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RESEARCH ARTICLE

Ginger Extract Exhibits Systemic Antifibrotic Potential through miR-21-5p and miR-29b Modulation in BALB/c Mice

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Abstract Ginger is rich in various phytochemicals known for their potential antifibrotic properties, largely attributed to their anti-inflammatory effects. Although the anti-inflammatory benefits of ginger are well established, the underlying molecular mechanisms, particularly those involving microRNAs (miRNAs) at the post-transcriptional level, remain inadequately understood. This study investigated the antifibrotic effects of ginger extract in mice by examining its influence on the modulation of miR-21-5p and miR-29b, along with their respective target genes. The BALB/c mice were allocated into two separate groups at random, with each group consisting of six mice: one serving as the control group and the other as the treatment group. Both groups received standard chow and water; the treatment group was additionally given ginger extract via oral gavage, while the control group received water. Throughout the treatment period, two mice from each group succumbed. The remaining animals were subsequently utilized for downstream analyses. After three weeks, blood samples were collected for miRNA expression analysis. TargetScan was utilized to identify the target genes of both miRNAs, and their expression levels were confirmed through real time quantitative PCR (RT-qPCR). *Smad7* and *Col1a1* were confirmed as target genes of miR-21-5p and miR-29b, respectively. *Smad7* is recognized for its antifibrotic role, while *Col1a1* is a key marker associated with extracellular matrix formation. RT-qPCR results showed that ginger treatment led to the upregulation of miR-21-5p accompanied by increased *Smad7* expression, while miR-29b levels were also elevated, suggesting activation of antifibrotic regulatory pathways. Although changes in *Col1a1* expression were less pronounced, the observed modulation of key antifibrotic miRNAs highlights ginger's potential as a natural modulator of fibrosis-related pathways and supports further investigation of its therapeutic benefits in fibrotic disorders.

Keywords: Ginger extracts, miR-21-5p, miR-29b, *Smad7*, *Col1a1*.

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Introduction

MicroRNAs (miRNAs) are non-coding RNA species that play essential roles in regulating gene expression. Typically measuring between 20 and 25 nucleotides in length, these highly conserved sequences are found across a wide range of species, including eukaryotes, and some viruses. Since their discovery in the early 1990s, miRNAs have become key elements in cellular regulatory networks, primarily functioning at the post-transcriptional level to control gene activity [1]. They typically exert their effects by binding to the 3' untranslated regions of mRNAs, which results in either the inhibition of translation or cleavage of the mRNA, thereby preventing protein synthesis [2].

miRNAs are crucial regulators of physiological and developmental processes such as growth, apoptosis, and immune function. They are initially transcribed by RNA polymerase II to primary miRNAs (pri-miRNAs), processed into precursor miRNAs in the nucleus, and further matured in the cytoplasm. Mature miRNAs associate with the Argonaute and are capable of silencing the mRNA [2, 3]. Notable examples

include miR-21-5p, encoded by *MIR21* on chromosome 17q23.2, which is evolutionarily conserved and implicated in inflammation and fibrosis [4]. Another important group is the miR-29 family, comprising miR-29a, miR-29b-1, miR-29b-2, and miR-29c. miR-29 was transcribed from loci on chromosomes 7q32.3 and 1q32.2. The miR-29 family plays key roles in apoptosis, cell proliferation, immune response, and fibrosis regulation [5]. Conservation of the seed sequence (nucleotides 2 to 7) within these miRNAs is crucial for their specific interaction with target messenger RNAs and is maintained across humans, mice, and rats [6].

Recent findings suggest that nutritional intake can influence miRNA expression, adding a new dimension to the relationship between diet and gene regulation [7]. Variations in diet, whether vegetarian, vegan, or omnivorous, can affect the expression of specific miRNAs in biological samples such as blood and stool. For instance, miRNAs such as miR-1277, miR-144-3p, and miR-652-3p have been shown to respond to dietary factors. Dairy consumption, along with nutrients like casein, and calcium has also been observed to modulate miRNA profiles [8].

