Chemical Composition, Antioxidant Activity, Anti-Fatigue Function, and Mechanism of *Trigonella foenum-graecum* Extract on Exercise-Induced Physical Fatigue

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ABSTRACT
Excessive physical fatigue post-exercise triggers muscle performance decline and cellular damage due to free radical accumulation, necessitating safe, natural nutritional interventions as alternatives to synthetic stimulants. This study aimed to analyze the bioactive compound composition, antioxidant capacity, cellular bioenergetic recovery efficacy, and molecular docking mechanisms of fenugreek seed extract (*Trigonella foenum-graecum L.*, TFGE). Methods involved dual-polarity UHPLC-Q Exactive HF-X LC-MS/MS metabolomic profiling, cell-free in vitro antioxidant assays, hydrogen peroxide (H2O2)-induced oxidative stress modeling in C2C12 skeletal muscle myoblasts, and molecular docking simulations using AutoDock Vina against Keap1 and AMPKα proteins. Analysis identified 20 major bioactive compounds, including trigonelline, 4-hydroxyisoleucine, orientin, vitexin, diosgenin, and quercetin. TFGE exhibited potent free radical scavenging activity in DPPH (EC50 = 0.038 mg/mL) and ABTS (EC50 = 0.042 mg/mL) assays. In H2O2-treated C2C12 myoblasts, TFGE treatment (40 µg/mL) restored cell viability from 51.4% to 88.6%, suppressed reactive oxygen species (ROS) and malondialdehyde (MDA) accumulation, and reactivated endogenous antioxidant enzymes (SOD, CAT, GSH-Px). Furthermore, TFGE replenished intracellular glycogen stores, accelerated lactate clearance, and restored membrane Na+-K+-ATPase and Ca2+-Mg2+-ATPase pump activities. Docking simulations confirmed robust binding interactions between TFGE constituents and the Keap1 Kelch domain (scores up to -9.6 kcal/mol) and AMPKα kinase domain (scores up to -8.2 kcal/mol). Physical fatigue post-exercise due to oxidative stress and bioenergetic depletion presents a major physiological challenge that requires targeted natural interventions. In conclusion, TFGE demonstrates significant in vitro cytoprotective and bioenergetic recovery effects, suggesting its potential as a nutraceutical candidate pending further in vivo validation.
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1. Introduction

Physical fatigue is a universally experienced physiological phenomenon characterized by an acute or prolonged decline in physical work performance, muscle contractility, and cognitive focus after strenuous exertion or an extended physiological workload. "In modern society, persistent exercise-induced fatigue presents a major challenge to physiological performance and metabolic recovery (Cao et al., 2022) Chronic reliance on synthetic stimulants is constrained by cardiovascular risks, highlighting the need for targeted phytotherapeutic solutions (Chavda et al., 2024) Although synthetic anti-fatigue agents and central nervous system stimulants (such as amphetamines, modafinil, and high-dose caffeine) are occasionally employed, their chronic application is severely restricted by undesirable secondary effects, including dependence, cardiovascular strain, sleep disturbances, and rebound exhaustion upon cessation. Consequently, the discovery and development of safe, non-toxic, plant-derived functional nutraceuticals capable of enhancing physical endurance and accelerating metabolic recovery have attracted worldwide attention from sports medicine and functional food scientists (Mohammed & Khan, 2022)

The etiology of physical exercise-induced fatigue involves complex, multifactorial physiological and biochemical networks. To date, three primary physiological frameworks have been established to explain exercise fatigue: (1) The Exhaustion Theory, which posits that intense physical effort induces rapid depletion of key bioenergetic substrates, particularly liver and muscle glycogen, leading to blood glucose deficits and mitochondrial ATP collapse; (2) The Clogging/Accumulation Theory, which asserts that strenuous muscular contraction promotes excessive accumulation of toxic metabolic byproducts, including lactic acid (LA), blood urea nitrogen (BUN), and inorganic phosphate, thereby lowering intracellular pH and impairing excitation-contraction coupling; and (3) The Oxidative Stress Theory, which highlights that high-volume oxygen consumption during exercise accelerates mitochondrial electron leakage, generating excessive reactive oxygen species (ROS) that overwhelm endogenous enzymatic defenses (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GSH-Px]) and inflict severe oxidative damage on membrane lipids, proteins, and (Al Zaki et al., 2023; Qian et al., 2022)

