



***Ipomoea Parasitica* AERIAL-PART SOXHLET FRACTIONS: ANTIDIABETIC EFFICACY IN STREPTOZOTOCIN-INDUCED RATS AND REVERSAL OF PALMITATE-DRIVEN INSULIN RESISTANCE IN L6 MYOTUBES AND HEPG2 HEPATOCYTES**

Uootukuru Ashwini^{1*}, Jayapal Reddy Gangadi² and Varun Dasari³

¹Research Scholar, Faculty of Pharmaceutical Sciences, Motherhood University, Roorkee, Uttarakhand, India

²Research Supervisor and Professor, Faculty of Pharmaceutical Sciences, Motherhood University, Roorkee, Uttarakhand, India

³Professor and Principal, Sri Indu Institute of Pharmacy, Sheriguda, Ibrahimpatnam, Hyderabad, India

*Corresponding Author Email: mahalakshmi30091991@gmail.com

ABSTRACT

Standardized Soxhlet fractions of *Ipomoea parasitica* (Convolvulaceae) aerial parts hexane (HE), ethyl acetate (EAE), ethanol (EE), and hydroalcoholic (HAE) were evaluated for acute oral toxicity and antidiabetic activity. Acute toxicity screening by OECD 423 Acute Toxic Class method returned no mortality at 2000 mg/kg p.o. for EAE, EE, and HAE in Swiss albino mice, establishing LD₅₀ > 2000 mg/kg (GHS Category 5). A 28-day in vivo study in streptozotocin-induced (55 mg/kg i.p.) Wistar rats (n = 6/group) dosed all three fractions at 400 mg/kg p.o. daily against metformin 150 mg/kg p.o. as comparator. Day-28 fasting blood glucose reductions versus the diabetic control: EE –57.7% (p < 0.001), EAE –45.5% (p < 0.001), HAE –35.5% (p < 0.01). EE and metformin (–61.2%) were not statistically separable by Tukey HSD at n = 6. Body weight tracked the glucose response: EE gained +9.9% against a diabetic-control loss of –16.4%. In vitro, MTT cytotoxicity established ≤100 µg/mL as the safe working ceiling (≥80% viability) for EE and EAE in both L6 myotubes and HepG2 hepatocytes, and ≤50 µg/mL for HAE. Palmitate-induced insulin-resistant L6 myotubes (300 µM, 16 h) recovered 2-NBDG uptake to 167 ± 7% of unchallenged basal with EE 100 µg/mL (versus metformin 1 mM: 178 ± 6%; insulin-resistant baseline: 96 ± 5%). In HepG2 hepatocytes (250 µM palmitate, 24 h), EE 100 µg/mL reached 152 ± 7% versus metformin 158 ± 6%. EE EC₅₀ values were 22 µg/mL in L6 and 31 µg/mL in HepG2. The cellular reversal ranking EE > EAE > HAE mirrored the polyphenol-load hierarchy established in the companion phytochemistry study. These findings constitute the first paired in vivo–in vitro antidiabetic record for *I. parasitica* at the aerial-part level and position EE as a lead fraction warranting mechanistic extension.

Key words:

Ipomoea parasitica; streptozotocin diabetic rat; L6 myotubes; HepG2; palmitate insulin resistance; 2-NBDG glucose uptake; quercetin; rutin; AMPK.

1. INTRODUCTION

Type 2 diabetes mellitus is sustained by the convergence of peripheral insulin resistance and progressive β -cell dysfunction, producing a state of chronic hyperglycemia whose downstream metabolic consequences dyslipidemia, oxidative stress, end-organ damage drives its global morbidity burden (Reaven, 1988). Current pharmacotherapy, anchored by metformin and SGLT-2 inhibitors, is effective but leaves a residual population for whom combination strategies or complementary agents

remain clinically relevant. Plant polyphenols have attracted mechanistic interest at multiple insulin-signalling nodes: AMPK activation in skeletal muscle (Dhanya et al., 2017), insulin receptor kinase potentiation (Hsu et al., 2014), and hepatic gluconeogenesis modulation (Huang et al., 2016) a pleiotropic profile that single-target synthetic agents replicate only incompletely (Xiao & Högger, 2015). The sister-species *Ipomoea aquatica* carries the most developed antidiabetic literature within the genus. Malalavidhane et al. (2000, 2003) established aerial-part

extract hypoglycemic activity in both animal models and type 2 diabetic subjects. Vijayakumar et al. (2012) subsequently reported fasting glucose reductions of 40–55% in alloxan-diabetic rats at 200–400 mg/kg ethanolic extract. Sajak et al. (2017) extended this to a metabolomic characterization of pathway remodeling in STZ-Wistar rats at 500 mg/kg. None of these studies paired *in vivo* efficacy with mechanistic *in vitro* data in insulin-resistant cells, leaving the cellular basis of the genus antidiabetic activity unanchored.

The companion study to this paper (Manuscript 1 of this series) established a complete chemical characterization of *I. parasitica* aerial-part Soxhlet fractions: EE carries 78.3 mg GAE/g TPC, 56.9 mg QE/g TFC, and 6.64 mg/g combined gallic acid, rutin, and quercetin — a flavonoid- and phenolic-acid-dominated profile consistent with the antidiabetic-active stream of the genus — and is reproducible across independent harvest batches at inter-batch RSDs of 2.2–4.4%. No acute toxicity, *in vivo* antidiabetic, or cell-based mechanistic data existed for *I. parasitica* aerial parts before the present study.

The specific objectives are: (i) to determine acute oral toxicity of EAE, EE, and HAE by OECD 423; (ii) to evaluate 28-day antidiabetic efficacy of all three fractions at 400 mg/kg p.o. daily in streptozotocin-induced diabetic Wistar rats against metformin 150 mg/kg; and (iii) to test whether EE restores insulin-stimulated glucose uptake in palmitate-induced insulin-resistant L6 rat skeletal myotubes and HepG2 human hepatoma-derived hepatocytes through the 2-NBDG glucose-uptake assay. The integrated design — phytochemistry from Manuscript 1 feeding *in vivo* efficacy and *in vitro* mechanism produces the first paired antidiabetic record for the species at the aerial-part level.

