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# Thai Kratom extracts ameliorate MAFLD through multi-target mechanism in FFA-induced HepG2 cells

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**ABSTRACT**

Metabolic dysfunction-associated fatty liver disease (MAFLD), characterized by excessive lipid accumulation, is a significant global health concern that is strongly associated with metabolic dysregulation, including insulin resistance and chronic inflammation. Thai kratom (*Mitragyna speciosa*) extracts exhibit diverse biological activities; however, their effects on hepatic lipid and glucose metabolism in MAFLD remain poorly documented. This study investigated the therapeutic effects of ethanol-extracted red and green kratom and its major alkaloid, mitragynine, in an *in vitro* MAFLD model using free fatty acid (FFA)-exposed HepG2 cells. Thai Kratom extracts significantly reduced FFA-induced lipid accumulation by activating the AMP-activated protein kinase pathway and downregulating lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase, key in fatty acid biosynthesis. Thai kratom extract and mitragynine enhanced glycogen production by increasing AKT and GSK3 phosphorylation, reducing precursors for fatty acid synthesis. Additionally, the extract demonstrated anti-inflammatory properties by decreasing p38 MAPK phosphorylation and downregulating key inflammatory mediators (TLR4, c-Jun, CCL2, and CCL21). These results highlight the multifaceted effects of Thai kratom extract and mitragynine on lipid metabolism, insulin signaling, and inflammation, suggesting their potential as therapeutic agents for MAFLD. Future *in vivo* studies are essential to elucidate the mechanisms of action, paving the way for safe and effective kratom-based MAFLD interventions.

## INTRODUCTION

Metabolic-associated fatty liver disease (MAFLD), a redefined term for nonalcoholic fatty liver disease (NAFLD) [1], encompasses a spectrum of liver conditions ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). This condition represents a critical global health challenge, frequently associated with common metabolic disorders such as obesity and metabolic syndrome [2–4]. The pathogenesis of MAFLD is multifactorial, with insulin resistance playing a pivotal role in initiating hepatic steatosis [5,6]. The excess influx of free fatty acids (FFAs) into hepatocytes, combined with heightened *de novo* lipogenesis and impaired lipid export, leads to the hallmark lipid accumulation characteristic of MAFLD [7,8]. Chronic inflammation and cellular stress are key drivers of MAFLD progression from simple steatosis to NASH, leading to further complications [9].

Excess FFA influx under high-energy conditions inhibits AMP-activated protein kinase (AMPK), a central energy sensor that normally suppresses *de novo* lipogenesis [10]. The AMPK-activating properties of quercetin have been extensively documented across various cell lines. Lee *et al.* [11] demonstrated that quercetin activates AMPK in cancer cells, resulting in the suppression of cyclooxygenase-2 and the induction of apoptosis. Similarly, Dhanya *et al.* [12] reported that quercetin activates AMPK in skeletal muscle cells by increasing the AMP/ATP ratio, thereby enhancing glucose uptake through the AMPK-p38 MAPK signaling pathway. Quercetin shares a mechanism similar to that of the well-known drug metformin, underscoring its potential as a promising compound for managing type 2 diabetes [11,12]. Inactive AMPK fails to

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phosphorylate and inhibit acetyl-CoA carboxylase (ACC), allowing ACC to catalyze malonyl-CoA production. Elevated malonyl-CoA activates fatty acid synthase (FAS), driving unchecked lipid synthesis while simultaneously suppressing mitochondrial β-oxidation via carnitine palmitoyltransferase 1 inhibition. Concurrently, the PI3K/AKT pathway exacerbates lipid accumulation by enhancing glucose uptake and glycolytic flux, thereby providing acetyl-CoA substrates for FAS-mediated lipogenesis. This dual dysregulation of AMPK inhibition and PI3K/AKT hyperactivation creates a metabolic imbalance that amplifies hepatic lipid deposition, disrupts glucose homeostasis, and accelerates insulin resistance [13,14]. Targeting these pathways, along with reducing inflammation and cellular stress, has emerged as a promising therapeutic approach for MAFLD and related metabolic disorders.

Traditional medicinal plants offer a rich reservoir of potential therapeutic agents for the management of MAFLD and are associated with lower evidence of side effects than conventional pharmaceuticals. Several plant-derived compounds have demonstrated efficacy in mitigating MAFLD symptoms by modulating lipid metabolism pathways [15]. Thai kratom (*Mitragyna speciosa*), traditionally used for its stimulant and analgesic properties, has recently gained attention for its potential metabolic benefits [16]. Preliminary evidence suggests the anti-lipogenic effect of *M. speciosa* [17–19]; however, the underlying mechanisms remain unexplored.

This study aimed to elucidate the effects of Thai kratom extracts (red and green vein varieties) and their major alkaloid mitragynine and quercetin on lipid metabolism, insulin signaling, and inflammation in an *in vitro* HepG2 cell model of MAFLD. Our findings demonstrate that kratom extract and mitragynine attenuate FFA-induced lipid accumulation, potentially through AMPK activation, and downregulation of lipogenic enzymes. Additionally, kratom extract enhanced glycogen synthesis by increasing AKT and GSK3 phosphorylation, reducing the availability of precursors for *de novo* lipogenesis, and subsequently leading to decreased fat accumulation in HepG2 cells. Furthermore, kratom extract exhibited anti-inflammatory effects by decreasing p38 MAPK phosphorylation and downregulating key inflammatory mediators.

These results provide novel insights into the molecular mechanisms underlying the potential therapeutic effects of Thai kratom on MAFLD and its related metabolic complications. Further research, including *in vivo* studies, is needed to validate the clinical applications of kratom and its active constituents.

## MATERIALS AND METHODS

### Thai Kratom extracts

Red and green kratom leaves were obtained from a community participation project in Nam Pu Sub-district, Ban Na San district, Surat Thani province, Thailand, as documented in a previous study [18]. This study documents the traditional use of these kratom varieties. The ground and dried powder of the leaves was extracted using a previously described method [18] with slight modifications: 100 g of each kratom powder (red and green) was soaked in 1 l of 95% ethanol for 24 hours. This step was repeated twice. After removing ethanol by rotary evaporation, crude ethanolic extracts of red and green kratom were obtained. LC-MS/MS analysis was performed to identify the major compounds in the extracts**.** Analysis of the ground and dried powders of red Thai kratom (RTK) and green Thai kratom (GTK) revealed significant differences in their major compounds. A previous publication from our research group found that GTK exhibited mitragynine levels (63%) that were 2 times higher than those found in RTK (37%), with quercetin content ranging from 10.2 to 17.4 mg/g (EtOH extract) [18].