Ginger *(Zingiber officinale),* widely used both as a culinary ingredient and in traditional medicine, is well known for its bioactive compounds and associated health benefits. It contains various phytochemicals such as gingerol, shogaol, zingerone, gingerdiol, and flavonoids, which contribute to its therapeutic properties [9]. The therapeutic effects of ginger, particularly its anti-inflammatory and antifibrotic potential, are increasingly being recognized [10]. However, the specific molecular pathways through which ginger exerts these benefits, especially involving miRNAs linked to fibrosis, remain to be fully clarified. miR-21-5p has been recognized as a pro-fibrotic factor, whereas miR-29b plays a protective, antifibrotic role by regulating gene associated with extracellular matrix formation [11]. The extent to which ginger influences the expression and activity of these miRNAs and their downstream targets remains poorly defined. This study seeks to determine how ginger extract affects the levels of miR-21-5p and miR-29b. It also aims to evaluate the potential implications of these effects for fibrotic regulation.

Materials and Methods

Preparation of Ginger Extract

Ginger extract in tablet form (Herlia Naturals Ginger Vita) was purchased from a local market. The tablet was made from ginger originating in Bentong, Malaysia (https://shorturl.at/vWkk0). According to the product label, each tablet weighs approximately 500 mg and contains 125 mg of gingerol. The tablets were first crushed and finely ground using a mortar and pestle. For administration via oral gavage, the resulting powder was then dissolved in distilled water to achieve the required concentration.

Animal Study and Experimental Design

Animal experiment was performed following the ethical standards approved by the Universiti Teknologi Malaysia Research Ethics Committee (UTMREC) (UTMREC-2024-52). Twelve four-week-old female BALB/c mice were procured from Sapphire A Company, Kuala Lumpur. The mice were housed in stainless steel wire mesh cages under controlled environment (12-hour light/dark cycle; room temperature). After five days acclimatization period, the mice were randomly assigned to two groups of six: a control group and a ginger-treated group [12]. Both groups were provided with unlimited access to a standard diet and water. The treatment group received 500 mg/kg body weight of ginger extract administered via oral gavage, while the control group was given distilled water by the same method. The treatment period took place for three weeks. The selected ginger extract dose was based on prior studies demonstrating its efficacy in modulating gene expression without inducing toxicity [13]. The weights of the mice in both groups throughout the treatment period were recorded at three-day intervals.

Sample Collection

Following the three-week treatment period, all mice were anesthetized using an intraperitoneal injection of ketamine/xylazine (0.1 mL per 100 g body weight). Blood was obtained by performing a cardiac puncture and then transferred into tubes lined with EDTA and stored at −20°C [14]. Circulating miRNAs in the blood are increasingly recognized as non-invasive biomarkers that reflect systemic fibrotic activity and regulatory mechanisms [15].

Total RNA Extraction

Total RNA extraction from the blood samples were performed using TRIzol™ LS Reagent (Invitrogen). For each blood sample, 250 µL was combined with 750 µL of TRIzol™ LS Reagent (Invitrogen) and incubated at room temperature for 5 minutes. Then, 200 µL of chloroform was added per 750 µL of TRIzol™ LS, followed by a 3-minute incubation. After that, the sample is centrifuged at 12,000 × g for 15

minutes at 4°C, and the upper aqueous phase was carefully transferred to a new microcentrifuge tube. Next, RNA was precipitated by adding 500 µL of isopropanol and incubating for 10 minutes, then centrifuged again at 12,000 × g for 10 minutes at 4°C. Finally, the resulting RNA pellet was washed, air-dried, and kept at −80°C refrigerator until further use.

cDNA Synthesis

RevertAid Reverse Transcriptase (Thermofisher) was used to synthesize the cDNA. The reaction was initiated using 300 µg of RNA. The detailed procedure has been described in our previous work [16].

Target Gene Prediction

TargetScan version 8.0 was used to predict the target genes of both miRNAs (https://www.targetscan.org/vert_80/) [17]. The software identifies potential miRNA targets by aligning 6-mer, 7-mer, and 8-mer seed sequences with complementary regions in the 3′ untranslated region (UTR) of mRNAs. Predicted targets are ranked based on context++ scores, reflecting site type, position, and evolutionary conservation [18].