2. MATERIALS AND METHODS

2.1 Extract preparation

HE, EAE, EE, and HAE fractions were prepared by successive Soxhlet extraction of dried *I. parasitica* aerial-part powder following the full protocol and three-tier standardization described in the companion publication (Manuscript 1, this series). Working suspensions for all biological assays were prepared fresh daily in 1% w/v carboxymethylcellulose (CMC) in sterile water (*in vivo*) or in 0.1% v/v DMSO in cell culture medium (*in vitro*), immediately before dosing.

2.2 Acute oral toxicity (OECD 423)

Female Swiss albino mice, nulliparous and non-pregnant (20–25 g, 8–10 weeks), were acclimatized 7 days. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC Protocol No.

1447/PO/Re/S/11/CPCSEA) and conforms to CPCSEA (2003) guidance; reporting follows ARRIVE 2.0 (Percie du Sert et al., 2020). HE was excluded from toxicity testing on the basis of its near-baseline phenolic content and lipid-dominant qualitative profile established in Manuscript 1. EAE, EE, and HAE were tested separately by the OECD 423 Acute Toxic Class stepwise procedure (OECD, 2002). The starting dose was 2000 mg/kg p.o. in 1% CMC (10 mL/kg by oral gavage); absence of mortality in the first triplet (n = 3) triggered a confirmatory triplet at the same dose. Animals were observed continuously for 4 h post-dose, hourly for the next 4 h, then daily for 14 days; body weight was recorded on days 0, 7, and 14. Gross necropsy was performed on day 14.

2.3 *In vivo* antidiabetic study-streptozotocin model

2.3.1 Animals and ethics

Adult male Wistar rats (180–220 g, 8–10 weeks) were acclimatized 7 days before STZ induction. The full protocol was reviewed by the IAEC (Protocol No. 1447/PO/Re/S/11/CPCSEA). Male rats were chosen to remove cyclical hormonal confounders, in line with dominant convention in the published STZ-rat literature.

2.3.2 Drugs and reagents

Streptozotocin (STZ; Sigma-Aldrich, ≥98% HPLC) was dissolved in 0.1 M citrate buffer pH 4.5 within 15 min of use (Furman, 2021). Metformin hydrochloride (Sigma-Aldrich, analytical grade) was suspended in 1% CMC. Glucose monitoring used an Accu-Chek Active glucometer with strip lots verified against control solution at the start of each measurement day.

2.3.3 Diabetes induction

After a 12-h overnight fast, baseline fasting blood glucose (FBG) was measured by tail-vein puncture. STZ was administered i.p. at 55 mg/kg in citrate buffer (1 mL/kg). The mechanism GLUT2-mediated β -cell uptake, DNA alkylation, NAD⁺ depletion via PARP overactivation, ROS generation, and β -cell ablation — was established by Lenzen (2008). To prevent early post-induction hypoglycemia from transient insulin release, all rats received 5% glucose drinking water for 24 h post-STZ. FBG was measured at 72 h; animals with FBG ≥ 250 mg/dL entered the study. Of 36 STZ-injected animals, 35 met the criterion (97% induction rate); one was excluded at FBG 178 mg/dL.

2.3.4 Group allocation and treatment

Thirty confirmed diabetic rats and six STZ-naive normal-control rats were randomized to six groups (n = 6) using a computer-generated sequence stratified on day-3 FBG. Power calculation targeted 80% power to detect ≥30% FBG difference (SD 25% of mean, α = 0.05, two-tailed),

yielding minimum $n = 5$ –6/group, rounded to 6. Day-0 group FBG means across G2–G6 (304–312 mg/dL) were not significantly different (one-way ANOVA, $p > 0.5$). The animal handler performing daily dosing was not

blinded; the glucometer technician was blinded to group assignment via recoded cage labels (ARRIVE 2.0 partial blinding). Treatment ran for 28 consecutive days by oral gavage at 08:00–10:00 daily.

Table 1: Group allocation, treatment, dose, route, and sample size.

Group	Label	Treatment	Dose	Route	n	Day-0 FBG (mg/dL)
G1	Normal control	Vehicle (1% CMC)	1 mL/kg	p.o.	6	88 ± 3
G2	Diabetic control	STZ + vehicle	STZ 55 mg/kg i.p.; CMC p.o.	i.p. + p.o.	6	310 ± 10
G3	Standard	STZ + metformin	150 mg/kg p.o.	p.o.	6	304 ± 9
G4	EAE	STZ + EAE	400 mg/kg p.o.	p.o.	6	308 ± 10
G5	EE	STZ + EE	400 mg/kg p.o.	p.o.	6	312 ± 11
G6	HAE	STZ + HAE	400 mg/kg p.o.	p.o.	6	307 ± 10

2.3.5 Endpoints

FBG was measured by tail-vein nick after a 12-h overnight fast on days 0, 7, 14, 21, and 28. Two readings per animal were averaged; if they differed by >10%, a third was taken and the median used. Body weight was recorded in parallel. On day 29, animals were euthanized by pentobarbital sodium overdose (200 mg/kg i.p.) followed by exsanguination.

2.4 In vitro studies cell lines and assays

2.4.1 L6 myotubes

L6 rat skeletal myoblasts (passage 8–18 from laboratory master vial; Yaffe, 1968) were maintained in DMEM-HG (4.5 g/L glucose) + 10% FBS + antibiotics at 37 °C, 5% CO₂. Differentiation was induced by switching to 2% horse serum at 80–90% confluence for 6 days; ≥70% myotube morphology by day 6 was the inclusion criterion. Mycoplasma testing (PCR) was performed every fifth passage. The BSA–palmitate conjugate (300 μM, 16 h for L6) was prepared at 6:1 palmitate:BSA molar ratio per Listenberger et al. (2003) and Powell et al. (2004), sterile-filtered, and used same-day.

2.4.2 HepG2 hepatocytes

HepG2 (human hepatocellular carcinoma-derived; Aden et al., 1979; Knowles et al., 1980) was maintained in DMEM-HG + 10% FBS at 37 °C, 5% CO₂, passages 5–25 from laboratory master vial. Serum was reduced to 1% FBS for 24 h before mechanistic assays. BSA–palmitate conjugate (250 μM, 24 h for HepG2) was prepared identically per Ishii et al. (2015).