### HepG2 cell culture

The human hepatocellular carcinoma cell line HepG2 (HB-8065) was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM high-glucose medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% $CO_2$.

### MTS assay

To establish a non-cytotoxic dose range for subsequent experiments, the viability of HepG2 cells following treatment with red or green Thai kratom (*M. speciosa*) extract and pure mitragynine was assessed using the MTS assay (Promega, Madison, WI, USA). HepG2 cells were seeded at a density of $1 \times 10^4$ cells/well in 96-well plates and allowed to adhere overnight. Cells were then treated with varying concentrations of red or green kratom extract (0–200 µg/ml) or mitragynine (0–400 µM) for 24 or 48 hours. The mitragynine dose range was selected based on previous studies reporting $IC_{50}$ values in HepG2 cells of 42–92.85 µM, with non-cytotoxicity in in HL-7702 cells at concentrations >200 µM [20,21]. Kong *et al.* [21]. To capture the full dose-response, mitragynine was tested at 0, 6.25, 12.5, 25, 50, 100, 200, and 400 µM. Since mitragynine constitutes ~40% of Thai kratom extracts [22], the highest kratom extract dose (200 µg/ml) corresponds to an approximate mitragynine-equivalent concentration of 80 µg/ml (~200 µM), which exceeds the mitragynine $IC_{50}$. After 24 and 48 hours of incubation at 37°C and 5% $CO_2$, cell viability was determined using the MTS assay. Twenty microliters of MTS solution were added to each well, followed by incubation for an additional 1 hour at 37°C and 5% $CO_2$. The absorbance of the formazan product was measured at 490 nm using a microplate reader (BioTek, Winooski, VT, USA). Cell viability in the treatment groups was normalized to that of the untreated control group (0 µM), which was designated as 100% viability.

### Assessment of the impact of Kratom extracts on FFA-induced lipid accumulation in HepG2

To investigate the effect of the test compounds on lipid accumulation, HepG2 cells ($4 \times 10^5$ cells/well) were plated onto coverslips 24 hours before co-treatment with 500 µM free fatty acids (FFA, mixture of oleic and palmitic acids, 3:1 ratio) and varying concentrations of green and red kratom crude ethanol extracts (0–200 µg/ml), mitragynine (0–200 µM), and quercetin (50 µM) for 24 hours. This co-treatment aimed to induce a fatty

liver phenotype while simultaneously evaluating the effects of the test compounds on lipid accumulation. Lipid accumulation was assessed using Oil Red O (ORO) staining. HepG2 cells were fixed with 4% formaldehyde for 10 minutes at room temperature and rinsed with 60% isopropanol. The cells were then stained with ORO solution for 1 hour. The coverslips were then rinsed with 60% isopropanol, washed with PBS three times, and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Lipid accumulation was quantified from the captured images using the ImageJ software (version 2.14.0/1.54f, National Institutes of Health, Bethesda, MD, USA).

### Immunoblotting analysis

Western blot analysis was performed as previously described [23], with minor modifications. Briefly, equal amounts of protein from each sample were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked and incubated with primary antibodies against several target proteins shown in Supplementary Table S1. Following primary antibody incubation, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Immunoreactive proteins were detected using an enhanced chemiluminescence substrate (Pierce, IL, USA) and visualized using a chemiluminescent imaging system. Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as an internal loading control for normalization.

### RNA isolation and RT-qPCR

Total RNA was extracted from samples using the TRIzol reagent as previously described [23]. cDNA was synthesized from 1 µg of RNA using an iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). To assess the transcript abundance, real-time PCR with SYBR Green was performed on an ABI Prism 7,500 Sequence Detection System (Applied Biosystems, CA, USA). Target gene expression was normalized to 18S rRNA, and relative expression was quantified using the Pfaffl method [24]. Primer sequences are listed in Supplementary Table S2.

### Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) from three independent replicate experiments ($n$ = 3) for all assays. Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, CA, USA). Differences between treatment groups were compared using a one-way analysis of variance (ANOVA), followed by an appropriate post-hoc test. Student's $t$-test was used to compare the two groups. Statistical significance was defined as a $p$-value < 0.05.

## RESULTS

### Effects of red and green Kratom extracts and mitragynine on HepG2 cell viability

To assess the potential cytotoxicity of the red and green kratom extracts, HepG2 cells were treated with varying concentrations (0–200 µg/ml) of each extract for 24 and 48 hours. Cell viability was determined using the MTS assay. Both extracts exhibited dose-dependent cytotoxicity, significantly reducing HepG2 cell viability at 100 and 200 µg/ml after 48 hours of treatment (Fig. 1A and 1B). To minimize cytotoxic effects in subsequent experiments, lower concentrations and a 24-hour incubation period were used. Mitragynine, the major alkaloid in Thai kratom extracts, was also evaluated for its effect on cell viability. Concentrations below 50 µM did not significantly affect HepG2 cell viability. However, mitragynine exhibited dose-dependent cytotoxicity at higher concentrations after both 24- and 48-hour incubations, with calculated $IC_{50}$ values of 125.5 µM and 115.2 µM, respectively (Fig. 1C). The