RT-qPCR of miRNAs Toward Target Genes

Primers for miR-21-5p, miR-29b, and their predicted target genes were designed based on previous studies (Table 1) [19, 20]. U6 and GAPDH were used as internal reference genes for miRNA and target genes, respectively [21]. Real-time PCR was carried out using Maxima SYBR Green Master Mix on a Bio-Rad CFX96 with cycling conditions of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 60 seconds (annealing and extension). Relative expression of each gene was determined using the Livak method (2^−ΔΔCt method) [22].

Table 1 List of primers used for gene analysis in this study

Gene	Primer sequence
miR-21-5p	F: 5'- TGTTGAGTCGTATCCAGT GCAA -3' R: 5'- GTATCCAGTGCGTGTCGT GG -3'
Smad7	F: 5'- TTCCTCCGCTGAAACAGGG -3' R: 5'- CCTCCCAGTATGCCACCAC-3'
miR-29b	F: 5'- TAGCACCATTTGAAATCAGT -3' R: 5'- GCGAGCACAGAATTAATACGAC -3'
Col1a1	F: 5'- GTCCCAACCCCCAAAGAC -3' R: 5'- CATCTTCTGAGTTTGGTGATACGT -3'
U6	F: 5'- CTCGCTTCGGCAGCA CA -3' R: 5'- AAC GCT TCACGAATTTGC GT -3'
GAPDH	F: 5'- ATTCCATGGCACCGTCAA GGC TGA -3' R: 5'- TTCTCCATGGTGGTGAAG ACGCCA -3'

Results and Discussion

Changes in Mouse Body Weight Following Ginger Extract Treatment

The weights of mice in both the control and treatment groups were recorded throughout the treatment period (Figure 1). On average, mice in the ginger-treated group showed a slightly lower body weight compared to those in the control group. A reduction in body weight among treated mice may be attributed to multiple factors. First, the oral gavage procedure, though a commonly used method of administration,

can be a source of significant physiological stress. Previous studies have indicated that stress from oral gavage may contribute to weight loss or, in extreme cases, mortality in laboratory animals [23]. In the current study, two mice from each group were removed due to fatal injuries, likely associated with the gavage procedure, leaving four mice per group for downstream analyses. Beyond procedural stress, ginger itself may contribute to weight modulation. Ginger's active constituents, particularly gingerols and shogaols, have been shown to exert metabolic effects that could explain the observed reduction in body weight. Notably, ginger is known to enhance thermogenesis and increase energy expenditure, thereby promoting calorie utilization [24]. Additionally, ginger's bioactive compounds are believed to regulate appetite by promoting satiety, potentially through central nervous system pathways or modulation of gut peptides [25]. These effects likely reduce food intake and contribute further to weight loss. Together, these observations suggest that the administration of ginger extract may influence body weight through a combination of stress-related and metabolic mechanisms. However, the small final sample size limits the statistical power of the findings and highlights the need for refined administration techniques and larger cohorts in future studies.