2.4.3 MTT cytotoxicity

Differentiated L6 (~30,000 cells/well) or HepG2 (~15,000 cells/well) in 96-well plates were exposed to EAE, EE, or HAE at 1–500 μg/mL in 0.1% DMSO for 24 h. After washing, MTT (0.5 mg/mL, 4 h, 37 °C) formazan was dissolved in DMSO and absorbance read at 570/630 nm.

Viability ≥80% of vehicle control defined the safe working window (Mosmann, 1983).

2.4.4 2-NBDG glucose-uptake assay

Cells were serum-starved 4 h in glucose-free DMEM + 0.1% BSA. Three experimental arms were run: (i) basal uptake 100 μM 2-NBDG ± extract, 30 min; (ii) insulin-stimulated — 100 nM insulin pre-treatment 20 min, then 100 μM 2-NBDG 30 min ± extract; (iii) palmitate–insulin-resistance reversal — 16 h (L6) or 24 h (HepG2) palmitate ± extract co-incubation, then 4-h serum-starvation, then insulin-stimulated 2-NBDG arm. After uptake, cells were washed three times with ice-cold PBS, lysed in 0.1 M NaOH, and fluorescence read at excitation 465/emission 540 nm (Yamada et al., 2007). Data expressed as percentage of unchallenged basal. Each condition: 6 replicate wells, ≥3 independent biological experiments. EC₅₀ for EE restoration of glucose uptake was derived from a four-parameter logistic fit.

2.5 Statistical analysis

In vivo data are presented as mean ± SEM ($n = 6$ /group); in vitro data as mean ± SD ($n = 3$ –4 biological replicates). For in vivo FBG and body weight: one-way ANOVA followed by Dunnett's multiple-comparison test against G2 diabetic control (Dunnett, 1955); Tukey HSD for pairwise comparisons (Tukey, 1949). For in vitro MTT and 2-NBDG: one-way ANOVA followed by Tukey HSD. $p < 0.05$ was the significance threshold throughout. Outliers were identified by Grubbs' test ($\alpha = 0.05$) and retained in primary analyses except in documented instrumental-error cases. Analyses were performed in GraphPad Prism v9.0.

3. RESULTS AND DISCUSSION

3.1 Acute oral toxicity — safety profile and dose selection

No mortality occurred in any extract group across 14 days (Table 2). Body weights tracked the age-matched Swiss albino growth curve in all three groups, remaining within $\pm 5\%$ of ad libitum control throughout. No tremor, convulsion, hyperactivity, sedation, ptosis, or autonomic sign was observed at any time point. Gross necropsy on day 14 was unremarkable in all animals. Zero mortality at 2000 mg/kg p.o. across two confirmatory triplets places each fraction in GHS Category 5 / unclassified with a cut-off LD50 > 2000 mg/kg (OECD, 2002).

This safety profile is consistent with the broader pattern for polar aerial-part extracts of *Ipomoea* species. Vijayakumar et al. (2012) reported no acute toxicity at up to 2000 mg/kg for *I. aquatica* ethanolic leaf extract in acute oral rodent models; Malalavidhane et al. (2003) noted no adverse effects in human subjects administered aqueous *I. aquatica* preparations at therapeutic doses. A single acute-dose panel, however, warrants an explicit scope note: it addresses only single-dose acute lethality, not repeated-

dose target-organ toxicity, not chronic-exposure margins, and not reproductive endpoints. The 14-day observation with gross necropsy supports only the narrow claim that a single oral exposure to 2000 mg/kg of EAE, EE, or HAE produced no observable acute toxicity adequate for setting an in vivo efficacy dose ceiling, not a translational safety assertion. Repeated-dose sub chronic toxicity data remain an identified gap for follow-on work.

From this ceiling, 400 mg/kg p.o. was selected as the working efficacy dose. Two considerations governed the choice above the conventional 1/10 starting point (200 mg/kg): a two-fold margin above 1/10 of the no-mortality ceiling remains comfortably below any toxicity signal while leaving headroom for a detectable pharmacological effect, and the published *I. aquatica* aerial-part ethanolic-extract literature has reported efficacy specifically in the 200–500 mg/kg range (Malalavidhane et al., 2003; Vijayakumar et al., 2012; Sajak et al., 2017), making 400 mg/kg the methodologically continuous choice.

Table 2: OECD 423 acute oral toxicity outcomes for EAE, EE, and HAE in Swiss albino mice.

Extract	Dose (mg/kg)	Step 1 (n)	Step 2 (n)	Mortality (14 d)	GHS class	Cut-off LD50
EAE	2000	3	3	0/6	Category 5 / unclassified	>2000 mg/kg
EE	2000	3	3	0/6	Category 5 / unclassified	>2000 mg/kg
HAE	2000	3	3	0/6	Category 5 / unclassified	>2000 mg/kg

3.2 Diabetes induction and baseline characterization

STZ induction at 55 mg/kg i.p. succeeded in 35/36 animals (97%). The one excluded animal measured FBG 178 mg/dL at 72 h, suggesting incomplete β -cell ablation rather than model failure; this exclusion rate (3%) is within the range reported for single-dose STZ-Wistar protocols of this type (Goyal et al., 2016; Furman, 2021). Day-0 FBG across the five diabetic groups (304–312 mg/dL, one-way ANOVA $p > 0.5$) confirmed comparable baseline glycaemia after stratified randomization. G1 normal animals measured 88 ± 3 mg/dL, well within the accepted Wistar fasting range of 75–105 mg/dL.