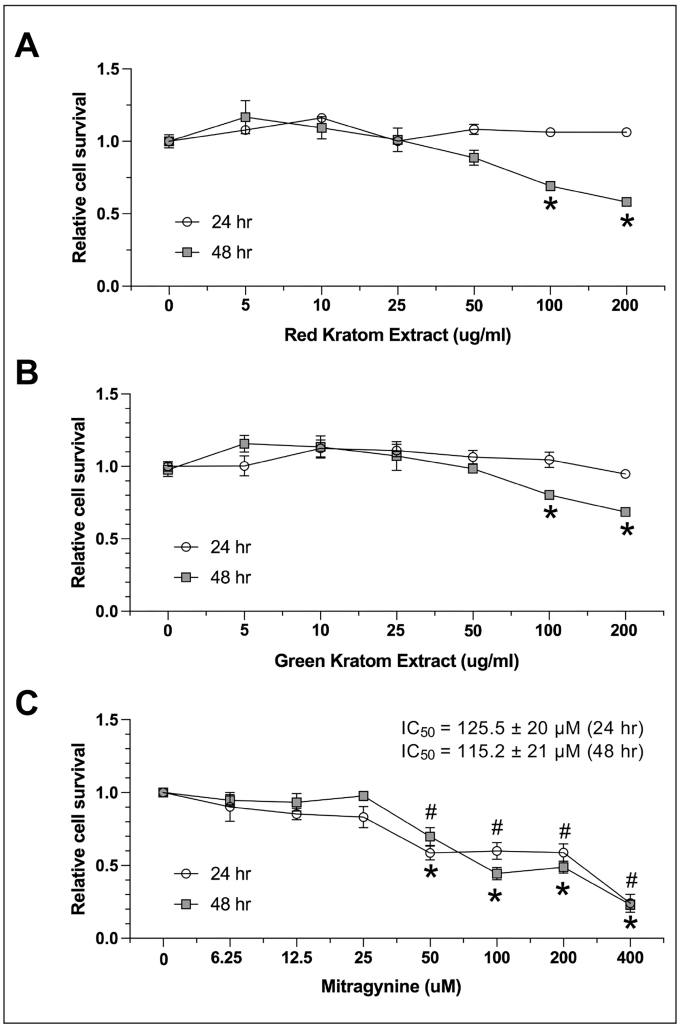


**Figure 1.** Cytotoxicity of red and green kratom extracts and mitragynine on HepG2 cells. HepG2 cells were treated with increasing concentrations (0–200 µg/ml) of red (A) and green (B) kratom extract for 24 and 48 hours. Cell viability was measured using the MTS assay and expressed as a percentage relative to untreated control cells. Additionally, HepG2 cells were treated with increasing concentrations (0–400 µM) of mitragynine for 24 or 48 hours, and cell viability was measured similarly. $IC_{50}$ values were calculated to determine cytotoxic effects. Data are presented as mean ± SEM of three independent experiments ($n$ = 3). Statistical significance was determined using one-way ANOVA followed by Turkey's post-hoc test: $^{\#}p < 0.05$ and $^{*}p < 0.05$ compared to the untreated control at 24 and 48 hours, respectively.

maximum concentration combined with a 24-hour incubation period was determined to mitigate cytotoxicity in subsequent experiments. 0–200 µg/ml of the extract showed no significant toxicity. Given the $IC_{50}$ of mitragynine of 125 µM, 50 µM was deemed safe for subsequent experiments.

**Thai Kratom extracts and constituents attenuate FFA-induced lipid accumulation**

Previous studies have suggested the potential anti-lipidemic effects of Thai kratom consumption [18,19]. To investigate the effects of Thai kratom on hepatic lipid accumulation,

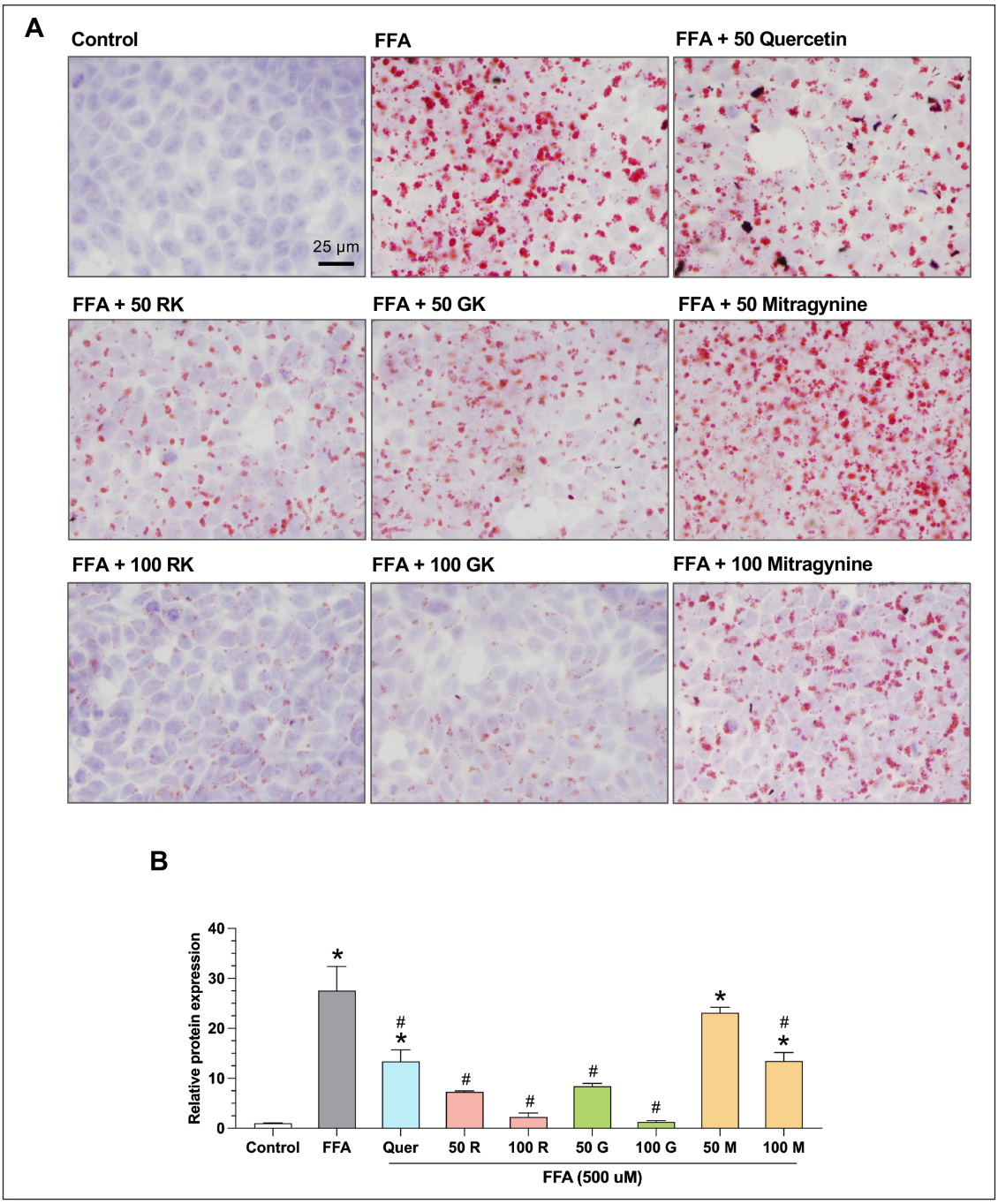


**Figure 2.** Effects of kratom extract and mitragynine on intracellular lipid accumulation in HepG2 cells. (A) Representative Oil Red O-stained images of HepG2 cells treated with palmitic and oleic acid (FFA) to induce lipid accumulation. Cells were then co-treated with quercetin (Q), red kratom extract (RK), green kratom extract (GK), or mitragynine (M) at the indicated concentrations. Lipid droplets were stained using Oil Red O. Scale bar = 25 µM. (B) Quantification of the lipid accumulation. The relative intensity of Oil Red O staining was analyzed using ImageJ software. Data are presented as mean ± SEM of three independent experiments ($n$ = 3). Statistical significance was determined using one-way ANOVA followed by Turkey's post-hoc test: $^{*}p < 0.05$, compared to the control group, $^{\#}p < 0.05$ compared to the FFA-treated group.

