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Aqueous extract of *Protaetia brevitarsis* larvae increases mTOR-mediated growth rate in zebrafish larvae

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ABSTRACT

Objective: To evaluate the effects of an aqueous extract of *Protaetia brevitarsis* (AEPB) on the growth of zebrafish and preosteoblast MC3T3-E1 cells.

Methods: The effects of AEPB on the linear growth and the expression of growth-related genes in zebrafish and MC3T3-E1 cells were assessed using various molecular techniques. Furthermore, the involvement of the mammalian target of rapamycin (mTOR) pathway in AEPB-induced growth was investigated by employing the mTOR inhibitor rapamycin.

Results: AEPB administration led to a significant and dose-dependent increase in zebrafish larvae growth over time. Additionally, AEPB treatment upregulated the expression of growth hormone-1 (*GH-1*), insulin-like growth factor-1 (*IGF-1*), growth hormone receptor-1 (*GHR-1*), and cholecystokinin-a (*CCKA*) in zebrafish. Similarly, AEPB stimulated the expression and release of IGF-1 and accelerated mTOR expression in MC3T3-E1 cells. In addition, rapamycin hindered AEPB-induced linear growth in zebrafish larvae and suppressed the expression of growth-promoting genes by inhibiting mTOR activation.

Conclusions: AEPB shows growth-promoting effects by upregulating growth-related genes and activating the mTOR signaling pathway. Further investigations are warranted to elucidate its mechanisms of action and explore its potential application in the development of growth-enhancing supplements for various purposes.

KEYWORDS: *Protaetia brevitarsis*; Growth rate; Growth hormone; Insulin-like growth factor; mTOR; MC3T3-E1

1. Introduction

The ingestion of nutrients, such as carbohydrates, proteins, and lipids, stimulates the secretion of cholecystokinin (CCK), leptin, and ghrelin from the gastrointestinal tract, adipose tissue, and stomach, respectively. These hormones, in turn, promote growth hormone (GH) release from the pituitary gland[1]. GH therapy has been shown to have short-term and long-term effects on height increase in children with idiopathic short stature (ISS)[2]. Treatment

Significance

Insect-derived supplements are gaining attention due to their rich nutritional profile, containing proteins, lipids, and vitamins, making them promising for applications in animal husbandry and aquaculture. The results revealed that the aqueous extract of *Protaetia brevitarsis* promoted the growth rate of zebrafish and MC3T3-E1 cells and upregulated growth-related genes. This suggests that the aqueous extract of *Protaetia brevitarsis* could be a valuable nutritional supplement to improve productivity in animal husbandry and aquaculture. Additionally, it may have practical implications in the development of growth-enhancing supplements, particularly for individuals requiring additional essential nutrients for optimal growth, such as children.

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with recombinant human GH has been found to effectively increase adult height in ISS patients by elevating insulin-like growth factor-1 (IGF-1) levels, and it has demonstrated a positive safety profile[3]. Clinical trials have also reported that recombinant human GH treatment improved height velocity in ISS patients, accompanied by an increase in IGF-1 and IGF-binding protein-3 levels, with no significant safety concerns[4]. GH has demonstrated its ability to enhance growth rate and appetite in various animal species, including poultry[5,6], fish[7], and frogs[8]. Furthermore, GH has been shown to improve bone growth and mineral density, contributing to osteoporosis prevention[9,10]. However, excessive or prolonged GH treatment can lead to several side effects, such as scoliosis, insulin resistance, arthralgia, and hypertension in children, adolescents, and healthy adults[11,12]. Micronutrients and macronutrients, including carbohydrates, proteins, and lipids, play crucial roles in maintaining metabolic homeostasis and supporting anabolic activity through the GH-IGF-1 axis[13]. Understanding the specific contributions of carbohydrates, proteins, and lipids in each metabolic pathway and their role in triggering growth *via* GH-IGF-1 activation is challenging due to the interconnectedness and physiological coordination of these pathways.