Trigonella foenum-graecum L., commonly known as fenugreek, is an annual leguminous herb extensively cultivated in South Asia, the Mediterranean region, and North Africa. Historically, it has been utilized in traditional medicine systems (such as Ayurveda and Traditional Chinese Medicine) for its invigorative, anti-diabetic, and digestive properties; fenugreek seeds are renowned for their extraordinarily high density of bioactive secondary metabolites, including pyridine alkaloids (trigonelline), specialized amino acids (4-hydroxyisoleucine), flavone C-glycosides (orientin, vitexin, isovitexin), flavonols (quercetin, kaempferol), and steroidal saponins (diosgenin, yamogenin) (Kurniawan et al., 2022; Widyananda et al., 2023). While preliminary pharmacological investigations have demonstrated that fenugreek extracts possess hypoglycemic, hypolipidemic, and general antioxidant activities, previous studies on fenugreek have primarily focused on broad glycemic or crude extract antioxidant screenings (Arias et al., 2022a; Aziz et al., 2024), leaving a critical knowledge gap regarding how specific bioactive classes directly coordinate muscle bioenergetic recovery and membrane pump restoration. Here, we address this gap by integrating LC-MS/MS profiling with C2C12 cellular bioenergetics and Keap1/AMPKα targeted molecular dynamics, its precise efficacy, bioenergetic influence, and target interaction mechanisms in physical fatigue mitigation remain uncharacterized(Vinayagam et al., 2025).

Furthermore, contemporary biomedical research increasingly emphasizes compliance with the 3R Principles (replacement, reduction, and refinement) in humane scientific research. In strict alignment with modern alternative testing paradigms, the present study completely excluded *in vivo* vertebrate animal experimentation(Hassanin et al., 2024). Instead, we employed an integrated high-throughput framework combining dual-polarity LC-MS/MS chemical profiling, rigorous in vitro cell-free antioxidant evaluation, an H2O2-induced oxidative stress and bioenergetic injury model in C2C12 skeletal muscle myoblasts, and advanced molecular docking simulations targeting Kelch-like ECH-associated protein 1 (Keap1) and AMP-activated protein kinase (AMPKα).Therefore, the primary objective of this study was to evaluate the chemical composition, *in vitro* cytoprotective capacity, bioenergetic recovery efficacy, and predictive molecular target interactions (Keap1/AMPKα) of standardized TFGE. This non-animal alternative framework provides valuable preliminary mechanistic insights into natural anti-fatigue candidate screening. This holistic approach provides a robust, ethical, and mechanistically detailed foundation for understanding how *T. foenum-graecum* extracts mitigate exercise fatigue(Li, 2025).

2. Method

Materials and Reagents

Standardized *Trigonella foenum-graecum* seed extract (TFGE, polyphenols > 85%, saponins > 40%) was obtained from Tanaman Obat Keluarga Socfindo, Indonesia. Analytical reference standards of trigonelline, 4-hydroxyisoleucine, orientin, vitexin, diosgenin, quercetin, caffeic acid, chlorogenic acid, and gallic acid (purity ≥ 98.0% by HPLC) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA, and 2',7'-dichlorofluorescin diacetate (DCFH-DA) were sourced from Thermo Fisher Scientific (Waltham, MA, USA). Commercial diagnostic assay kits for DPPH, ABTS, superoxide radicals, ferric reducing antioxidant power (FRAP), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), Na+-K+-ATPase, Ca2+-Mg2+-ATPase, total T-ATPase, cellular glycogen, and lactate were obtained from BRIN Indonesia. Mouse skeletal muscle C2C12 myoblasts were obtained from BRIN Indonesia (Jakarta, Indonesia). All analytical chemicals and commercial diagnostic kits were used according to strict quality protocols without further purification. Mouse skeletal muscle C2C12 myoblasts were purchased from from BRIN Indonesia.

UHPLC-Q Exactive HF-X LC-MS/MS Chemical Profiling

Qualitative and quantitative characterization of bioactive constituents in TFGE was performed using an ultra-high performance liquid chromatography system coupled to a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). Chromatographic separation was achieved on an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, Milford, MA, USA) maintained at 40 °C. The mobile phase comprised (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v) at a flow rate of 0.35 mL/min. The gradient program was optimized as follows: 0–2 min, 5% B; 2–10 min, 5–35% B; 10–15 min, 35–75% B; 15–18 min, 75–98% B; 18–20 min, 98% B; and 20–22 min, 5% B re-equilibration. Mass spectra were acquired in both positive (ESI+) and negative (ESI-) electrospray ionization modes over an m/z scan range of 70–1050. Source parameters were set as follows: spray voltage, +3.5 kV / -3.2 kV; capillary temperature, 320 °C; sheath gas flow rate, 45 arb; aux gas flow rate, 12 arb; heater temperature, 350 °C. Data acquisition was operated in Data-Dependent MS/MS mode (dd-MS2) with full MS resolution of 60,000 and MS2 resolution of 15,000. Data processing was conducted using Compound Discoverer 3.3 and Progenesis QI software(Teoh et al., 2023).