an FFA-induced fatty liver HepG2 cell line was used. HepG2 cells were treated with 0, 50, and 100 µg/ml of red and green kratom for 24 hours, and lipid accumulation was determined by ORO staining. Quantification of lipid accumulation revealed that FFA treatment significantly increased intracellular lipid content compared to the control, resulting in a 27.54 ± 4.83-fold increase. However, red and green kratoms at 50 and 100 µg/ml, respectively, significantly and dose-dependently reduced FFA-induced lipid accumulation compared with FFA treatment alone. Additionally, 50 µM quercetin, a well-known compound that reduces hepatic fat accumulation [25], and 100 µM mitragynine significantly decreased intracellular fat accumulation compared to the FFA-treated group (Fig. 2A and 2B). These findings indicate that Thai kratom extracts and their constituents, particularly mitragynine and quercetin, have the potential to mitigate FFA-induced lipid accumulation in HepG2 cells.

### Effects of Thai Kratom extracts and mitragynine on lipid metabolism

As shown in Figure 3A–D, co-treatment with FFA and red or green kratom extracts led to a dose-dependent reduction in the protein expression of both ACC and FAS. In contrast, mitragynine (50–200 µM) and quercetin (50 µM) did not significantly affect ACC and FAS protein levels. Hepatic *de novo* lipogenesis is often dysregulated in MAFLD [26] and is known to be suppressed by AMPK, a key regulator of lipid metabolism. AMPK achieves this by inhibiting key enzymes the ACC and FAS [27,28]. We observed that FFA treatment significantly decreased AMPK phosphorylation (0.79 ± 0.07-fold), while both red and green Thai kratom extracts increased the p-AMPK/AMPK ratio. Red kratom extract at 100 and 200 µg/ml increased the ratio by 1.18 ± 0.14-fold and 1.35 ± 0.19-fold, respectively, while green kratom extract induced a 1.44 ± 0.08-fold and 1.71 ± 0.28-fold increase (Fig. 3D). AMPK activation was associated with a 50% reduction in ACC and FAS protein levels, indicating dose-dependent suppression of lipogenic enzymes (Fig. 3B and C). Notably, treatment with the pure compounds mitragynine at lower doses (50 and 100 µM) and quercetin did not significantly alter the p-AMPK/AMPK ratio compared to that in the FFA-treated group. However, a higher dose of mitragynine (200 µM) significantly decreased the p-AMPK/AMPK ratio compared to that in the FFA-treated group. These findings suggest that red and green Thai kratom extracts may have a stronger potential to attenuate *de novo* lipogenesis and mitigate lipid accumulation in HepG2 cells than their constituents mitragynine and quercetin. This effect may be mediated, at least in part, by AMPK activation.

### Effects of Thai Kratom extracts on glucose homeostasis and insulin signaling

Our study investigated the effects of Red and Green Thai Kratom extracts, their primary alkaloid mitragynine, and

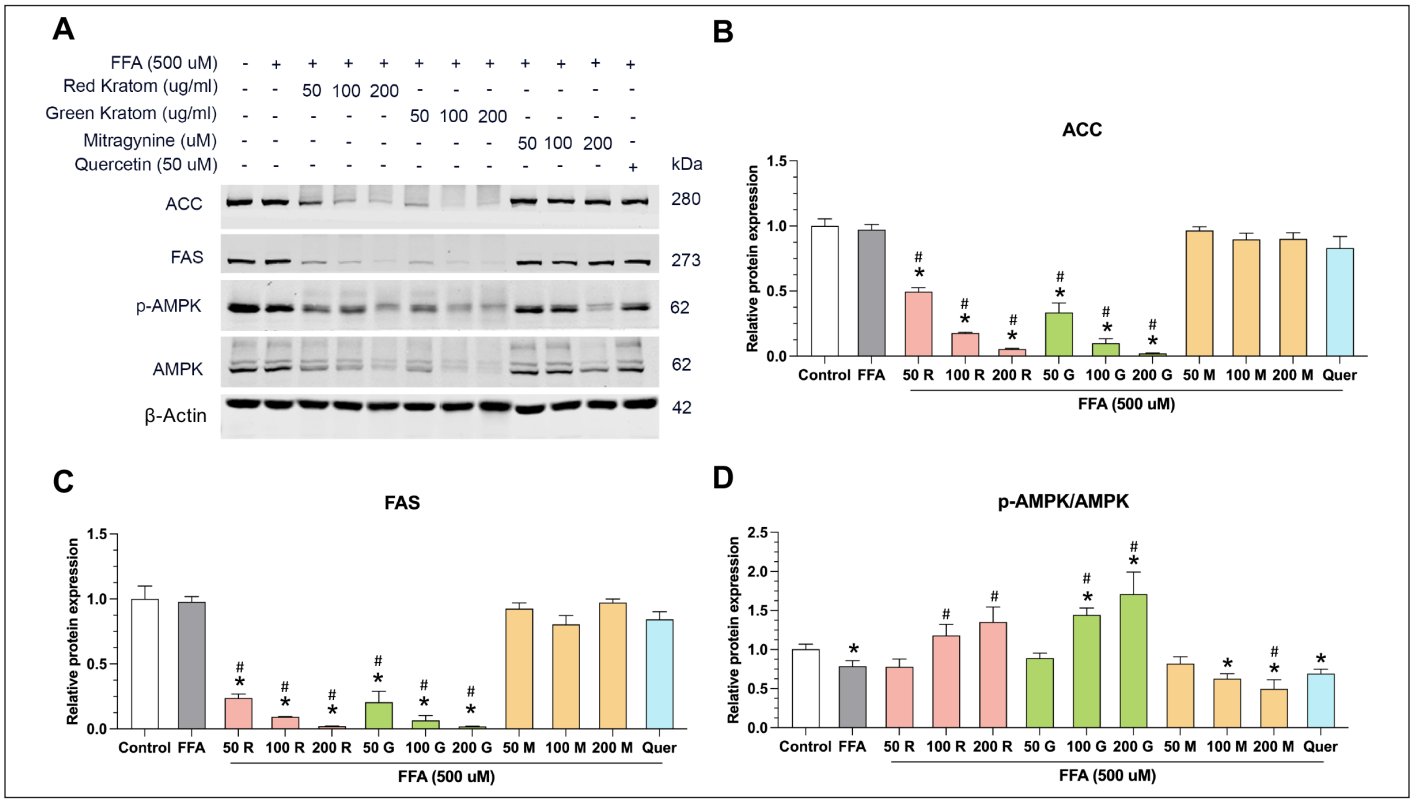


**Figure 3.** Effects of kratom extract and mitragynine on lipid metabolism in HepG2 cells. (A) Representative Western blots showing the expression of ACC, FAS, p-AMPK, AMPK, and β-actin (loading control) in HepG2 cells treated with FFA alone or in combination with red kratom extract (R), green kratom extract (G), mitragynine (M), or quercetin (Quer) at the indicated concentrations for 24 hours. Quantification of ACC (B) and FAS (C) protein expression levels relative to β-actin. (D) Quantification of the p-AMPK/AMPK ratio. Data are presented as mean SEM of three independent experiments ($n = 3$). Statistical significance was determined using one-way ANOVA followed by Turkey's post-hoc test: $^{*}p < 0.05$ compared to control, $^{\#}p < 0.05$ compared to the FFA-treated group.