GH is synthesized and released from the pituitary gland, circulating in the body and primarily binding to the GH receptor (GHR) in the liver[1]. This leads to the activation of the Janus kinase 2-signal transducer and activator of transcription 5 signaling pathway, ultimately resulting in IGF-1 release[14]. The GH-IGF-1 axis plays a critical role in promoting growth, as mutations in *GHR* and IGF-1 receptor (*IGF-1R*) cause growth retardation[15,16]. Upon binding of GH and IGF-1 to their respective receptors, the mammalian target of rapamycin (mTOR) pathway is initiated. Specifically, mTOR complex 1 (mTORC1) is activated, promoting cell growth[17,18]. Due to its central role in nutrient signaling and the regulation of cell and tissue growth, mTOR is considered a key target in the nutrient signaling pathway[19]. Milk amino acids, such as tryptophan and leucine, activate the GH-IGF-1-mTORC1 axis, leading to enhanced cell growth and metabolic regulation[20–22]. Therefore, investigating the effects of nutrients on the GH-IGF-1-mTORC1 axis represents a promising avenue of research.

Lentjes[23] emphasized the significance of maintaining a balanced diet comprising both food and dietary supplements to reduce the risk of chronic diseases and promote overall health. Insect-derived nutritional supplements have drawn attention as a potential solution to address malnutrition due to their rich composition of essential nutrients, including proteins, fatty acids, vitamins, and minerals[24]. Among various insect species, *Protaetia brevitarsis* (*P. brevitarsis*) larvae have been traditionally used in Asian countries for medicinal purposes, primarily due to their rich nutritional profile containing amino acids such as histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, and valine, as well as fatty acids such as palmitic acid, palmitoleic acid, and oleic acid[25,26]. Furthermore, *P. brevitarsis* larvae have demonstrated safety for consumption, with no toxicity or genotoxicity observed when orally administered to rats for 13 weeks[27]. Recent studies have also highlighted the potential benefits of *P. brevitarsis*, including their anti-benign prostatic hyperplasia[28], anti-obesity[29], and hepatoprotective effects[30]. Moreover, an aqueous extract of *P. brevitarsis* larvae stimulated osteoblast differentiation and bone formation by activating β-catenin in a recent study[31]. However, the effects of the aqueous extract of *P. brevitarsis* larvae on growth rate have not yet been investigated. Therefore, the present study aims to explore the potential of the aqueous extract of *P. brevitarsis* larvae in enhancing its ability to stimulate the expression of growth-promoting genes in both zebrafish larvae and preosteoblast MC3T3-E1 cells.

2. Materials and methods

2.1. Preparation of the aqueous extract of *P. brevitarsis* larvae

Freeze-dried *P. brevitarsis* larvae from Huimang-Gonchung Farm (Hapcheon, Gyeongsangnam-do, Republic of Korea) were authenticated and deposited at the Nakdonggang National Institute of Biological Resources (Sangju, Gyeongsangbuk-do, Republic of Korea). Preparation of the aqueous extract of *P. brevitarsis* larvae was conducted following established protocols[31,32], resulting in an approximately 23% yield.

2.2. Reagents and antibodies

Fetal bovine serum, an antibiotic mixture, and Minimum Essential Medium Alpha Modification were purchased from WELGENE (Gyeongsan, Gyeongsangbuk-do, Republic of Korea). β-Glycerophosphate (GP), rapamycin, and tricaine methanesulfonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against mTOR (sc-517464, 1∶1 000 dilution) and peroxidase-labeled anti-mouse immunoglobulins (sc-516102, 1∶3 000 dilutions) were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.3. Maintenance of zebrafish embryos

The adult zebrafish were obtained by the Nakdonggang National Institute of Biological Resources (Sanju, Gyeongsangbuk-do, Republic of Korea) and raised at 28.5℃ with a light-to-dark cycle of 14∶10 h. The fertilized eggs were cultured and supplemented with 200 μg/100 mL of methylene blue. The E3 embryo media were

replenished every 3 days, along with appropriate treatment.

2.4. Evaluation of growth rate of zebrafish larvae

To assess the growth rate of zebrafish larvae, their total length was measured at 12 days post-fertilization (dpf). The larvae were subjected to various concentrations of the aqueous extract of *P. brevitarsis* larvae (0-200 μg/mL) or 4 mM GP. The culture medium was replaced with the appropriate treatment every 3 days. In a parallel experiment, the larvae were treated with an mTOR inhibitor, rapamycin (20 μM), for 2 h, before 10-day treatment with the aqueous extract of *P. brevitarsis* larvae (200 μg/mL). The larvae growth rate was then assessed and compared among the different treatment groups.