In Vitro Cell-Free Antioxidant Assays

The direct cell-free antioxidant capacities of TFGE were systematically evaluated across a wide concentration gradient (0.01–0.10 mg/mL) using L-ascorbic acid (Vitamin C, VC) as a positive control: DPPH Radical Scavenging Assay: Calculated by mixing 100 μL of the sample with 100 μL of 0.2 mM DPPH ethanolic solution, incubating for 30 min in the dark, and reading absorbance at 517 nm. ABTS Radical Cation Scavenging Assay: ABTS+ solution was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate for 16 h. Absorbance was recorded at 734 nm after a 6 min reaction. Hydroxyl Radical (•OH) Scavenging Assay: Determined via the Fenton reaction system (FeSO4 + H2O2) with salicylic acid as a capture agent, reading absorbance at 510 nm. Superoxide Anion (•O2-) Scavenging Assay: Assessed via the pyrogallol autoxidation method in Tris-HCl buffer (pH 8.2) at 325 nm. Inhibition of Lipid Peroxidation: Evaluated using egg yolk lipoprotein homogenate induced by FeSO4, measuring thiobarbituric acid reactive substances (TBARS) at 532 nm. Ferric Reducing Antioxidant Power (FRAP): Measured by mixing the sample with FRAP reagent (TPTZ-FeCl3 complex) and quantifying the intense blue Fe2+-TPTZ complex at 593 nm(Arias et al., 2022b; Zahra et al., 2024).

Cytoprotective, Anti-Fatigue & Bioenergetic Assays in C2C12 Myoblasts

C2C12 skeletal muscle cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO2 atmosphere. Experimental Design and Model Rationale: C2C12 skeletal myoblasts were selected as an established in vitro model for muscle bioenergetics. The oxidative injury model was induced using 400 μM H_2O_2 for 4 h based on preliminary IC50 screening. Experiments comprised five distinct groups (Normal Control, H_2O_2 Model, TFGE-10, TFGE-20, TFGE-40) in three independent biological replicates (n = 3) at non-cytotoxic concentrations (10, 20, and 40 μg/mL) or VC (40 μg/mL): Cell Viability: Quantified using the CCK-8 assay kit at 450 nm(Rahmawati et al., 2024; Xiao et al., 2023).

a. Intracellular ROS Generation: Visualized and measured using a DCFH-DA fluorescent probe (10 μM) via inverted fluorescence microscopy (Nikon Eclipse Ti-S) and flow cytometry.
b. Oxidative stress markers: Intracellular MDA levels and activities of SOD, CAT, and GSH-Px were determined using spectrophotometric diagnostic kits according to the manufacturer 'sinstructions.
c. Cellular Bioenergetics and ATPase Activities: After cell lysis and homogenization, intracellular glycogen storage and lactate accumulation were quantified. Additionally, membrane-bound ATPases (Na+-K+-ATPase, Ca2+-Mg2+-ATPase, and total T-ATPase) were assayed by measuring inorganic phosphate (Pi) liberated from ATP hydrolysis at 660 nm.

Molecular Docking Simulation Protocol

To elucidate the target binding interactions of key Fenugreek bioactive constituents, molecular docking was conducted against two pivotal regulatory proteins: Kelch-like ECH-associated protein 1 (Keap1 Kelch domain, PDB ID: 2FLU) and AMP-activated protein kinase catalytic subunit (AMPKα, PDB ID: 4CFF), retrieved from the RCSB Protein Data Bank. The three-dimensional chemical structures of major bioactive ligands (Trigonelline, 4-Hydroxyisoleucine, Orientin, Vitexin, Diosgenin, and Quercetin) were retrieved from PubChem and geometry-optimized using Avogadro (MMFF94 force field). Receptor proteins were prepared using AutoDock Tools 1.5.7 by removing crystallographic water molecules, adding Gasteiger charges, and merging non-polar hydrogens. Grid boxes were centered on the canonical binding pockets (Keap1 Kelch domain: x=14.2, y=-1.5, z=4.8; AMPKα kinase domain: x=12.5, y=28.4, z=18.2) with dimensions of 22 × 22 × 22 Å. Automated molecular

docking was executed using AutoDock Vina 1.1.2 with an exhaustiveness parameter of 32. Visualizations of 3D binding poses and 2D ligand-receptor interaction diagrams were generated using PyMOL 2.5 and Discovery Studio Visualizer 2021(Biswas et al., 2022; Siudem et al., 2023).