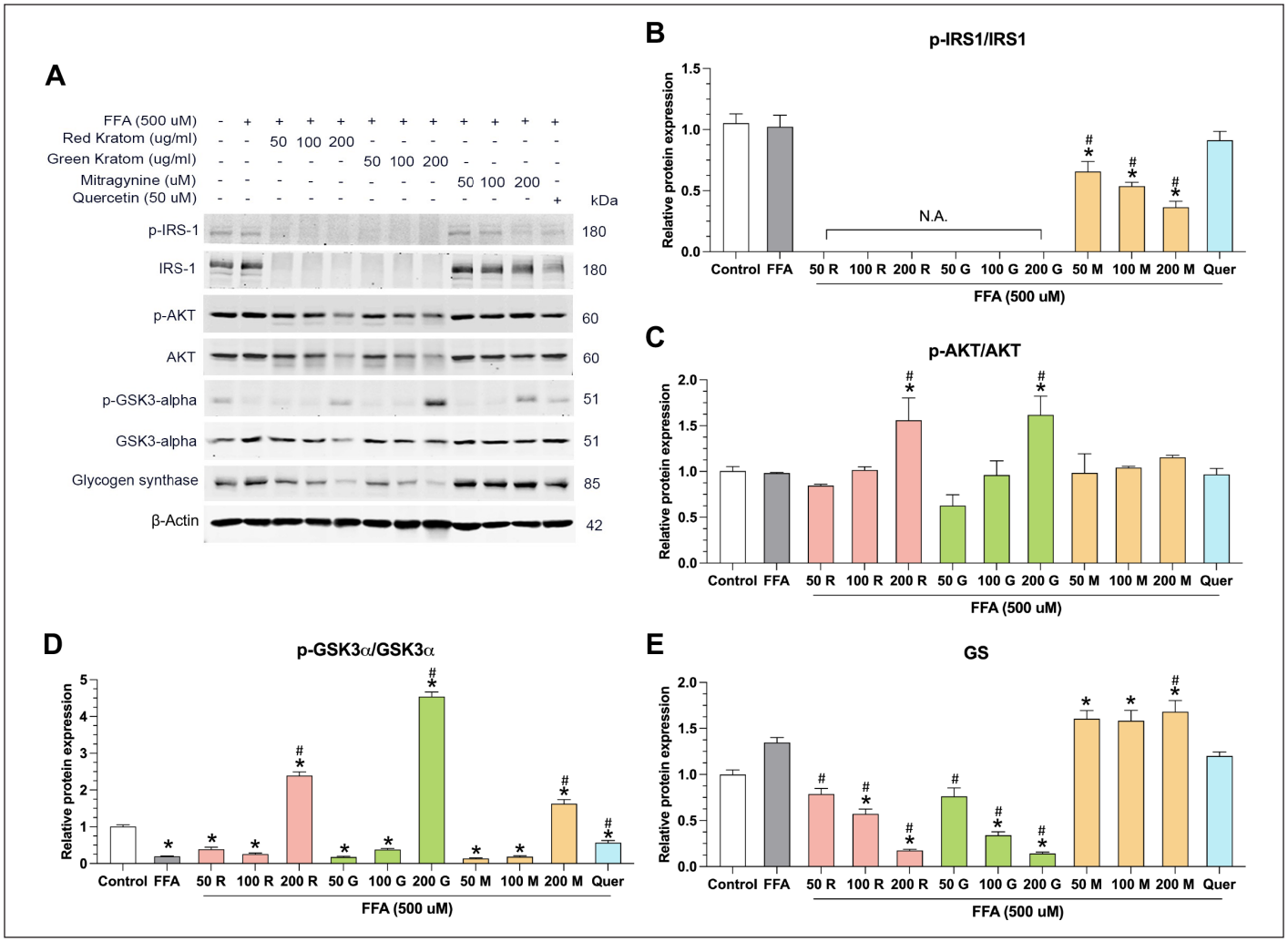


**Figure 4.** Effects of thai kratom extracts, mitragynine, and quercetin on insulin signaling and glycogen synthesis in HepG2 cells. (A) representative western blots showing the protein expression of phosphorylated (p-) and total forms of (B) IRS-1, (C) AKT, (D) GSK3a, and (E) Glycogen synthase (GS). HepG2 cells were treated with FFA alone or in combination with Red Thai Kratom (RTK), Green Thai Kratom (GTK), mitragynine, or quercetin at the indicated concentration. β-actin serves as a loading control. "N.A." indicates that the signal was not detectable. Data are presented as mean ± SEM of three independent experiments ($n$ = 3). Statistical significance was determined using one-way ANOVA followed by Turkey's post-hoc test: *$p < 0.05$ compared to the control group and $^{\#}p < 0.05$ compared to the FFA-treated group.

quercetin (known to enhance insulin signaling and glucose uptake [29]) on insulin signaling and glucose metabolism in HepG2 cells, as shown in Figure 4A–E. We observed a dose-dependent decrease in the p-IRS-1/IRS-1 ratio following mitragynine treatment and a complete disappearance of IRS-1 bands upon kratom extract treatment. This suggests a potential inhibitory effect on IRS-1, possibly through increased degradation or decreased expression [30]. Interestingly, despite this potential inhibition at the IRS-1 level, high doses of kratom extract enhanced downstream insulin signaling by increasing AKT phosphorylation. This suggests that the activation of AKT through alternative pathways is independent of IRS-1. Furthermore, both kratom extracts and mitragynine counteracted the negative effects of FFA treatment on glycogen synthesis, as evidenced by the increased p-GSK3a/GSK3a ratios. However, kratom extract decreased glycogen synthase (GS) levels, whereas mitragynine increased GS levels, suggesting that only mitragynine enhanced GS activity and increased glycogen storage in the liver. This discrepancy suggests that other components within kratom extract may influence GS expression, potentially through complex interactions with signaling pathways or regulatory mechanisms. Notably, quercetin exerted its effects without altering p-AKT/AKT levels, highlighting its distinct mechanisms of action compared to kratom extracts.