2.5. Cell culture

MC3T3-E1 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured at 37℃ in a humidified atmosphere with 5% CO_2. The cells were maintained in Minimum Essential Medium Alpha Modification supplemented with 10% fetal bovine serum and an antibiotic mixture. Initially, the cells were treated with various concentrations of the aqueous extract of *P. brevitarsis* larvae (0-20 μg/mL) or rapamycin (20 μM) for 7 d. The culture medium was refreshed every 2 days by adding fresh medium containing the aqueous extract of *P. brevitarsis* larvae or rapamycin.

2.6. Enzyme–linked immunosorbent assay (ELISA) for IGF–1 release

MC3T3-E1 cells were treated with varying concentrations of the aqueous extract of *P. brevitarsis* larvae (0-20 μg/mL) for 3 d. Following the treatment, the cell culture media were collected and centrifuged (12 000 ×*g*) to obtain cell-free supernatants. The levels of IGF-1 in the supernatants were quantified using an IGF-1 ELISA kit (K033225, KOMA BIOTECH, Seoul, Republic of Korea).

2.7. Reverse transcription polymerase chain reaction (RT–PCR)

For MC3T3-E1 cells, various concentrations of the aqueous extract of *P. brevitarsis* larvae (0-20 μg/mL) were administered, and on day 7, total RNA was extracted using the Easy-BLUE Total RNA Extraction Kit (iNtRON Biotechnology, Sungnam, Gyeonggi-do, Republic of Korea). In the case of zebrafish larvae at 3 dpf, they were treated with varying concentrations of the aqueous extract of *P. brevitarsis* larvae (0-200 μg/mL) or 4 mM GP. Culture media were refreshed every 3 days with either the aqueous extract of *P. brevitarsis* larvae or GP treatment. At 12 dpf, the larvae were anesthetized using a 0.002% tricaine methanesulfonate solution. Total RNA was extracted using an Easy-BLUE Total RNA Extraction Kit. Subsequently, cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase (Bioneer, Daejeon, Republic of Korea). The target genes were amplified using specific primers (Table 1). The results were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to *β–actin* in zebrafish and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in mice.

2.8. Western blotting analysis

Total cellular proteins were extracted using RIPA Lysis Buffer (Rockland Immunochemicals, Pottstown, PA, USA) supplemented with a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA,

Table 1. Primers used in the study for RT-PCR analysis.

Species	Gene	Primer sequences (5′→3′)	Amplicon size (bp)	Accession No.
Zebrafish	*GH–1*	F: 5′- GGT GGT GGT TAG TTT GCT GG-3′	157	NM_001020492.2
		R: 5′- CAA CTG TCT GCG TTC CTC AG-3′		
	IGF–1	F: 5′- GAG TAC CCA CAC CCT CTC AC-3′	213	NM_131825.2
		R: 5′- TGA AAG CAG CAT TCG TCC AC-3′		
	GHR–1	F: 5′- TCA GTC CGA CTC AGA AAC CG-3′	178	NM_001083872.1
		R: 5′- TTC TGA AGC ACG GGA CCA TA-3′		
	CCKA	F: 5′- GAT GAA GAA CCT CGC AGC AG-3′	154	NM_001386383.1
		R: 5′- GGC CCA AAT CCA TCC ATC CC-3′		
	β–actin	F: 5′- CGA GCG TGG CTA CAG CTT CA-3′	155	NM_131031.2
		R: 5′- GAC CGT CAG GCA GCT CAT AG-3′		
Mouse	*IGF–1*	F: 5′- GGA CCA GAG ACC CTT TGC GGG-3′	210	NM_010512.5
		R: 5′- GGC TGC TTT TGA CCC TTC AGT GG-3′		
	GAPDH	F: 5′- ACC ACA GTC CAT GCC ATC AC-3′	450	NM_001289726.2
		R: 5′- CAC CAC CCT GTT GCT GTA GC-3′		

GH–1: growth hormone-1; *IGF–1*: insulin-like growth factor-1; *GHR–1*: growth hormone receptor-1; *CCKA*: cholecystokinin a; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; bp: base pair.