Polydipsia, polyuria, and polyphagia were fully penetrant across all STZ-injected animals at 96 h water bottles emptying at 2.5–3 $\times$ the normal-control rate, urine output visibly elevated on absorbent bedding, food intake running 1.3–1.5 $\times$ normal. This triad, the classical read-out of insulin-deficient polyphagia-with-weight-loss, confirmed the STZ phenotype was active before treatment commenced. A brief mechanistic note is appropriate here. STZ 55 mg/kg single i.p. in adult rats produces a state dominated by β -cell loss and relative insulin deficiency mechanistically closer to late-stage type 1 diabetes than to the insulin-resistance-driven type 2 phenotype prevalent in clinical practice (Lenzen, 2008; Goyal et al., 2016). The in

vivo arm therefore reads efficacy against an insulin-secretion-deficient phenotype; the in vitro arm in Sections 3.4–3.6 directly addresses the insulin-resistance axis. The two arms are pharmacologically complementary, not redundant.

3.3 Fasting blood glucose trajectory and day-28 efficacy

FBG trajectories are presented in Table 3. G1 normal animals held a flat FBG between 88 and 91 mg/dL across all five time points (inter-timepoint CV < 2%), confirming that handling, fasting, and glucometer drift introduced negligible noise. G2 diabetic-control animals progressed from 310 ± 10 to 381 ± 15 mg/dL over 28 days (+22.9%) — a worsening trajectory consistent with published single-dose STZ-Wistar protocols, where residual β -cell function declines progressively through weeks two to four (Goyal et al., 2016; Furman, 2021).

Metformin (G3) produced the steepest descent: –23.7% at day 7, –41.4% at day 14, –53.3% at day 21, –61.2% at day 28 (118 ± 7 mg/dL). The day-28 value approaches the upper edge of the normal Wistar fasting range. Metformin's primary mechanism at this dose is hepatic gluconeogenesis suppression via AMPK-mediated and AMPK-independent pathways (Bailey & Turner, 1996; Rena et al., 2017); a 61% reduction is consistent with the

published window for 150 mg/kg p.o. daily dosing in this model.

EE (G5) ran second only to metformin at every time point: -21.8% at day 7, -38.5% at day 14, -50.0% at day 21, -57.7% at day 28 (132 ± 7 mg/dL). The day-7 EE reduction (-21.8%) nearly matched metformin's (-23.7%) — a near-equivalence in early-phase glucose-lowering velocity that argues against a purely hepatic mechanism for EE, since metformin's hepatic gluconeogenesis suppression typically achieves its maximum relative contribution by day 7. EE descent was monotonic across all five time points, suggesting a cumulative rather than acutely saturating effect over the 28-day window.

EAE (G4) sat consistently between EE and HAE, reaching 168 ± 8 mg/dL (-45.5%) at day 28. HAE (G6) produced the most modest response: 198 ± 10 mg/dL (-35.5%), with a trajectory that flattened from day 14 onward. Day-28 ranking: G3 metformin $\approx$ G5 EE > G4 EAE > G6 HAE > G2 diabetic control. The EE result 57.7% FBG reduction over 28 days, statistically indistinguishable from metformin at $n = 6$ (Tukey HSD, $p > 0.05$) sits at the upper end of the range documented for *I. aquatica* aerial-part ethanolic extracts: Malalavidhane et al. (2003) reported 25–40% reductions over 4–8 weeks; Vijayakumar et al. (2012) 40–55% at 200–400 mg/kg in alloxan-diabetic rats; Sajak et al. (2017) approximately 50–55% at 500 mg/kg

over 28 days. The comparison is positional rather than quantitative different doses, durations, and rodent strains but it confirms that *I. parasitica* aerial-part EE is not an outlier on the genus efficacy axis. And the present study adds what the *I. aquatica* papers lack: a paired in vitro mechanistic arm under insulin-resistant conditions, which contextualises the in vivo glucose-lowering in cellular terms.

The extract efficacy ordering EE > EAE > HAE tracks — almost too neatly — the polyphenol-loading ranking from Manuscript 1 (TPC: EE 78.3 > HAE 51.6 > EAE 42.7 mg GAE/g; HPTLC marker total: EE 6.64 > HAE 3.99 > EAE 2.32 mg/g). One position deviate: HAE outranks EAE on colorimetric TPC but underperforms it in vivo. The resolution lies in two non-exclusive effects. First, HAE's high carbohydrate and reducing-sugar load inflates the Folin–Ciocalteu reading (Sánchez-Rangel et al., 2013), so HAE's functional polyphenol concentration at systemic target tissues is lower than its TPC implies. Second, HAE's saponin-and-polysaccharide-heavy composition carries lower oral bioavailability than EAE's more lipophilic flavonoid aglycone pool. Taken together, the in vivo ordering is chemically coherent; it simply requires reading the HPTLC marker-total ranking (which is more structurally specific than colorimetric TPC) rather than the colorimetric TPC ranking.

Table 3: Fasting blood glucose trajectory (mg/dL, mean $\pm$ SEM, $n = 6$) across 28 days. ** $p < 0.01$, * $p < 0.001$ versus G2 diabetic control (Dunnett's post-hoc following one-way ANOVA).**

Group	Day 0	Day 7	Day 14	Day 21	Day 28
G1 Normal	88 ± 3	90 ± 4	91 ± 3	89 ± 4	90 ± 4
G2 Diabetic control	310 ± 10	348 ± 12	362 ± 13	374 ± 14	381 ± 15
G3 Metformin 150 mg/kg	304 ± 9	$232 \pm 10^{***}$	$178 \pm 9^{***}$	$142 \pm 8^{***}$	$118 \pm 7^{***}$
G4 EAE 400 mg/kg	308 ± 10	$268 \pm 11^{***}$	$224 \pm 10^{***}$	$192 \pm 9^{***}$	$168 \pm 8^{***}$
G5 EE 400 mg/kg	312 ± 11	$244 \pm 10^{***}$	$192 \pm 9^{***}$	$156 \pm 8^{***}$	$132 \pm 7^{***}$
G6 HAE 400 mg/kg	307 ± 10	$282 \pm 11^{**}$	$248 \pm 11^{**}$	$218 \pm 10^{**}$	$198 \pm 10^{**}$

3.4 Body weight trajectories and clinical observations

G1 normal animals gained 48 g (+24.0%) over 28 days, following the standard Wistar age-related growth curve. G2 diabetic-control animals lost 33 g (-16.4%) despite polyphagia the expected insulin-deficiency catabolic signature in which muscle-protein breakdown and lipolysis produce net negative nitrogen balance even in the face of elevated food intake (Wilcox, 2005). Body weight trajectories tracked the FBG response across all treatment arms: EE gained the most (+20 g, +9.9%), followed by metformin (+15 g, +7.5%), EAE (+11 g, +5.5%), and HAE (+6 g, +3.0%).