### Effects of Kratom extracts and mitragynine on cellular stress and inflammation

Exposure of HepG2 cells to FFA led to a marked elevation in the phosphorylation of p38 MAPK, a pivotal signaling molecule implicated in cellular stress and inflammatory responses. [31]. Interestingly, treatment with red and green kratom extracts mitigated this effect, decreasing p38 MAPK phosphorylation and suggesting potential anti-inflammatory

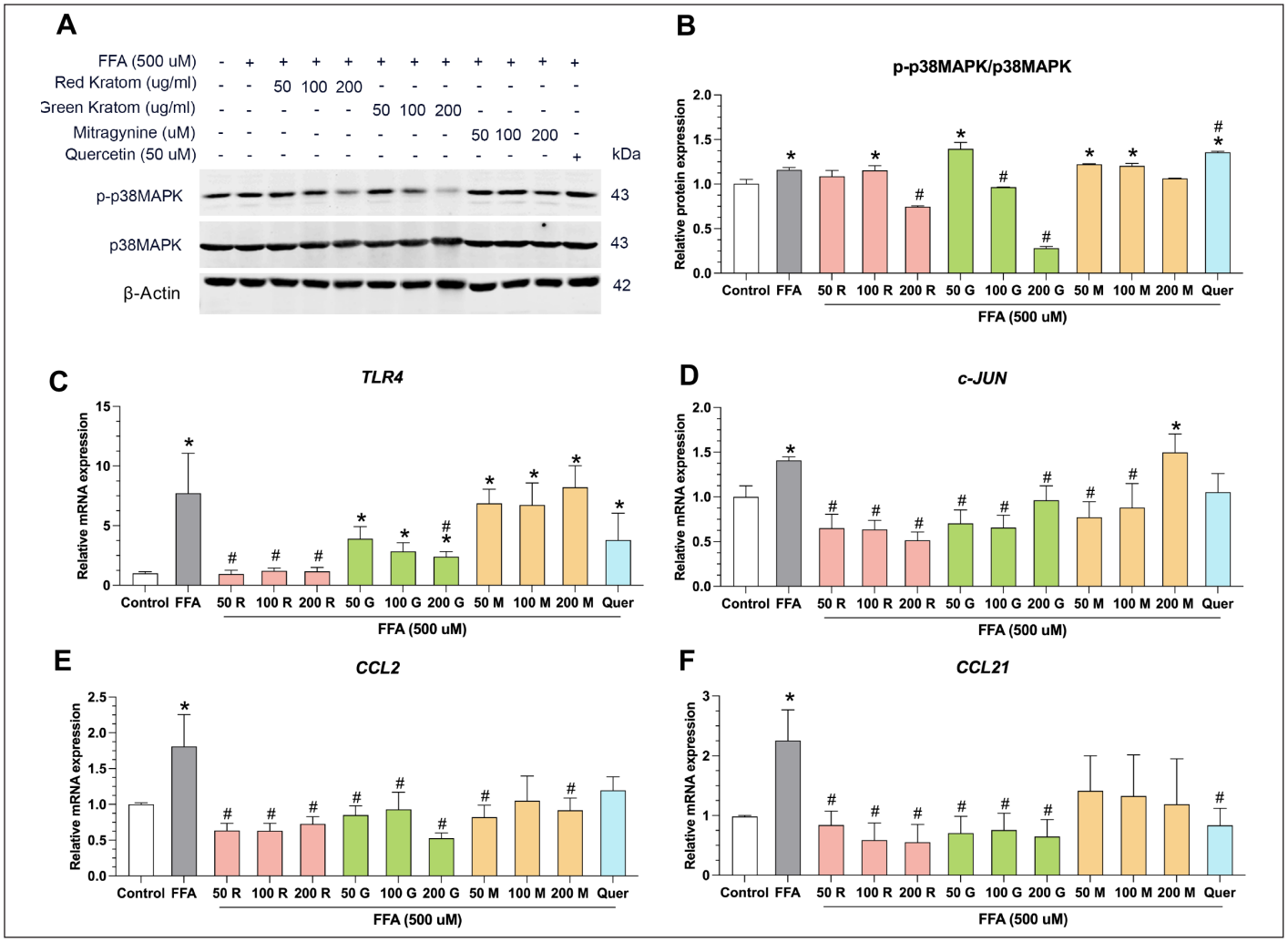


**Figure 5.** Effects of Thai kratom extracts and mitragynine on stress and inflammation in HepG2 Cells. (A) representative western blots showing the levels of phosphorylated (p-) and total p38 MAPK in HepG2 cells treated with FFA alone or in combination with Red Thai Kratom (RTK), Green Thai Kratom (GTK), mitragynine, or quercetin at the indicated concentrations. β-actin serves as a loading control. Quantification of stress and inflammatory markers (B) p-p38 MAPK/p38 MAPK ratio. Relative mRNA expression levels of (C) *TLR4*, (D) *c-JUN*, (E) *CCL2*, and (F) *CCL21*. Data are presented as mean ± SEM of three independent experiments ($n = 3$). Statistical significance was determined using one-way ANOVA followed by Turkey's post-hoc test: $^{*}p < 0.05$, compared with the control group, and $^{\#}p < 0.05$, compared with the FFA-treated group.

properties (Fig. 5A and 5B). In contrast, mitragynine did not significantly alter p-p38 MAPK levels, whereas quercetin, which has been previously reported to remarkably increase p38 MAPK phosphorylation [32], further augmented p38 MAPK phosphorylation compared to FFA-treated cells.

Hepatocellular function is intricately regulated by a network of signaling pathways, including TLR4, c-Jun, CCL2, and CCL21 [33]. TLR4 activation triggers signaling cascades that culminate in the induction of c-Jun, a transcription factor with diverse roles in cellular processes and inflammation [34]. The interplay between these pathways drives the expression of inflammatory chemokines, such as CCL2 and CCL21 [35]. Our study demonstrated that Thai kratom extract and mitragynine can modulate these pathways in HepG2 cells. Both Thai kratom extracts significantly downregulated the mRNA expression of *TLR4*, *c-Jun*, *CCL2*, and *CCL21*, indicating their potential anti-inflammatory effects (Fig. 5C–F). In contrast, mitragynine did not significantly alter *TLR4* and *CCL21* mRNA expression compared to that in FFA-treated cells.