USA), and 25 μg/mL protein was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. Subsequently, the proteins were separated by gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was then incubated with primary antibodies followed by secondary antibodies. Finally, the protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The results were quantified using ImageJ software and normalized to β-actin.

2.9. Statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA). The data are expressed as mean±SEM of at least three independent experiments. Significance between groups was determined using an unpaired one-way ANOVA test with Bonferroni correction, as appropriate. $P<0.05$ was considered significantly different.

2.10. Ethical statement

All animal experiments conducted in this study were approved by the Institutional Animal Care and Use Committee of Jeju National University (Jeju, Jeju Special Self-Governing Province, Republic of Korea; Approval No. 2023-0004). Zebrafish care followed the established guidelines.

3. Results

3.1. Aqueous extract of *P. brevitarsis* larvae promotes the linear growth of zebrafish larvae accompanied by high expression of growth-enhancing genes

In this study, the potential of the aqueous extract of *P. brevitarsis* larvae to improve the linear growth of zebrafish larvae and its effect on the expression of growth-promoting genes were investigated. Treatment with the aqueous extract of *P. brevitarsis* larvae at 100 and 200 μg/mL significantly increased the lateral length of zebrafish larvae compared to untreated larvae ($P<0.05$) (Figure 1A). The growth-promoting effect of the aqueous extract of *P. brevitarsis* larvae was similar to GP. However, the aqueous extract of *P. brevitarsis* larvae at 50 μg/mL or lower concentration did not result in a significant increase in lateral growth at 12 dpf. Subsequently, growth-promoting genes in zebrafish larvae were analyzed using RT-PCR. In untreated zebrafish larvae, the expression levels of *GH–1*, *IGF–1*, *GHR–1*, and *CCKA*, which are growth-promoting genes, were low (Figure 1B). Conversely, treatment with the aqueous extract of *P. brevitarsis* larvae led to a dose-dependent upregulation of these genes except *CCKA* ($P<0.05$). *IGF–1* displayed moderate expression at 25 μg/mL of the aqueous extract of *P. brevitarsis* larvae, but its expression was notably increased at 50 μg/mL of the aqueous extract of *P. brevitarsis* larvae ($P<0.05$). Additionally, *GHR–1* expression was significantly increased at all concentrations of the aqueous extract of *P. brevitarsis* larvae ($P<0.05$). These findings indicate that the aqueous extract of *P. brevitarsis* larvae enhances the linear growth