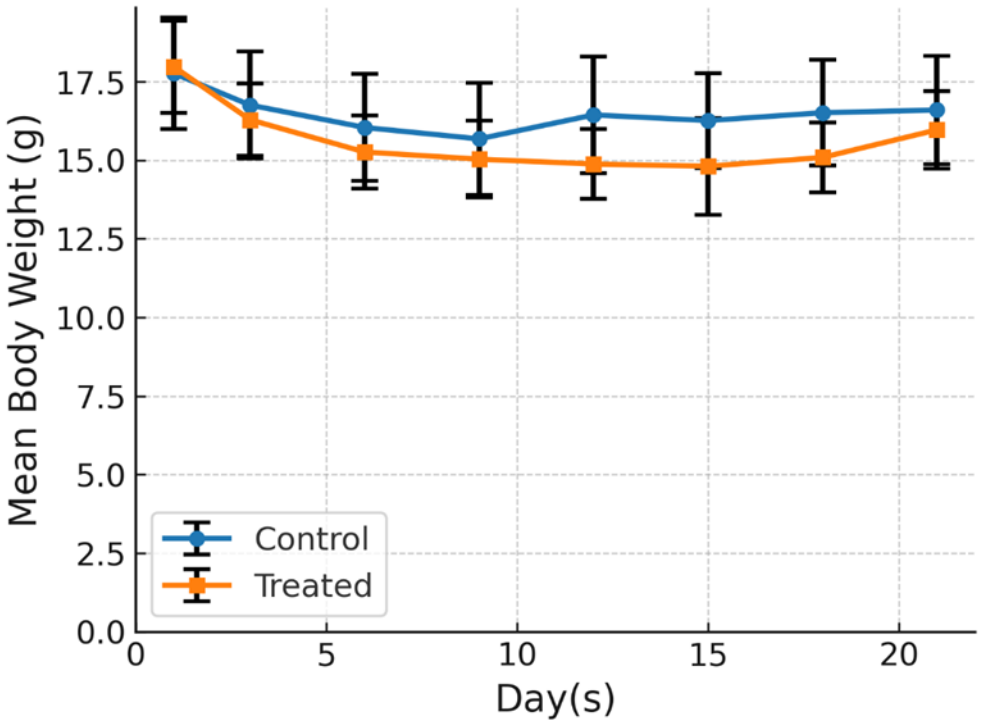

Figure 1 Data showing the mean weight of experimental and control mice within 3 weeks. Each time point represents data from four individual mice (n=4)

Determination of RNA Quantity and Quality

In this study, blood samples were used to evaluate systemic antifibrotic activity, as circulating miRNA are increasingly recognized as minimally invasive biomarkers. These biomarkers can reflect systemic fibrotic processes and provide insights into underlying regulatory mechanisms, supporting their potential for clinical monitoring and therapeutic assessment [15]. As shown in Table 2, RNA concentrations varied among the samples. The lowest RNA concentration was observed in sample 3C (53.8 ng/μL), while the highest was detected in sample 4C (142.1 ng/μL). This variation may reflect inherent differences in RNA yield from individual mice or minor inconsistencies during sample handling and processing.

Table 2. The quantity and quality of RNA extracted from each sample were assessed. Sample C represents the control, while Sample T represents the treatment

Samples	Concentration (ng/μL)	A260/280	A260/230
1C	77.0	1.84	0.26
2C	95.7	1.80	0.48
3C	53.8	1.69	0.40
4C	142.1	1.89	0.43
1T	76.1	1.90	0.25
2T	52.9	1.83	0.18
3T	60.7	1.83	0.20
4T	136.1	1.92	0.47

The A260/280 ratio provides an estimate of RNA purity, specifically the absence of protein and phenol contamination. Most samples showed A260/280 ratios close to the acceptable range of 1.8–2.0, indicating generally good RNA purity in this respect. Sample 3C, with a ratio of 1.69, had the lowest purity among all samples, suggesting possible protein contamination. The A260/230 ratio, which reflects contamination by carbohydrates, phenols, and other organic compounds, was below the ideal range of 2.0–2.2 for all samples. This suggests the presence of residual contaminants, likely from the TRIzol-based extraction method used. Incomplete phase separation during extraction may allow phenol and other organic solvents to carry over into the aqueous RNA phase, thereby lowering the A260/230 ratio. The phenolic components in TRIzol absorb at both 230 nm and ~270 nm, contributing to this interference [26, 27]. Despite suboptimal A260/230 values, the RNA samples were deemed suitable for downstream applications, including cDNA synthesis and quantitative PCR. This decision aligns with previous findings showing that low A260/230 ratios do not necessarily inhibit enzymatic reactions if RNA integrity is preserved [28].

Target Prediction Analysis

Target prediction analysis was conducted using TargetScan, which identified 303 transcripts with conserved sites for miR-21-5p, comprising 322 conserved and 106 poorly conserved sites in mice. The target prediction output has been deposited in a public database (https://data.mendeley.com/datasets/9f985t4wfc/1). For miR-29b, the analysis revealed 1012 transcripts with conserved target sites, including 1161 conserved and 240 poorly conserved sites for miR-29-3p. The output has also been deposited in the public database (https://data.mendeley.com/datasets/4n263zm8sk/1). These findings emphasize the biological relevance of conserved regions in miRNA–mRNA interactions, which are generally more functionally important than non-conserved regions.