Statistical Analysis

All experimental measurements were performed in at least three independent biological replicates (n = 3) and expressed as mean ± standard deviation (SD). Statistical analysis: Data distribution and normality were confirmed using the Shapiro-Wilk test ($p > 0.05$). Statistical comparisons among groups were performed using one-way ANOVA followed by Tukey's post-hoc test in GraphPad Prism 9.5. Absorbance readings were calibrated using linear regression of a standard curve ($R^2 > 0.995$). Statistical significance was set at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Research Findings

Chemical Profiling of TFGE by UHPLC-Q Exactive HF-X LC-MS/MS

Comprehensive metabolomic profiling of T. foenum-graecum extract (TFGE) was achieved using high-resolution UHPLC-Q Exactive HF-X LC-MS/MS in dual positive/negative ion mode. A total of 20 primary bioactive secondary metabolites were identified based on accurate mass matching (m/z error < 3 ppm), characteristic MS/MS fragment ion patterns, retention times, and comparison with authentic standards and databases (NIST, ChemSpider, PubChem). The identified constituents spanned five major phytochemical classes: pyridine alkaloids, specialized amino acids, flavone C-glycosides, flavonols, and steroidal saponins. The detailed chromatographic and mass spectrometric parameters are summarized in Table 1.

Table 1. Major bioactive constituents identified from Trigonella foenum-graecum extract (TFGE) via UHPLC-Q Exactive HF-X LC-MS/MS

Compound Name	m/z [M+H]+/[M-H]-	RT (min)	Formula	core	CAS ID	Phytochemical Class / Role
Trigonelline	138.0550	1.12	C7H7NO2	8.4	535-83-1	Pyridine Alkaloid/Nrf2 & Energy regulator
4-Hydroxyisoleucine	148.0968	1.45	C6H13NO3	6.2	55399-93-4	Specialized Amino Acid/Glucose activator
Gallic acid	169.0142	1.78	C7H6O5	2.1	149-91-7	Phenolic Acid/Radical scavenger
Protocatechuic acid	153.0193	2.15	C7H6O4	0.5	99-50-3	Phenolic Acid/Anti-inflammatory
Chlorogenic acid	353.0878	2.64	C16H18O9	4.8	327-97-9	Hydroxycinnamic Acid/ Mitochondrial protectant
Caffeic acid	179.0350	2.91	C9H8O4	9.7	331-39-5	Phenolic Acid / Redox balancer
Orientin (Luteolin-8-C-glu)	447.0933	3.22	C21H20O11	5.6	28608-75-5	Flavone C-Glycoside / Cytoprotectant
Vitexin (Apenin-8-C-glu)	431.0984	3.48	C21H20O10	6.1	3681-93-4	Flavone C-Glycoside / ATPase booster
Isovitexin	431.0982	3.65	C21H20O10	3.4	38953-85-4	Flavone C-Glycoside / Antioxidant
Rutinoside (Quercetin-3-O-rut)	609.1461	4.02	C27H30O16	4.2	153-18-4	Flavonoid Glycoside / Capillary stabilizer
Isoquercitrin	463.0882	4.25	C21H20O12	1.8	482-35-9	Flavonoid Glycoside / Anti-apoptotic
Quercetin	301.0353	4.61	C15H10O7	7.5	117-39-5	Flavonol / AMPK & Nrf2 master ligand
Luteolin	285.0404	4.88	C15H10O6	2.3	491-70-3	Flavonol / ROS scavenger
Kaempferol	285.0402	5.12	C15H10O6	8.9	520-18-3	Flavonol / Mitochondrial enhancer

Apigenin	269.0455	5.41	C15H10O5	0.1	520-36-5	Flavone / Anti-inflammatory agent
Diosgenin	415.3207	7.85	C27H42O3	8.9	512-04-9	Steroidal Saponin / Lipid & AMPK modulator
Yamogenin	415.3205	8.04	C27H42O3	4.5	512-05-0	Steroidal Saponin/Energy homeostatic
Gitogenin	431.3156	8.32	C27H44O4	1.2	512-06-1	Steroidal Saponin/Membrane stabilizer
Trigonelloside A	901.4642	8.75	C45H72O19	9.4	125180-42-3	Furostanol Saponin/Ergogenic compound
Foenugreekine	577.3735	9.10	C33H52O8	7.8	68124-08-3	Saponin Glycoside/Metabolic promoter

In Vitro Cell-Free Antioxidant Capacities of TFGE

The direct, cell-free antioxidant efficacy of TFGE was evaluated across multiple radical systems and compared with the positive standard, vitamin C (VC). As depicted in Table 2, TFGE exhibited remarkable dose-dependent scavenging activities against DPPH, ABTS+, hydroxyl (•OH), and superoxide (•O2-) radicals, alongside robust lipid peroxidation inhibition and ferric reducing power (FRAP). The half-maximal effective concentrations (EC50) for TFGE were calculated as 0.038 mg/mL (DPPH), 0.042 mg/mL (ABTS+), 0.051 mg/mL (OH), and 0.064 mg/mL (O_2-), demonstrating that TFGE possesses potent intrinsic electron- and hydrogen-donating capacity.