Collectively, these findings suggest that Thai kratom and its constituents, particularly its extracts, may possess protective properties against inflammation and glycogen accumulation in hepatic cells. Further research is warranted to elucidate the precise molecular mechanisms underlying these observations and to explore the potential therapeutic implications of kratom in inflammatory liver diseases.

## DISCUSSION

Our study provides evidence supporting the therapeutic benefits of Thai kratom extract and its major alkaloid mitragynine in the context of MAFLD prevention. Treatment with both red and green kratom extracts significantly reduced FFA-induced lipid accumulation in HepG2 cells, indicating their ability to mitigate hepatic steatosis, a hallmark of MAFLD. This

observation aligns with a previous study highlighting the anti-lipidemic effects of *M. speciosa* [19]. Notably, the direct comparison revealed that kratom extracts exerted a greater reduction in lipid accumulation than mitragynine alone, suggesting that additional bioactive compounds in the extracts may act synergistically to enhance this effect. These results suggest that Thai kratom, particularly its extracts, could serve as a promising natural therapeutic strategy for managing hepatic lipid accumulation, a key pathological feature of MAFLD.

The lipid-lowering effects of kratom extracts appear to be mediated, at least in part, by AMPK activation, a key regulator of cellular energy homeostasis and lipid metabolism [36–38]. AMPK activation suppresses *de novo* lipogenesis by inhibiting key enzymes such as ACC and FAS [39–41]. Our findings demonstrated that palmitic/oleic acid FFA decreased the relative protein levels of p-AMPK/AMPK, which is consistent with *in vivo* models [42]. However, kratom extracts significantly enhanced AMPK phosphorylation while concurrently downregulating ACC and FAS protein expression, suggesting a potential mechanism underlying its lipid-lowering effects (Fig. 3). Notably, although mitragynine also contributed to AMPK activation, the increase in the p-AMPK/AMPK ratio was more pronounced in kratom extract-treated cells compared to those treated with mitragynine. This finding reinforces the hypothesis that the diverse bioactive compounds in kratom extracts may exert stronger metabolic effects through synergistic interaction. In particular, non-mitragynine components such as quercetin, flavonoids, and other alkaloids may further enhance AMPK signaling, contributing to the superior efficacy of the extract in modulating lipid metabolism [43]. In the previous reported, quercetin also activates AMPK, a critical regulator of glucose uptake and metabolism. This mechanism is directly related to our investigation of the effects of kratom on the AMPK-mediated pathways. The ability of quercetin to enhance glucose uptake and insulin sensitivity in hepatic cells makes it an appropriate comparative standard for evaluating the metabolic effects of kratom [11,12,44–46]. Our findings are consistent with those of several studies demonstrating the potential of plant alkaloids to improve dyslipidemia. For example, *i*) berberine, an isoquinoline alkaloid found in various medicinal plants, has been shown to reduce cholesterol and triglyceride levels by regulating lipid metabolism pathways [47], and *ii*) nuciferine, derived from medicinal plants, reduces fat mass *in vivo* and improves dyslipidemia [48]. Both berberine and nuciferine also reduce *de novo* lipogenesis by suppressing ACC and FAS expression and have been demonstrated to enhance their overall therapeutic effects by improving MAFLD [49]. However, kratom differs mechanistically from berberine and nuciferine in its ability to modulate both the AMPK and insulin signaling pathways simultaneously, as demonstrated by its dual effect on AKT activation and glycogen synthesis. This distinction suggests that the therapeutic potential of kratom extends beyond AMPK activation alone and may influence broader metabolic pathways. Interestingly, mitragynine alone did not exhibit the same degree of AMPK activation as the whole extract, reinforcing the hypothesis that its efficacy may arise from its complex phytochemical composition, including other alkaloids and flavonoids, such as quercetin [18]. Further research should focus on isolating individual compounds to determine their contribution to the metabolic effects of kratom and their potential synergistic interactions.

An unexpected observation in our study was the lack of ACC and FAS induction by FFA treatment, despite a decrease in the p-AMPK/AMPK ratio. This could be due to compensatory mechanisms triggered by FFA-induced lipid accumulation, counteracting the decrease in AMPK activity, or the involvement of other signaling pathways, such as the mTORC1/SREBP-1c pathway, in regulating ACC and FAS expression [50]. Further investigation is needed to unravel the complex interplay between FFA-induced signaling and lipogenic enzyme regulation in HepG2 cells.

In addition to its impact on lipid metabolism, we observed complex modulation of insulin signaling by kratom extract and mitragynine. Previous studies have shown that FFA treatment increases IRS1 phosphorylation [51]. However, in our study, both kratom extract and mitragynine exhibited antagonistic effects on phosphorylation in the FFA-treated group. Although these treatments led to a decrease in IRS-1 protein levels, potentially inhibiting early insulin signaling, they also increased AKT phosphorylation, which is a key downstream effector of insulin signaling. AKT activation was observed primarily with kratom extract, with a non-significant trend towards increased p-AKT/AKT, which was also observed with mitragynine, but not with quercetin. This observation suggests that kratom extracts may activate AKT and improve insulin sensitivity in HepG2 cells through alternative pathways that bypass IRS-1 [52–54]. Recent evidence indicates that AMPK can activate AKT independently of IRS-1 by directly phosphorylating mTORC2, which in turn phosphorylates AKT at Ser473 [55]. The AMPK-mTORC2-AKT axis may explain our observations. The study by Lee *et al.* [11] illustrated that quercetin activates AMPK and presents compelling evidence that Compound C, by specifically inhibiting AMPK activity, effectively counteracts this quercetin-induced activation [11]. Additionally, Gao *et al.* [46] showed that inhibition of AMPK by Compound C prevented mTORC2-mediated AKT activation in HepG2 cells, further supporting the dependency of this pathway on AMPK activity. These studies highlight the complex interplay between AMPK, mTORC2, and AKT in the regulation of cellular responses to energetic stress [46]. Future studies incorporating selective AMPK inhibitors, such as Compound C, will be essential to validate the proposed AMPK-mTORC2-AKT mechanism in the context of kratom extract treatment. Such experiments would provide direct evidence for the involvement of AMPK in mediating AKT activation via mTORC2 and clarify its role in modulating insulin-signaling pathways. Additionally, other potential mechanisms may contribute to this effect, including *i*) the activation of other receptor tyrosine kinases that can also signal through PI3K to AKT, such as the insulin-like growth factor 1 receptor or epidermal growth factor receptor [56], and *ii*) crosstalk with G protein-coupled receptors that can phosphorylate and activate AKT independent of IRS-1 [57].