EE's slightly larger weight gain than metformin (9.9% vs 7.5%) despite a marginally higher day-28 FBG is

interpretively interesting. Metformin acts primarily through hepatic gluconeogenesis suppression without substantial direct effect on muscle protein synthesis; a polyphenol-rich extract may exert effects on multiple metabolic axes insulin signalling, oxidative stress, inflammation that together support net anabolism more effectively than gluconeogenesis suppression alone (Xiao & Högger, 2015). This is consistent with, not confirmed by, the 2-NBDG glucose-recovery data in Section 3.6.

Clinical observations at day 28 were concordant with the biochemical rankings. G1 and G5 (EE) were indistinguishable by day 28 alert, glossy fur, normal water intake. G3 (metformin) achieved comparable normalisation, slightly slower. G4 (EAE) showed fur

improvement from day 14 but water intake normalised only by day 28. G6 (HAE) showed partial improvement only: fur ruffling reduced but still present, water intake elevated, two of six animals still scored as mildly lethargic at day 28. No mortality occurred in any group throughout the 28-day window. The concordance between primary biochemical readout (FBG), secondary biochemical readout (body weight), and cage-side clinical observations across treatment arms strengthens the inference that what is being read is a real systemic metabolic response.

3.5 MTT cytotoxicity — working concentration selection for in vitro assays

EE was the best-tolerated fraction in both cell lines across the full 1–500 µg/mL range. In L6 myotubes at 100 µg/mL, EE preserved $90 \pm 4\%$ viability; EAE $86 \pm 4\%$; HAE $81 \pm 4\%$ — on the boundary. In HepG2 at 100 µg/mL: EE $88 \pm 4\%$, EAE $84 \pm 4\%$, HAE $79 \pm 4\%$ (below the 80% threshold). HAE viability fell to $64 \pm 5\%$ (L6) and $60 \pm 5\%$ (HepG2) at 250 µg/mL, consistent with saponin-mediated membrane perturbation at concentrations above 50–100 µg/mL a well-documented cytotoxic mechanism for saponin-rich fractions in cultured cells (Mosmann, 1983). The convergence of equivalent cytotoxicity windows across two cell lines (rat myotubes and human hepatoma cells) argues that the profile is intrinsic to the extract chemistry rather than to a cell-type-specific susceptibility. The 80% viability criterion translates operationally to working ceilings of ≤ 100 µg/mL for EAE and EE, and ≤ 50 µg/mL for HAE, in both cell lines. EE's higher cytocompatibility despite carrying the highest polyphenol mass is consistent with the view that flavonoid aglycones and glycosylated flavonoids are not particularly membrane-active at concentrations relevant to glucose-uptake assays. The ceiling was set conservatively 5% above the 75% threshold used in many published cytotoxicity workflows so that any glucose-uptake response in Sections 3.6–3.7 could be attributed to a pharmacological effect on insulin signalling rather than to a cytotoxic confound on mitochondrial reductase activity.

3.6 Model validation basal and insulin-stimulated 2-NBDG uptake

Insulin (100 nM, 20 min) stimulated 2-NBDG uptake to $184 \pm 7\%$ of unchallenged basal in L6 myotubes ($p < 0.001$ vs basal) and to $162 \pm 6\%$ in HepG2 hepatocytes ($p < 0.001$ vs basal). The larger L6 stimulation reflects skeletal muscle's dominant role in post-prandial whole-body glucose disposal and the well-characterized GLUT4 translocation response in differentiated L6 cells (Mitsumoto & Klip, 1992; Yamada et al., 2007). The more modest HepG2 stimulation is expected: hepatic glucose handling under insulin is dominated by gluconeogenic

suppression and glycogen synthesis rather than GLUT-mediated uptake at the plasma membrane. Both contrasts placed the models within their expected pharmacological envelopes, validating the assay system before the insulin-resistant challenge was imposed.

The 100 nM insulin concentration sits above the physiological postprandial peak (~ 0.5 – 1.0 nM in plasma) and is the conventional saturating concentration for in vitro stimulation; the 20-min stimulation window is similarly standard. This design maximises the dynamic range of the readout, which is what one needs when subsequent assays will look for partial restoration of uptake against an insulin-resistant baseline. A more physiological insulin concentration would compress the basal-to-insulin span and make any reversal signal harder to read above experimental noise. The trade-off is standard in the cellular insulin-resistance literature (Yamada et al., 2007).

3.7 Palmitate-induced insulin resistance and extract-mediated reversal

Palmitate (300 µM, 16 h, L6) flattened basal uptake at $96 \pm 5\%$ of unchallenged basal not statistically different from baseline and abolished the insulin response, producing the expected basal-flat insulin-resistant profile. The mechanistic basis is well established: ceramide accumulation from de novo synthesis driven by saturated fatty acid loading activates PKC ζ , which interrupts Akt phosphorylation downstream of the insulin receptor; in parallel, JNK activation through ceramide and mitochondrial ROS phosphorylates IRS-1 on serine residues, blocking tyrosine phosphorylation and breaking the insulin-signalling cascade (Powell et al., 2004; Chavez & Summers, 2003; Hirosumi et al., 2002; Yuan et al., 2001). Palmitate (250 µM, 24 h, HepG2) produced a different profile suppression of basal uptake to $88 \pm 5\%$ ($p < 0.05$ vs unchallenged basal) consistent with proteasomal degradation of insulin receptor substrates described by Ishii et al. (2015) and the mitochondrial-ROS-dependent insulin-resistance phenotype in hepatocyte models (Nakamura et al., 2009). The two cell-type-specific profiles basal-flat in L6, basal-suppressed in HepG2 are intrinsic features of how each cell type responds to lipotoxic stress and are independently documented in the cited primary literature.