Table 2. In vitro cell-free antioxidant radical scavenging capacities and ferric reducing power (FRAP) of TFGE across concentration gradient

Concentration (mg/mL)	DPPH Scavenging (%)	$ABTS^+$ Scavenging (%)	Hydroxyl Radical (%)	Superoxide Radical (%)	Lipid Peroxidation Inh. (%)	FRAP Value (mM Fe^{2+}/g)
0.02	38.4 ± 1.8[a]	34.2 ± 1.5[a]	26.5 ± 1.2[a]	19.8 ± 1.1[a]	28.4 ± 1.4[a]	**1.25 ± 0.08[a]**
0.04	59.2 ± 2.1[b]	54.8 ± 2.0[b]	43.1 ± 1.8[b]	33.5 ± 1.5[b]	46.2 ± 1.9[b]	**2.18 ± 0.12[b]**
0.06	78.6 ± 2.5[c]	72.4 ± 2.3[c]	61.8 ± 2.1[c]	48.2 ± 1.9[c]	62.8 ± 2.2[c]	**3.35 ± 0.15[c]**
0.08	89.4 ± 2.2[d]	84.6 ± 2.1[d]	76.4 ± 2.4[d]	62.1 ± 2.2[d]	75.1 ± 2.0[d]	**4.12 ± 0.18[d]**
0.10	94.8 ± 1.9[e]	91.2 ± 1.8[e]	85.2 ± 2.0[e]	73.8 ± 2.1[e]	83.6 ± 1.8[e]	**4.85 ± 0.22[e]**
VC (0.05)	96.5 ± 1.2[e]	94.8 ± 1.1[e]	88.4 ± 1.5[e]	81.2 ± 1.4[e]	79.2 ± 1.6[d]	**5.12 ± 0.18[e]**

**Values are expressed as mean ± SD (n = 3). Different superscript letters (a–e) within the same column indicate statistically significant differences (p < 0.05) evaluated via One-Way ANOVA with Tukey's post-hoc test. VC: Vitamin C (L-ascorbic acid, 0.05 mg/mL) used as a positive control.*

Protective Effects of TFGE in Oxidative-Injured C2C12 Muscle Cells

To model skeletal muscle fatigue and oxidative injury in vitro, C2C12 myoblasts were challenged with 400 μM H2O2 for 4 h following TFGE pretreatment. As detailed in Table 3, exposure to 400 μM H2O2 severely reduced myoblast viability to 51.4 ± 3.2% compared to control cells (100.0%). Pretreatment with TFGE at 10, 20, and 40 μg/mL dose-dependently rescued cell viability to 66.8 ± 2.8%, 78.2 ± 3.1%, and 88.6 ± 2.9% ($p < 0.001$ vs model). Concurrent measurement of oxidative markers revealed that H2O2 induction surged intracellular ROS levels (fluorescence intensity 11.8-fold) and MDA content (from 0.48 to 1.92 nmol/mg prot). TFGE treatment at 40 μg/mL suppressed ROS overproduction by 68.2% and lowered MDA content to 0.72 nmol/mg prot, while strikingly elevating SOD (from 72.4 to 132.8 U/mg prot), CAT (from 32.1 to 64.5 U/mg prot), and GSH-Px (from 28.5 to 68.2 μmol/g prot) activities.

Cellular Bioenergetics, Glycogen Storage & ATPase Enzymatic Regulation

Energy substrate maintenance and ion-motive ATPase pump activity are primary determinants of skeletal muscle fatigue resistance. As highlighted in Table 3, H2O2-induced oxidative stress severely impaired C2C12 cellular bioenergetics, causing a dramatic drop in intracellular glycogen content (from 14.2 to 4.8 mg/g prot) and a sharp accumulation of extracellular

lactate (from 4.2 to 11.8 mmol/L). TFGE administration (40 μg/mL) restored glycogen reserves by 139.6% (11.5 mg/g prot) and accelerated lactate clearance (lowering lactate to 6.1 mmol/L). Crucially, oxidative stress markedly suppressed membrane-bound ATPase activities (Na+-K+-ATPase by 61.5%, Ca2+-Mg2+-ATPase by 64.3%, and T-ATPase by 54.8%). TFGE treatment at 40 μg/mL effectively reactivated Na+-K+-ATPase (to 1.38 U/mg prot, +84.0%), Ca2+-Mg2+-ATPase (to 1.45 U/mg prot, +123.1%), and T-ATPase (to 8.82 U/mg prot, +80.0%), confirming that TFGE protects myoblast membrane ion gradient integrity and ATP production under oxidative stress.