The disappearance of p-IRS-1 and IRS1 protein expression upon kratom extract treatment is also noteworthy. This can be attributed to several mechanisms, including increased protein degradation or decreased protein synthesis.

Previous studies have shown that certain natural compounds can promote IRS-1 degradation via the ubiquitin–proteasome pathway [58]. Alternatively, kratom may interfere with IRS-1 gene expression or mRNA stability, leading to reduced protein levels. Further research is necessary to elucidate the precise mechanisms underlying this phenomenon.

An increase in the p-AKT/AKT ratio activates the AKT pathway, including downstream targets such as GSK3α and glycogen synthase, which promote glycogen synthesis [59]. This is consistent with the established role of the AKT pathway in enhancing glucose uptake and glycogen synthesis [60]. Activation of AKT also leads to several downstream effects that could contribute to reduced lipid accumulation, such as *i*) stimulation of glucose uptake by cells, thereby decreasing glucose availability for conversion into fatty acids [61], and *ii*) promoting glycogen storage as a non-lipid form of glucose [62]. These findings are consistent with those of previous studies on GSK3α-knockout mice, which exhibited increased hepatic glycogen deposition [63]. Additionally, *in vitro* studies using GSK3 inhibitors have demonstrated reduced glucose production in hepatic cells by lowering the expression of gluconeogenesis-related genes (PEPCK and G6Pase) independent of glycogen synthesis. This suggests that the mechanisms by which kratom extract and mitragynine activate the AKT pathway may involve the inhibition of GSK3 activity [64].

Chronic inflammation is a hallmark of MAFLD progression [65]. Our study underscores the anti-inflammatory potential of kratom extract. Treatment with these extracts led to a decrease in p38 MAPK phosphorylation, a key mediator of cellular stress and inflammation [31]. Moreover, both kratom extract and quercetin downregulated the expression of the inflammatory mediators *TLR4, c-JUN, CCL2*, and *CCL21*, suggesting their ability to suppress inflammatory responses in hepatic cells. These findings align with those of previous reports on the anti-inflammatory properties of kratom and its constituents [66]. The ability to mitigate inflammation is crucial for preventing the progression of MAFLD to NASH, highlighting another potential therapeutic avenue for kratom extracts. This observation is consistent with the established anti-inflammatory properties of quercetin, a component of Thai kratom [67,68].

The findings of this study may provide a mechanistic basis for the observed effects of kratom extract and mitragynine on HepG2 cells. This first report suggests that both kratom and mitragynine can reduce fat accumulation in the HepG2 cell line by *i*) converting glucose to glycogen via the AKT-GSK3 signaling pathway, thereby suppressing *de novo* fatty acid synthesis from glucose precursors [69]; *ii*) decreasing *de novo* fatty acid synthesis by decreasing ACC and FAS through AMPK activation, which correlates with several herbal compounds [70,71]; and *iii*) anti-inflammatory activity to prevent NASH progression, consistent with several alkaloid compounds [72,73]. Notably, compared to quercetin, a well-established potential anti-obesity agent, the effects of kratom extract and mitragynine on the AKT pathway appear to be more pronounced [74]. This observation underscores the possibility that kratom extracts may have stronger therapeutic potential by more effectively modulating this signaling pathway. In addition to the MAFLD model, we also observed a 1% ethanol-induced reduction in fat accumulation in cells treated with red and green kratoms (50 and 100 µg/ml) to reduce fat induction (Supplementary Fig. S1). Although this study offers valuable insights derived from cell culture, further *in vivo* investigations using animal models are necessary to validate these anti-obesity effects and thoroughly assess the therapeutic potential of Thai kratom against MAFLD. Furthermore, the potential therapeutic application of Kratom in MAFLD necessitates addressing safety concerns, particularly given its classification by the US FDA as potentially harmful. The FDA has issued warnings about the safety of kratom, highlighting concerns about its association with multiorgan toxicity, including cardiovascular effects. These effects are primarily linked to mitragynine and 7-hydroxy mitragynine, which exhibit opioid-like properties [75]. Although our study focused on the metabolic benefits of kratom, its safety profile, particularly its cardiovascular effects, remains a critical consideration. A comprehensive review by Leong Bin Abdullah and Singh [75] documented that the most common adverse cardiovascular effects of kratom include tachycardia and hypertension, with possible dose-dependent effects on cardiac rhythm. *In vitro* studies have indicated that mitragynine can prolong QTc intervals, whereas case reports have described ventricular arrhythmias and cardiopulmonary arrest, although often in cases involving polysubstance use. At physiologically relevant doses, kratom extracts may have different safety profiles than those from isolated high-concentration alkaloids. These findings suggest that standardizing kratom dosage and optimizing extraction methods to maintain beneficial components while minimizing potential cardiotoxic effects may be crucial for therapeutic development. To mitigate these risks, future strategies should isolate or modify specific bioactive compounds, such as mitragynine, to reduce toxicity while retaining therapeutic efficacy. These modifications can involve structural optimization or combination with synergistic compounds to enhance safety profiles. Rigorous preclinical and clinical testing is essential to validate the safety and efficacy of kratom-derived substances. Comprehensive safety assessments, including dose-response studies and long-term evaluations, are critical for establishing Kratom's viability as a therapeutic option for metabolic disorders.

This study had some limitations. The use of HepG2 cells, an immortalized hepatocellular carcinoma cell line, may not fully replicate the metabolic and signaling dynamics of primary hepatocytes. HepG2 cells exhibit altered lipid metabolism, inflammatory responses, and insulin signaling, which may limit the translational relevance of our findings. Additionally, although *in vitro* FFA treatment simulates aspects of fatty liver disease, it does not replicate the complexity of *in vivo* conditions. Finally, the complexity of kratom extracts makes it difficult to pinpoint the specific molecules responsible for the observed effects, warranting further research to isolate and characterize the active compounds.

## CONCLUSION

This study presents compelling evidence that Thai kratom extracts, particularly mitragynine, may offer a promising natural approach to managing MAFLD by modulating lipid

metabolism, enhancing insulin sensitivity, and reducing inflammation. While further *in vivo* research is necessary to validate these findings and assess the safety and efficacy of kratom-derived therapies, this study provides the groundwork for the future exploration of kratom as a potential treatment for metabolic disorders.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## CONFLICTS OF INTEREST

The author reports no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## DATA AVAILABILITY

All data generated and analyzed are included in this research article.

## PUBLISHER'S NOTE

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## USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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## SUPPLEMENTARY MATERIAL

The supplementary material can be accessed at the link here: [https://japsonline.com/admin/php/uploadss/4590_pdf.pdf]