Glucose uptake restoration results are presented in Tables 4 and 5. In L6 myotubes, EE 100 µg/mL recovered 2-NBDG uptake to $167 \pm 7\%$ of unchallenged basal ($p < 0.001$ vs insulin-resistant baseline) 90% of the metformin response ($178 \pm 6\%$) and 91% of the unchallenged insulin-stimulated value ($184 \pm 7\%$). EAE 100 µg/mL produced intermediate recovery ($138 \pm 6\%$, $p < 0.01$ vs insulin-resistant baseline); HAE 50 µg/mL produced the weakest

statistically significant restoration ($124 \pm 6\%$, $p < 0.05$ vs insulin-resistant baseline). In HepG2, EE $100 \mu\text{g/mL}$ reached $152 \pm 7\%$ ($p < 0.001$ vs insulin-resistant baseline), within experimental noise of metformin ($158 \pm 6\%$). EAE $100 \mu\text{g/mL}$ produced meaningful but weaker reversal ($126 \pm 6\%$, $p < 0.01$ vs insulin-resistant baseline). HAE $50 \mu\text{g/mL}$ reached only $114 \pm 5\%$ and did not separate statistically from the insulin-resistant baseline in HepG2. The HAE null in HepG2 is the sharpest evidence that HAE's modest in vivo signal does not arise from direct insulin-sensitizing action at the hepatocyte plasma membrane. Rather, HAE's 35.5% in vivo FBG reduction is

most plausibly attributable to mechanisms the 2-NBDG uptake assay does not address: α -glucosidase inhibition at the intestinal brush border (a plausible saponin-mediated mechanism given HAE's qualitative saponin profile), intestinal carbohydrate-absorption modulation, or gut-microbiota-mediated effects on glucose metabolism. These are testable hypotheses a parallel α -glucosidase inhibition assay on HAE alongside saponin enrichment characterization would distinguish intestinal from systemic contributions and they represent the natural next analytical step for this fraction.

Table 4: 2-NBDG glucose uptake in L6 myotubes under palmitate insulin-resistance reversal conditions (% of unchallenged basal, mean $\pm$ SD, n = 4).

Condition	Uptake (% of basal)	SD	Significance vs IR baseline
Basal	100	4	—
Insulin 100 nM	184	7	*** $p < 0.001$ vs basal
Palmitate $300 \mu\text{M} \times 16 \text{ h}$ (IR)	96	5	ns vs basal
IR + EAE $100 \mu\text{g/mL}$	138	6	** $p < 0.01$
IR + EE $100 \mu\text{g/mL}$	167	7	*** $p < 0.001$
IR + HAE $50 \mu\text{g/mL}$	124	6	* $p < 0.05$
IR + Metformin 1 mM	178	6	*** $p < 0.001$

Table 5: 2-NBDG glucose uptake in HepG2 hepatocytes under palmitate insulin-resistance reversal conditions (% of unchallenged basal, mean $\pm$ SD, n = 4).

Condition	Uptake (% of basal)	SD	Significance vs IR baseline
Basal	100	4	—
Insulin 100 nM	162	6	*** $p < 0.001$ vs basal
Palmitate $250 \mu\text{M} \times 24 \text{ h}$ (IR)	88	5	* $p < 0.05$ vs basal
IR + EAE $100 \mu\text{g/mL}$	126	6	** $p < 0.01$
IR + EE $100 \mu\text{g/mL}$	152	7	*** $p < 0.001$
IR + HAE $50 \mu\text{g/mL}$	114	5	ns
IR + Metformin 1 mM	158	6	*** $p < 0.001$

The EE dose-response under palmitate insulin-resistance conditions was monotonic in both cell lines, with inflection between 10 and $25 \mu\text{g/mL}$ and approach to plateau by 50 – $100 \mu\text{g/mL}$. EC₅₀: L6 $\sim 22 \mu\text{g/mL}$, HepG2 $\sim 31 \mu\text{g/mL}$. Hill slopes close to unity (1.05 in L6, 0.98 in HepG2) are consistent with a multi-component extract acting through several independent agonists converging on the same downstream phenotype, rather than cooperative or threshold behaviour. A steeper Hill slope (≥ 2) would have argued for cooperativity; a shallower one (≤ 0.5) for multiple sites with very different affinities. Neither was observed. The near-unity slopes argue against a single dominant marker driving the uptake response and in favour of a composite, additive contribution from multiple polyphenol classes a reading aligned with the pleiotropic

antidiabetic mechanism synthesis of Xiao and Högger (2015).

The three HPTLC-quantified markers of EE supply distinct mechanistic threads at the molecular level. Quercetin (3.12 mg/g in EE) activates AMPK in skeletal muscle (Dhanya et al., 2017; Eid & Haddad, 2017; Jiang et al., 2019), providing an insulin-independent route to GLUT4 translocation that bypasses the ceramide/PKC ζ /IRS-1 block induced by palmitate. The L6 EC₅₀ ($\sim 22 \mu\text{g/mL}$) is mechanistically consistent with an AMPK-pathway contribution from quercetin-class flavonoids at the concentrations delivered by EE. Rutin (2.34 mg/g) potentiates insulin receptor kinase activity and GLUT4 translocation (Hsu et al., 2014) and produces antihyperglycemic and antioxidant effects in STZ-Wistar rats (Kamalakkannan & Prince, 2006) the antioxidant co-

effect addressing the mitochondrial-ROS arm of the palmitate-induced insulin-resistance pathway (Nakamura et al., 2009). Gallic acid (1.18 mg/g) improves hepatic carbohydrate metabolism and gluconeogenic enzyme expression in metabolically challenged rodents (Huang et al., 2016); the HepG2 EC₅₀ (~31 µg/mL) is mechanistically consistent with a gallic-acid-class contribution to hepatic glucose handling alongside any quercetin-mediated AMPK activation in hepatocytes.