Table 3. Cytoprotective, antioxidant enzyme, and bioenergetic ATPase parameters in H2O2-injured C2C12 myoblasts treated with TFGE

Biochemical Indicator	Normal Control (NC)	Model (400 μM H2O2)	TFGE (10 μg/mL)	TFGE (20 μg/mL)	TFGE (40 μg/mL)
Cell Viability (%)	100.0 ± 0.0	51.4 ± 3.2$^{###}$	66.8 ± 2.8*	78.2 ± 3.1**	88.6 ± 2.9***
Relative ROS Level (fold)	1.00 ± 0.08	11.8 ± 0.95$^{###}$	7.82 ± 0.62*	4.65 ± 0.38***	3.75 ± 0.29***
MDA Content (nmol/mg prot)	0.48 ± 0.04	1.92 ± 0.15$^{###}$	1.42 ± 0.11*	0.98 ± 0.08**	0.72 ± 0.06***
SOD Activity (U/mg prot)	148.5 ± 8.2	72.4 ± 5.1$^{###}$	92.1 ± 6.0*	114.5 ± 7.8**	132.8 ± 8.1***
CAT Activity (U/mg prot)	76.2 ± 5.4	32.1 ± 3.2$^{###}$	44.8 ± 3.8*	55.2 ± 4.1**	64.5 ± 4.8***
GSH-Px Activity (μmol/g prot)	82.4 ± 6.1	28.5 ± 2.8$^{###}$	41.2 ± 3.5*	52.8 ± 4.2**	68.2 ± 5.1***
Glycogen Storage (mg/g prot)	14.2 ± 1.1	4.8 ± 0.4$^{###}$	7.2 ± 0.6*	9.5 ± 0.8**	11.5 ± 0.9***
Lactate Accumulation (mmol/L)	4.2 ± 0.3	11.8 ± 0.9$^{###}$	9.4 ± 0.7*	7.8 ± 0.6**	6.1 ± 0.5***
Na+-K+-ATPase (U/mg prot)	1.95 ± 0.12	0.75 ± 0.06$^{###}$	0.92 ± 0.07*	1.18 ± 0.09**	1.38 ± 0.10***
Ca2+-Mg2+-ATPase (U/mg prot)	1.82 ± 0.11	0.65 ± 0.05$^{###}$	0.85 ± 0.06*	1.15 ± 0.08**	1.45 ± 0.10***
T-ATPase (U/mg prot)	10.8 ± 0.8	4.9 ± 0.4$^{###}$	6.2 ± 0.5*	7.4 ± 0.6**	8.8 ± 0.7***

*Data are expressed as mean ± SD (n = 3). ### $p < 0.001$ vs. Normal Control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Model group.*

Molecular Docking Interactions of Fenugreek Bioactives with Keap1 Protein

To explore the computational target engagement mechanism underlying Nrf2 antioxidant pathway activation, automated molecular docking was conducted between the Keap1 Kelch domain (PDB ID: 2FLU) and the key bioactive constituents of fenugreek. The canonical binding pocket (Kelch domain) of Keap1 comprises five sub-pockets (P1–P5) rich in arginine and glycine residues (Arg415, Gly462, Gly367, and Val512), which are essential for anchoring Nrf2. As detailed in Table 4, all evaluated fenugreek constituents exhibited robust negative binding energy scores (ranging from -6.8 to -9.6 kcal/mol). Quercetin (-9.6 kcal/mol), Orientin (-9.2 kcal/mol), and Vitexin (-8.8 kcal/mol) formed extensive networks of direct hydrogen bonds with Arg415, Gly462, and Val512, effectively steric-hindering the Keap1-Nrf2 interface and facilitating Nrf2 escape for nuclear translocation.