Interestingly, mmu-miR-21 aligned with multiple miRNA families, with miR-21-5p showing broader evolutionary conservation than miR-21-3p, whose target sites were mostly poorly conserved. This difference may reflect the greater functional relevance of miR-21-5p in key cellular processes such as development, differentiation, and disease progression, including various fibrotic and oncogenic pathways [29]. Similarly, both miR-29b and miR-29-3p displayed high conservation across species, suggesting evolutionary pressure to maintain their sequence and regulatory function. This conservation often results in overlapping gene targets, a characteristic observed in previous studies within miR-29 family [30].

From the pool of predicted targets, *Smad7* and *Col1a1* were selected for further investigation as representative targets of miR-21-5p and miR-29b, respectively. These target genes were selected due to their well-documented roles in fibrosis-related pathways, including kidney fibrosis [21]. Both selected target genes possess 8-mer seed matches with their corresponding miRNAs, representing the highest confidence category for miRNA binding. The seed region, spanning nucleotides 2–7 from the 5′ end of the miRNA, is essential for target recognition and repression efficiency [2]. *Smad7* functions as an

inhibitor of the TGF-β signaling pathway, which contributes in regulating inflammation, immune responses, and fibrosis development [31]. In contrast, *Col1a1* encodes the collagen type I, an important protein component of the extracellular matrix in tissues such as skin, tendon, and bone. Overexpression of *Col1a1* is a hallmark of fibrotic conditions [32]. These miRNA–target interactions may also be modulated by ginger, which has been reported to exert renoprotective effects, possibly through anti-inflammatory and antifibrotic mechanisms [33]. However, the molecular basis of ginger's protective action, particularly its impact on miRNA expression and target gene regulation, remains underexplored.

Real-time PCR of miR-21-5p and miR-29b toward *Smad7* and *Col1a1*

Ginger treatment resulted in an upregulation of both miR-21-5p and its target gene *Smad7* compared with the control group, as shown by the RT-qPCR analysis. The expression of miR-21-5p increased approximately three-fold in treated mice, while *Smad7* exhibited an even greater elevation, approaching a ten-fold increase. Notably, the treated groups displayed considerable variability, suggesting possible inter-individual differences in response to ginger. These findings indicate that ginger may modulate the miR-21-5p/*Smad7* axis, potentially contributing to its regulatory effects on pathways associated with fibrosis; however, the high variability warrants further investigation with larger sample sizes to confirm the consistency of this response.

Smad7 is a well-characterized inhibitory SMAD protein that antagonizes TGF-β signaling, a main pathway in onset of fibrogenesis. Elevated levels of miR-21-5p have been associated to fibrotic progression in various tissues, including the kidney, through suppression of *Smad7*, thereby enhancing pro-fibrotic TGF-β activity. In the present study, ginger extract supplementation appears to downregulate miR-21-5p expression, allowing for the accumulation of *Smad7*, which in turn may attenuate TGF-β-driven fibrosis. This suggests that ginger may exert antifibrotic effects at the molecular level by affecting the relationship of miR-21-5p and *Smad7*. These results are in agreement with emerging evidence highlighting the renoprotective properties of ginger, particularly through the modulation of profibrotic signaling pathways [34].