EE-mediated reversal of palmitate-induced insulin resistance could in principle act at three distinct intervention points along the impaired signalling cascade: reducing de novo ceramide accumulation; attenuating JNK activation through antioxidant quenching of upstream mitochondrial ROS (Chavez & Summers, 2003; Hirosumi et al., 2002); or activating AMPK in parallel to insulin signalling, bypassing the JNK/IRS-1 serine-phosphorylation block (Hardie et al., 2016; Kjøbsted et al., 2018). The 2-NBDG uptake assay reads a downstream phenotypic output and cannot distinguish these upstream intervention points. Western-blot characterization of AMPK-T172, Akt-S473, JNK, and IRS-1 serine/tyrosine phosphorylation status, paired with membrane GLUT4 fractionation, would resolve the mechanistic question and is the natural next experimental step. Equally, running quercetin, rutin, and gallic acid individually at their EE-loaded concentrations (0.31, 0.23, and 0.12 µg/mL respectively at 100 µg/mL EE) would establish whether their combined contribution accounts for the observed uptake restoration or whether the broader unassigned phenolic envelope contributes substantively.

The cross-tissue response asymmetry is a small but interpretively useful observation. Expressed as a fraction of the unchallenged insulin response, EE achieved 91% uptake reversal in L6 (167/184) and 94% in HepG2 (152/162) — marginally higher fractional reversal in liver than in muscle. The same pattern did not hold for EAE (75% in L6, 78% in HepG2) or HAE (67% in L6, statistically non-significant in HepG2). The cross-tissue uniformity of the EE response across two pharmacologically distinct models is one of the more interpretively robust observations from the *in vitro* arm: it argues that EE's restorative chemistry is not restricted to a single tissue's insulin-signalling architecture.

4. CONCLUSION

The first paired *in vivo-in vitro* antidiabetic record for *Ipomoea parasitica* aerial-part Soxhlet extracts establishes that EAE, EE, and HAE are acutely non-toxic at 2000 mg/kg p.o. (LD₅₀ > 2000 mg/kg, GHS Category 5). EE at 400 mg/kg p.o. daily for 28 days reduced fasting blood

glucose by 57.7% in STZ-Wistar rats statistically indistinguishable from metformin 150 mg/kg at n = 6 while reversing diabetic weight loss and normalizing cage-side clinical signs. At the cellular level, EE 100 µg/mL reversed palmitate-induced insulin resistance in both L6 myotubes (167% of unchallenged basal, EC₅₀ ~22 µg/mL) and HepG2 hepatocytes (152% of unchallenged basal, EC₅₀ ~31 µg/mL), with near-unity Hill slopes arguing for composite, additive contributions from multiple polyphenol classes. The cellular reversal ranking — EE > EAE > HAE mirrors the polyphenol-load hierarchy from the companion phytochemistry study, with the EAE/HAE rank reversal attributable to the higher oral bioavailability of EAE's mid-polarity flavonoid aglycone pool relative to HAE's saponin-dominated composition. HAE's failure to restore uptake in HepG2 at its cytotoxicity-limited ceiling points toward intestinal rather than systemic mechanisms for that fraction's modest *in vivo* activity. These findings place *I. parasitica* aerial-part EE on the antidiabetic axis of the *Ipomoea* genus for the first time and supply a mechanistically anchored scientific basis for the follow-on work Western-blot pathway characterization, marker-additivity dissection, primary-cell confirmation, and dose-response extension that would convert this pharmacological prior into a fuller mechanistic account.

REFERENCES

- Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., & Knowles, B. B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature*, 282(5739), 615–616.
<https://doi.org/10.1038/282615a0>
- Bailey, C. J., & Turner, R. C. (1996). Metformin. *New England Journal of Medicine*, 334(9), 574–579.
<https://doi.org/10.1056/NEJM199602293340906>
- Chavez, J. A., & Summers, S. A. (2003). Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Archives of Biochemistry and Biophysics*, 419(2), 101–109.
<https://doi.org/10.1016/j.abb.2003.08.020>
- CPCSEA. (2003). CPCSEA guidelines for laboratory animal facility. *Indian Journal of Pharmacology*, 35(4), 257–274.
- Dhanya, R., Arun, K. B., Syama, H. P., & Nisha, P. (2017). Rutin and quercetin enhance glucose uptake in L6 myotubes. *Frontiers in Pharmacology*, 8, 336.
<https://doi.org/10.3389/fphar.2017.00336>
- Dunnett, C. W. (1955). A multiple comparison procedure for comparing several treatments with a control. *Journal of the American Statistical Association*, 50(272), 1096–1121.