Table 4. Molecular docking binding parameters of major Fenugreek bioactive constituents complexed with Keap1 protein (PDB ID: 2FLU)

Ligand Compound	Binding Score (kcal/mol)	Hydrogen Bonded Residues	Electrostatic / Hydrophobic Interactions	Binding Pocket Location
Quercetin	-9.6	Gly367,	Val512, Ala366, Val418	(Pi- P1 & P2 Sub-

		Gly462, Arg415, Leu557	Alkyl)	pockets
Orientin	-9.2	Arg415, Gly462, Val512, Ile559, Ser602	Ala366, Val463 (Pi-Sigma)	P1 Canonical Pocket
Vitexin	-8.8	Arg415, Gly367, Val465, Gly605, Val606	Ala510, Val418 (Pi-Alkyl)	P1 & P3 Pockets
Diosgenin	-8.4	Val512, Gly558, Leu365	Ala366, Ile461, Val418 (Alkyl)	Hydrophobic Core
Trigonelline	-7.2	Arg415, Gly462, Val512	Ala366 (Electrostatic)	P1 Central Pocket
Caffeic Acid	-6.8	Arg415, Gly558, Ile559, Gly367	Ala366 (Pi-Alkyl)	P2 Pocket

Molecular Docking Interactions of Fenugreek Bioactives with AMPK Protein

AMP-activated protein kinase (AMPK) serves as the master cellular energy sensor regulating mitochondrial biogenesis and fatty acid oxidation. Molecular docking targeting the catalytic kinase domain of AMPKα (PDB ID: 4CFF) demonstrated that Fenugreek bioactive constituents engage in stable hydrogen bonding and electrostatic interactions with key active site residues (Leu189, Leu206, Arg188, Asp252). As shown in Table 5, Quercetin (-8.2 kcal/mol), Diosgenin (-7.9 kcal/mol), Orientin (-7.6 kcal/mol), and Vitexin (-7.4 kcal/mol) exhibited favorable binding affinities, providing a strong structural basis for allosteric AMPK activation and downstream bioenergetic pathway upregulation.

Table 5. Molecular docking binding parameters of major Fenugreek bioactive constituents complexed with AMPKα catalytic subunit (PDB ID: 4CFF)

Ligand Compound	Binding Score (kcal/mol)	Hydrogen Bonded Residues	Electrostatic / Hydrophobic Forces	Target Domain
Quercetin	-8.2	Leu189, Ile205, Leu206, Arg188	Pro208, Pro253, Asp252	AMPKα Kinase Domain
Diosgenin	-7.9	Leu206, Asp252, Val11	Pro182, Pro208 (Alkyl)	Allosteric Hydrophobic Pocket
Orientin	-7.6	Arg188, Leu189, Asp252, Glu183	Pro208, Pro253 (Pi-Cation)	ATP Binding Cleft
Vitexin	-7.4	Leu206, Ile205, Arg188	Pro208, Pro182 (Pi-Alkyl)	Kinase Active Site
Trigonelline	-6.5	Leu189, Arg188	Asp252 (Electrostatic)	Catalytic Core
4-Hydroxyisoleucine	-5.9	Leu206, Asp252, Glu183	Pro208 (Salt Bridge)	Substrate Binding Channel

Discussion

Physical exercise-induced fatigue is a highly complex, multi-systemic physiological state driven by the confluence of oxidative overload, depletion of intracellular energy stores, and disruption of cellular ion transport pumps. Finding non-toxic, plant-derived functional supplements capable of simultaneously enhancing antioxidant defense and cellular bioenergetics is a primary objective in modern sports science and nutraceutical research. In this study, we systematically investigated *Trigonella foenum-graecum L*. (TFGE) extract (TFGE), combining LC-MS/MS metabolomic profiling, cell-free antioxidant assays, an in vitro C2C12 myoblast oxidative injury model, and computational molecular docking without relying on vertebrate animal models. In agreement with previous phytopharmacological reports

(Nagulapalli Venkata et al., 2017), trigonelline and 4-hydroxyisoleucine were confirmed as signature chemical markers. However, unlike earlier studies that predominantly focused on crude polyphenol fractions, our high-resolution UHPLC-Q Exactive profiling revealed a unique co-occurrence of flavone C-glycosides and steroidal saponins directly modulating glucose uptake and ion pump kinetics.

Our chemical characterization identified 20 major bioactive constituents in TFGE. Among these, the pyridine alkaloid trigonelline and the specialized amino acid 4-hydroxyisoleucine are hallmark markers of fenugreek. Trigonelline has been reported to modulate metabolic homeostasis, suppress neuroinflammation, and enhance mitochondrial function (Kadam & Kulkarni, 2026). 4-Hydroxyisoleucine plays a crucial role in stimulating glucose transport and cellular insulin sensitivity (Sellami et al., 2021; Tian et al., 2022). Additionally, flavone C-glycosides (orientin, vitexin, isovitexin), flavonols (quercetin, luteolin, kaempferol), and steroidal saponins (diosgenin, yamogenin) exhibit potent ROS-scavenging capacities and membrane-stabilizing properties (Maulydia et al., 2023). The synergistic action among these multi-class phytochemicals likely provides the molecular basis for TFGE's comprehensive anti-fatigue effects.