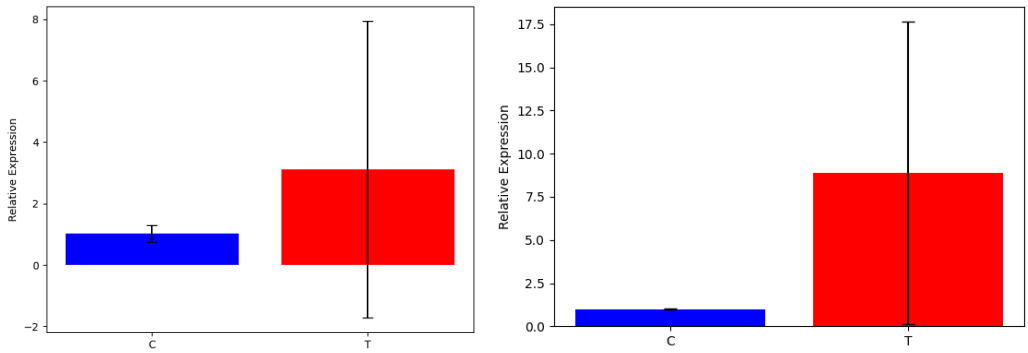

Figure 2. Relative expression (2RT-qPCR) of miR-21-5p (A) and its target gene *Smad7* (B) in control (C) and ginger-treated (T) mice. Vertical lines represent the standard deviation

In addition, studies have shown that mice with *Smad7* gene deletion in the fibrotic model develop severe tubulointerstitial fibrosis driven by enhanced TGF-β/SMAD2/3 signaling. Concurrently, sustained activation of the NF-κB pathway contributes to increased inflammation, as evidenced by the infiltration of macrophage and elevated renal expression of ICAM-1, MCP-1, osteopontin, and TNF-α [34, 35]. Furthermore, during the progression of renal fibrosis, the immunoreactivity of phosphorylated SMAD2 and SMAD3 increases significantly, while *Smad7* expression is diminished due to enhanced ubiquitin-dependent degradation. This reduction in *Smad7* protein contributes to the amplification of fibrotic signaling pathways. Notably, in a type 1 diabetic mouse model, it was confirmed that TGF-β1 could ameliorate diabetic kidney disease by modulating the the interaction of Arkadia and *Smad7*, further highlighting the protective role of *Smad7* [34, 36].

As shown in Figure 3A, administration of ginger extract resulted in a marked increase in miR-29b expression in the treatment group relative to the control group. miR-29b is well known for its role as a negative regulator of extracellular matrix (ECM) components, especially collagen genes such as *Col1a1*,

primarily through post-transcriptional mechanisms that decrease mRNA stability or inhibit translation [21]. Given this established regulatory function, it would typically be expected that elevated miR-29b expression would lead to suppressed *Col1a1* mRNA levels. However, in this study, *Col1a1* transcript levels remain similar or even slightly increased in the ginger-treated mice, albeit with considerable variation (Figure 3B). This apparent discrepancy between increased miR-29b expression and non-suppressed *Col1a1* mRNA levels could arise from several plausible factors. First, the temporal dynamics of miRNA regulation may play a role. The inhibitory effect of miR-29b effect could be time-dependent, requiring prolonged exposure or acting more prominently at specific stages of fibrosis progression. It is also possible that ginger's bioactive compounds, including gingerols and shogaols, activate other signaling pathways that upregulate ECM-related genes, thereby transiently increasing *Col1a1* transcription despite the presence of miR-29b. Second, miRNAs often suppress gene expression at the translational level, meaning they may inhibit protein synthesis without degrading the mRNA [2]. Therefore, while *Col1a1* transcripts may still be detectable at the mRNA level, protein output could be significantly reduced, which was not assessed in this study. This aligns with previous observations where miR-29b suppressed collagen protein production even in the absence of significant mRNA downregulation.

Taken together, while the elevated miR-29b expression observed in ginger-treated mice supports its potential antifibrotic role, the unchanged *Col1a1* mRNA levels highlight the need for further investigation into post-transcriptional regulation and collagen protein expression. Future studies incorporating protein-level validation would provide deeper insight into the functional impact of miR-29b modulation by ginger extract in kidney fibrosis models.