- Eid, H. M., & Haddad, P. S. (2017). The antidiabetic mechanisms of quercetin. *Current Medicinal Chemistry*, 24(4), 355–364.
<https://doi.org/10.2174/0929867323666160909153707>
- Furman, B. L. (2021). Streptozotocin-induced diabetic models in mice and rats. *Current Protocols*, 1(4), e78.
<https://doi.org/10.1002/cpz1.78>
- Goyal, S. N., Reddy, N. M., Patil, K. R., et al. (2016). Challenges and issues with streptozotocin-induced diabetes. *Chemico-Biological Interactions*, 244, 49–63.
<https://doi.org/10.1016/j.cbi.2015.11.032>
- Hardie, D. G., Schaffer, B. E., & Brunet, A. (2016). AMPK: An energy-sensing pathway with multiple inputs and outputs. *Trends in Cell Biology*, 26(3), 190–201.
<https://doi.org/10.1016/j.tcb.2015.10.013>
- Hirosumi, J., Tuncman, G., Chang, L., et al. (2002). A central role for JNK in obesity and insulin resistance. *Nature*, 420(6913), 333–336.
<https://doi.org/10.1038/nature01137>
- Hsu, C. Y., Shih, H. Y., Chia, Y. C., et al. (2014). Rutin potentiates insulin receptor kinase to enhance insulin-dependent glucose transport activity. *Molecular Nutrition & Food Research*, 58(6), 1168–1176.
<https://doi.org/10.1002/mnfr.201300691>
- Huang, D. W., Chang, W. C., Wu, J. S. B., Shih, R. W., & Shen, S. C. (2016). Gallic acid ameliorates hyperglycemia and improves hepatic carbohydrate metabolism in rats fed a high-fructose diet. *Nutrition Research*, 36(2), 150–160. <https://doi.org/10.1016/j.nutres.2015.10.001>
- Ishii, Y., Maeda, A., Tani, K., & Akagawa, M. (2015). Palmitate induces insulin resistance in human HepG2 hepatocytes by enhancing ubiquitination and proteasomal degradation of key insulin signaling molecules. *Archives of Biochemistry and Biophysics*, 566, 26–35.
<https://doi.org/10.1016/j.abb.2014.12.009>
- Jiang, S., Young, J. L., Wang, K., Qian, Y., & Ai, L. (2019). Quercetin-induced AMPK activation: A potential treatment strategy for type 2 diabetes. *Scientific Reports*, 9, 38989.
<https://doi.org/10.1038/s41598-019-38989-7>
- Kamalakkannan, N., & Prince, P. S. M. (2006). Antihyperglycaemic and antioxidant effect of rutin in streptozotocin-induced diabetic Wistar rats. *Basic & Clinical Pharmacology & Toxicology*, 98(1), 97–103.
<https://doi.org/10.1111/j.17427843.2006.pto>
- Kjøbsted, R., Hingst, J. R., Fentz, J., et al. (2018). AMPK in skeletal muscle function and metabolism. *FASEB Journal*, 32(4), 1741–1777.
<https://doi.org/10.1096/fj.201700442R>
- Knowles, B. B., Howe, C. C., & Aden, D. P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*, 209(4455), 497–499.
<https://doi.org/10.1126/science.6248960>
- Lenzen, S. (2008). The mechanisms of alloxan- and streptozotocin-induced diabetes. *Diabetologia*, 51(2), 216–226. <https://doi.org/10.1007/s00125-007-0886-7>
- Listenberger, L. L., Han, X., Lewis, S. E., et al. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *PNAS*, 100(6), 3077–3082.
<https://doi.org/10.1073/pnas.0630588100>
- Malalavidhane, T. S., Wickramasinghe, S. M., & Jansz, E. R. (2000). Oral hypoglycaemic activity of *Ipomoea aquatica*. *Journal of Ethnopharmacology*, 72(1–2), 293–298.
[https://doi.org/10.1016/S0378-8741\(00\)00207-X](https://doi.org/10.1016/S0378-8741(00)00207-X)
- Malalavidhane, T. S., Wickramasinghe, S. M., Perera, M. S., & Jansz, E. R. (2003). Oral hypoglycaemic activity of *Ipomoea aquatica* in streptozotocin-induced, diabetic Wistar rats and type II diabetics. *Phytotherapy Research*, 17(9), 1098–1100.
<https://doi.org/10.1002/ptr.1345>
- Mitsumoto, Y., & Klip, A. (1992). Development regulation of GLUT1 and GLUT4 glucose transporters during myogenesis of L6 muscle cells. *Journal of Biological Chemistry*, 267(7), 4957–4962.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival. *Journal of Immunological Methods*, 65(1–2), 55–63.
[https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
- Nakamura, S., Takamura, T., Matsuzawa-Nagata, N., et al. (2009). Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. *Journal of Biological Chemistry*, 284(22), 14809–14818.
<https://doi.org/10.1074/jbc.M901488200>
- OECD. (2002). Test No. 423: Acute oral toxicity Acute toxic class method. OECD Publishing.
<https://doi.org/10.1787/9789264071001-en>
- Percie du Sert, N., Hurst, V., Ahluwalia, A., et al. (2020). The ARRIVE guidelines 2.0. *PLOS Biology*, 18(7), e3000410.
<https://doi.org/10.1371/journal.pbio.3000410>
- Powell, D. J., Turban, S., Gray, A., Hajduch, E., & Hundal, H. S. (2004). Intracellular ceramide synthesis and protein kinase C ζ activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochemical Journal*, 382, 619–629.
<https://doi.org/10.1042/BJ20040139>
- Reaven, G. M. (1988). Role of insulin resistance in human disease. *Diabetes*, 37(12), 1595–1607.
<https://doi.org/10.2337/diab.37.12.1595>
- Rena, G., Hardie, D. G., & Pearson, E. R. (2017). The mechanisms of action of metformin. *Diabetologia*, 60(9), 1577–1585.
<https://doi.org/10.1007/s00125-017-4342-z>
- Sánchez-Rangel, J. C., Benavides, J., Heredia, J. B., Cisneros-Zevallos, L., & Jacobo-Velázquez, D. A. (2013). The Folin–Ciocalteu assay revisited. *Analytical Methods*, 5(21), 5990–5999.
<https://doi.org/10.1039/C3AY41125G>

- Sajak, A. A. B., Abas, F., & Ismail, A. (2017). Ipomoea aquatica ameliorates glucose metabolism. *Phytomedicine*, 33, 1–8. <https://doi.org/10.1016/j.phymed.2017.10.011>
- Tukey, J. W. (1949). Comparing individual means in the analysis of variance. *Biometrics*, 5(2), 99–114.
- Vijayakumar, S., Presannakumar, G., & Vijayalakshmi, N. R. (2012). Antidiabetic activity of Ipomoea aquatica in alloxan-induced diabetic rats. *Journal of Ethnopharmacology*, 139(2), 396–400. <https://doi.org/10.1016/j.jep.2012.02.005>
- Wilcox, G. (2005). Insulin and insulin resistance. *Clinical Biochemist Reviews*, 26(2), 19–39.
- Xiao, J., & Högger, P. (2015). Dietary polyphenols and type 2 diabetes. *Current Medicinal Chemistry*, 22(1), 23–38. <https://doi.org/10.2174/0929867321666140706130807>
- Yaffe, D. (1968). Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *PNAS*, 61(2), 477–483. <https://doi.org/10.1073/pnas.61.2.477>
- Yamada, K., Saito, M., Matsuoka, H., & Inagaki, N. (2007). A real-time method of imaging glucose uptake in single, living mammalian cells. *Nature Protocols*, 2(3), 753–762. <https://doi.org/10.1038/nprot.2007.76>
- Yuan, M., Konstantopoulos, N., Lee, J., et al. (2001). Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikk β . *Science*, 293(5535), 1673–1677. <https://doi.org/10.1126/science.1061620>

***Corresponding author Email address:**
mahalakshmi30091991@gmail.com