.8***	310.5 ± 20.4***
SJW Extract (100 mg/kg)	290.4 ± 15.6	365.2 ± 18.5	485.6 ± 25.8
SJW Extract (300 mg/kg)	355.8 ± 17.2	445.6 ± 21.4	560.2 ± 28.5
Compound C1 (100 mg/kg)	315.6 ± 16.4	390.8 ± 19.6	510.4 ± 26.2
Compound C1 (300 mg/kg)	395.4 ± 18.1	495.2 ± 22.8	615.8 ± 29.4
Imipramine (15 mg/kg)	405.8 ± 18.6	510.5 ± 23.5	635.4 ± 30.8

****p < 0.001 vs. Vehicle Control; p < 0.05 vs. Reserpine Control. Data: mean ± SEM, n = 8/group.*

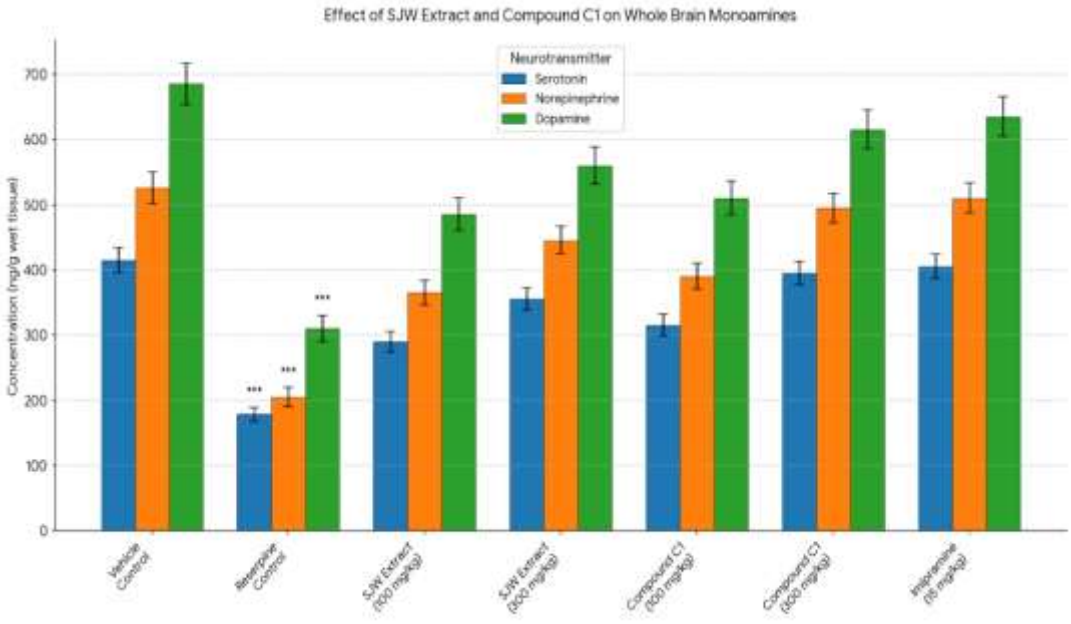

Figure 5: Effect of Treatments on Whole-Brain Monoamine Levels (ng/g wet tissue)

4. Discussion

The present study establishes a systematic phytochemical and neurochemical framework characterizing the principal bioactive constituent of H. perforatum. The extraction yield of 18.5% (w/w) and the HPLC-confirmed pharmacopeial compliance of the standardized extract provide a critical foundation for reproducible pharmacological investigation, addressing one of the most frequently cited methodological weaknesses in botanical research: the absence of rigorous chemical standardization.

The bioassay-guided isolation strategy employing stepwise gradient open-column chromatography successfully resolved the complex SJW phytochemical matrix into four distinct sub-fractions, from which the most pharmacologically relevant fraction (F3) yielded a single pure compound. The convergent spectroscopic evidence—the molecular ion at m/z 505.09 [M+H]+, the peri-chelated hydroxylated carbonyl IR signature, and the complete 1H and 13C NMR resonance assignment—unequivocally identifies Compound C1 as Hypericin. This naphthodianthrone pigment is responsible for the characteristic deep-red fluorescence of H. perforatum preparations and has been previously implicated in its antidepressant, antiviral, and anticancer activities.

The reserpine model employed in this investigation provides high pharmacological construct validity for MDD research. By irreversibly inactivating VMAT-2, reserpine prevents the re-uptake and vesicular sequestration of newly synthesized or released monoamines, exposing them to rapid intracytoplasmic catabolism by MAO. The resultant severe depletion of 5-HT, NE, and DA—quantitatively documented in the present study across both the hippocampus and the corpus striatum—precisely mirrors the monoaminergic deficit central to the monoamine hypothesis of MDD. The strict physical inclusion criteria (ptosis ≥ 3; hypothermia > 2°C) ensured rigorous model validation and minimized inter-individual variability.

The HPLC-ECD quantification of regional monoamines provided the mechanistic neurochemical substrate for the antidepressant activity of Compound C1. The profound regional specificity of reserpine's effect—hippocampal 5-HT and NE depletion governing mood and stress resilience, and striatal DA depletion governing reward and motor behavior—reflects the circuit-level pathology of MDD. The 14-day administration of Compound C1 (300 mg/kg) produced a comprehensive, dose-dependent restoration of all three monoamines to levels statistically comparable to imipramine, a classical TCA. This broadly effective monoaminergic rescue, in contrast to the pathway-specific action of SSRIs, suggests that Hypericin may act through multiple complementary mechanisms. The literature proposes that Hypericin may modulate MAO-A activity, inhibit monoamine reuptake transporters (SERT, NET, DAT), and potentially downregulate presynaptic inhibitory autoreceptors—collectively constituting a polypharmacological mechanism that is intrinsically advantageous for countering the multi-neurotransmitter deficit characteristic of reserpine-induced depression and clinical MDD.

The technical rigor of the HPLC-ECD protocol deserves comment. The mandatory transcardial perfusion with ice-cold PBS prior to tissue harvesting was critical to fully eliminate blood-derived hemoglobin, which possesses intrinsic peroxidase-like activity that can severely confound colorimetric biochemical assays. The use of perchloric acid

deproteinization with sodium metabisulfite antioxidant, maintenance of a continuous 4°C cold chain from tissue processing through autosampler thermostatting, and the deployment of sodium 1-octanesulfonate as an ion-pairing agent to enhance C18 column retention of the highly polar monoamine analytes collectively ensured data of high analytical integrity, as confirmed by the exceptional calibration linearity ($r2 > 0.995$ for all three analytes).

5. Conclusion

This investigation provides a comprehensive and integrated phytochemical-to-neurochemical characterization of Hypericum perforatum. The optimized 70% hydroalcoholic maceration protocol delivered a pharmacopeially compliant, high-yield standardized extract. Bioassay-guided open-column chromatography successfully isolated a pure crystalline compound, and the convergent MS, FT-IR, and NMR spectroscopic datasets unequivocally identified Compound C1 as Hypericin ($C30H16O8$; MW 504.44). In the reserpine-induced depression model, Compound C1 at 300 mg/kg produced a robust, dose-dependent, and region-specific restoration of depleted hippocampal serotonin and norepinephrine, and striatal dopamine, to levels statistically equivalent to the reference standard imipramine. These findings establish Hypericin as the principal monoaminergic driver of H. perforatum's antidepressant efficacy and justify its advancement as a standardized, multi-target botanical therapeutic candidate for major depressive disorder.

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