Oxidative stress is a primary driver of exercise fatigue. Strenuous physical exertion dramatically boosts mitochondrial oxygen consumption, accelerating superoxide radical generation and causing lipid peroxidation of skeletal muscle membranes (Machida & Imai, 2021; Riani et al., 2025). Our cell-free antioxidant evaluation demonstrated that TFGE possesses robust radical-scavenging properties. In silico docking indicates that quercetin and orientin occupy key residues in Keap1, which is predicted to favor Nrf2 stabilization, while 4-hydroxyisoleucine binding suggests a potential structural basis for enhancing cellular energy homeostasis. In oxidative-injured C2C12 skeletal muscle myoblasts, TFGE treatment significantly reduced intracellular ROS fluorescence intensity and lowered MDA accumulation, while up-regulating SOD, CAT, and GSH-Px activities. Mechanistically, molecular docking revealed that key TFGE constituents (quercetin, orientin, vitexin, and diosgenin) bind tightly to the Kelch domain of Keap1 (-6.8 to -9.6 kcal/mol), occupying key residues (Arg415, Gly462, Val512) required for Keap1-Nrf2 binding. This structural disruption releases Nrf2 for nuclear translocation, driving downstream expression of antioxidant response element (ARE) phase II enzymes (NQO1, HO-1(Khairani et al., 2023)

In addition to redox regulation, energy substrate preservation, and ion pump homeostasis are crucial for sustained muscular endurance. During prolonged muscular exertion, rapid glycogen depletion induces blood glucose drops and muscle power loss, while intracellular lactate buildup lowers cellular pH (Salehi et al., 2025; Watt & Goel, 2022). In C2C12 myoblasts, TFGE significantly restored glycogen reserves and reduced lactate accumulation. Furthermore, muscle contraction critically depends on membrane-bound ion pump ATPases (Na+-K+-ATPase and Ca2+-Mg2+-ATPase). Oxidative damage inhibits these ATPases, disrupting intracellular Na+/K+ and Ca2+ gradients, impairing membrane excitation, and inducing muscle fatigue. TFGE treatment remarkably reactivated Na+-K+-ATPase, Ca2+-Mg2+-ATPase, and total T-ATPase activities. Docking simulations confirmed that TFGE constituents bind directly to the catalytic kinase domain of AMPKα (-5.9 to -8.2 kcal/mol), suggesting that TFGE activates AMPK signaling to promote PGC-1α/PPAR-α mediated mitochondrial biogenesis, fatty acid β-oxidation, and ATP production (Ciucci et al., 2022; Liu et al., 2024; Sharifi-Rad et al., 2021)

4. Conclusion

This study provides a comprehensive, ethically rigorous, and mechanistically detailed evaluation of Trigonella foenum-graecum L. extract (TFGE) as a natural anti-fatigue functional agent. Through dual-polarity UHPLC-Q Exactive HF-X LC-MS/MS, 20 major bioactive

constituents were identified, including trigonelline, 4-hydroxyisoleucine, orientin, vitexin, diosgenin, and quercetin. In cell-free and C2C12 skeletal muscle myoblast models, TFGE demonstrated potent radical-scavenging capacity, suppressed ROS and MDA production, reactivated endogenous antioxidant enzymes (SOD, CAT, GSH-Px), enhanced intracellular glycogen storage, promoted lactate clearance, and restored membrane-bound ATPase pump activities (Na+-K+-ATPase, Ca2+-Mg2+-ATPase, T-ATPase). Molecular docking simulations confirmed strong target-binding interactions between fenugreek bioactives and key regulatory proteins Keap1 (PDB: 2FLU) and AMPKα (PDB: 4CFF). In conclusion, standardized TFGE demonstrates potent in vitro antioxidant, cytoprotective, and bioenergetic recovery properties in H_2O_2-stressed C2C12 myoblasts. Molecular docking provides preliminary structural evidence... Study Limitations & Future Recommendations: This study is limited to cell-free, in vitro, and computational models without direct in vivo physiological validation. Future research should focus on in vivo animal models and Western blot pathway validation.

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6. Conflict of Interest

The authors declare that they have no financial or personal relationships with other persons or organizations that could improperly influence our work, no professional or other personal interest of any kind in any product, service, and/or company that could be construed as influencing the position presented in, or review of, the manuscript.

7. Author Contributions

All authors confirm that they have read and approved the final version of this manuscript. The contributions of each author to the development of the research should be stated in a single paragraph, using only initials followed by a period. Percentages should be used to indicate each author's contribution (from a total of 100%).

8. Data Availability Statement

The authors state that the data supporting the findings of this study will be made available by the corresponding author [AMH: armansyahhrp@unimed.ac.id] upon reasonable request.

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