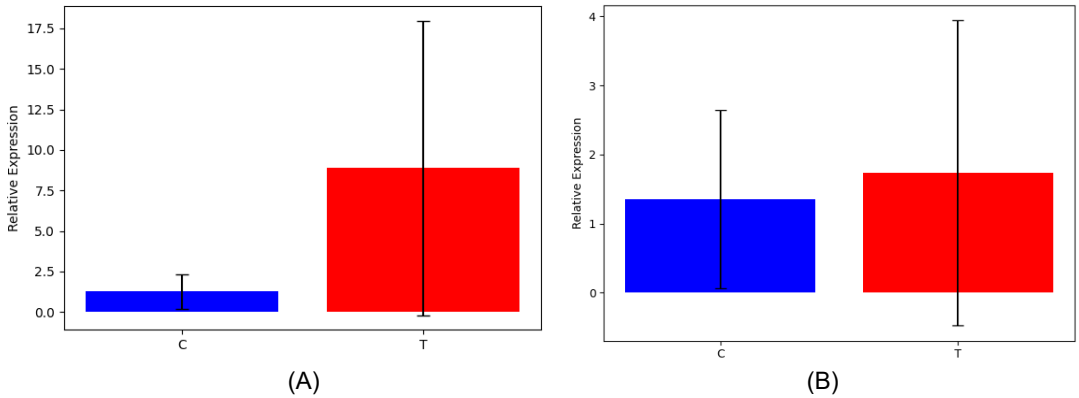

Figure 3. Relative expression (2RT-qPCR) of miR-29b (A) and its target gene *Col1a1* (B) in control (C) and ginger-treated (T) mice. Vertical lines represent the standard deviation

Additionally, it is important to confirm changes in collagen at the protein level, for example, by Western blotting or immunohistochemical staining, because mRNA data alone may be inconclusive when post-transcriptional regulation is involved. Such analyses would provide more definitive evidence of the functional consequences of miR-29b modulation on collagen synthesis and deposition.

Ginger and its constituents have been shown to possess significant biological activities and able to influence the expression of miRNAs and inflammatory signaling pathways [10]. These compounds may also activate or interact with other signaling cascades, such as TGF-β or NF-κB, that can counterbalance the inhibitory effect of miR-29b on *Col1a1* expression. This interaction could explain why *Col1a1* mRNA levels remain stable or are slightly increased, despite the upregulation of miR-29b in the treatment group. Hence, while the observed increase in miR-29b expression upon ginger treatment supports its putative antifibrotic role, the lack of a corresponding decrease in *Col1a1* mRNA levels underscores the complexity of gene regulation in vivo. Overall, this study provides promising but preliminary evidence that ginger extract may contribute to antifibrotic effects via upregulation of miR-29b. However, further research is required to establish its definitive influence on collagen production and tissue remodeling. A key limitation of this study is the absence of protein-level validation, which is essential for confirming the functional relevance of the observed transcriptional changes**.** Additionally, the mortality of two mice in each group reduced the sample size, which may have affected the statistical power and robustness of the observed differences. Future studies should incorporate protein expression analyses and ensure adequate sample sizes to strengthen the interpretation and reproducibility of the findings.

Conclusions

In conclusion, ginger extract demonstrates potential antifibrotic effects through the modulation of miR-21-5p and miR-29b expression in BALB/c mice. Ginger supplementation resulted decreased expression of miR-21-5p, which in turn led to the upregulation of *Smad7*, a key inhibitory regulator of TGF-β signaling. This suggests a mechanism by which ginger may mitigate fibrosis by dampening pro-fibrotic signaling pathways. Additionally, miR-29b was upregulated, consistent with its established role as a negative regulator of extracellular matrix genes, including *Col1a1*. However, *Col1a1* mRNA levels did not show a corresponding decrease, likely due to compensatory signaling pathways, time-dependent expression dynamics, or post-transcriptional regulatory mechanisms. These findings collectively support the hypothesis that ginger extract may exert protective effects against fibrosis by influencing miRNA-mediated regulatory networks. Nevertheless, further investigations are warranted, particularly to validate these effects at the protein level and in organ-specific fibrosis models. Moreover, studies in human models are necessary to fully establish the translational potential of ginger as a therapeutic agent for fibrotic diseases.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgment

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Ethics Approval

The *in vivo* animal studies were approved by the UTM Research Ethics Committee (UTMREC), Universiti Teknologi Malaysia, under approval number UTMREC-2024-52.

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