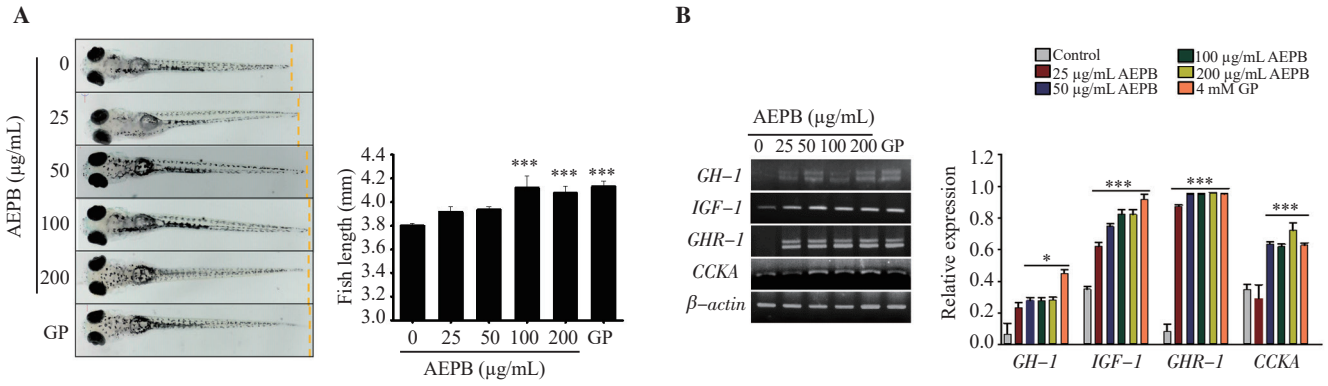

Figure 1. Aqueous extract of *Protaetia brevitarsis* larvae (AEPB) enhances the lateral growth of zebrafish larvae and upregulates growth-enhancing gene expression. (A) Zebrafish larvae (n=20) at 3 days post-fertilization (dpf) were exposed to different concentrations of AEPB (0-200 μg/mL) or 4 mM β-glycerophosphate (GP), and their average length was measured at 12 dpf using stereomicroscopy (×4). The graph represents the average length of zebrafish larvae. (B) At 12 dpf, total RNA was extracted and RT-PCR was performed to assess the expression of *GH–1*, *IGF–1*, *GHR–1*, and *CCKA*. *β–Actin* was used as a loading control. The graph shows the relative densities of each gene expression. The relative density was calculated using ImageJ. Statistical analysis was conducted using one-way ANOVA followed by the Bonferroni method to determine significant differences among the groups. The values represent mean±SEM of three independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* untreated zebrafish larvae.

of zebrafish larvae by upregulating growth-enhancing genes.

3.2. Aqueous extract of P. brevitarsis larvae enhances IGF–1 expression and release in preosteoblast MC3T3–E1 cells

The effect of the aqueous extract of *P. brevitarsis* larvae on growth enhancement was investigated by measuring IGF-1 expression and release in preosteoblast MC3T3-E1 cells. Treatment with 10 and 20 μg/mL of the aqueous extract of *P. brevitarsis* larvae significantly increased extracellular IGF-1 production (P<0.001) (Figure 2A). Moreover, the aqueous extract of *P. brevitarsis* larvae induced a concentration-dependent increase in *IGF–1* expression (P<0.001) (Figure 2B). The results of these studies suggest that the aqueous extract of *P. brevitarsis* larvae can directly affect the expression and release of IGF-1 in MC3T3-E1 cells.

3.3. Aqueous extract of P. brevitarsis larvae promotes a time–dependent growth rate and growth–enhancing gene expression in MC3T3–E1 cells

The effects of the aqueous extract of *P. brevitarsis* larvae on growth rate and growth-enhancing genes were evaluated in

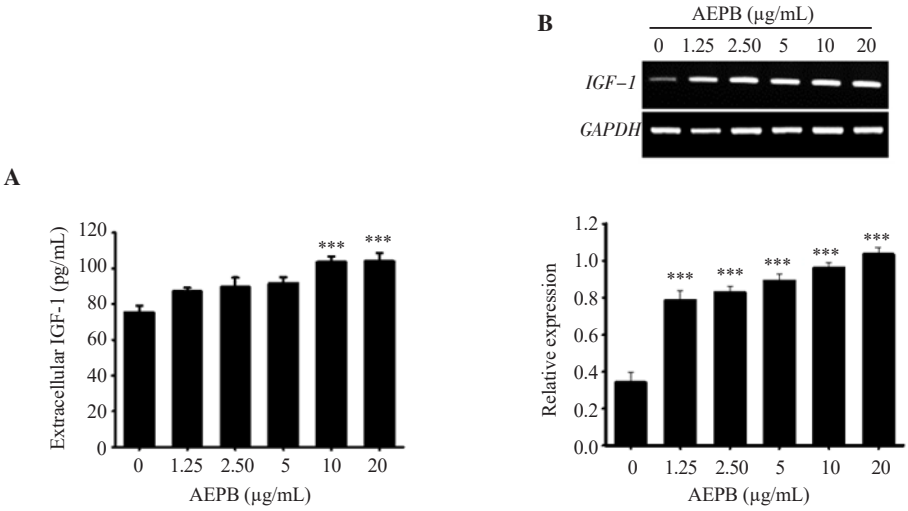

Figure 2. AEPB promotes IGF-1 expression and release in MC3T3-E1 preosteoblast cells. (A) MC3T3-E1 cells were treated with varying concentrations of AEPB (0-20 μg/mL) for 3 days, and extracellular IGF-1 levels were measured using an ELISA assay. (B) MC3T3-E1 cells were treated with AEPB (0-20 μg/mL) for 7 days. Total RNA was extracted, and RT-PCR was performed to evaluate *IGF–1* expression. *GAPDH* was applied as a loading control. The relative density for *IGF–1* was calculated using ImageJ software. Statistical analysis was performed using one-way ANOVA followed by the Bonferroni method. The values represent mean±SEM of three independent experiments. $^{***}P < 0.001$ *vs.* untreated cells.

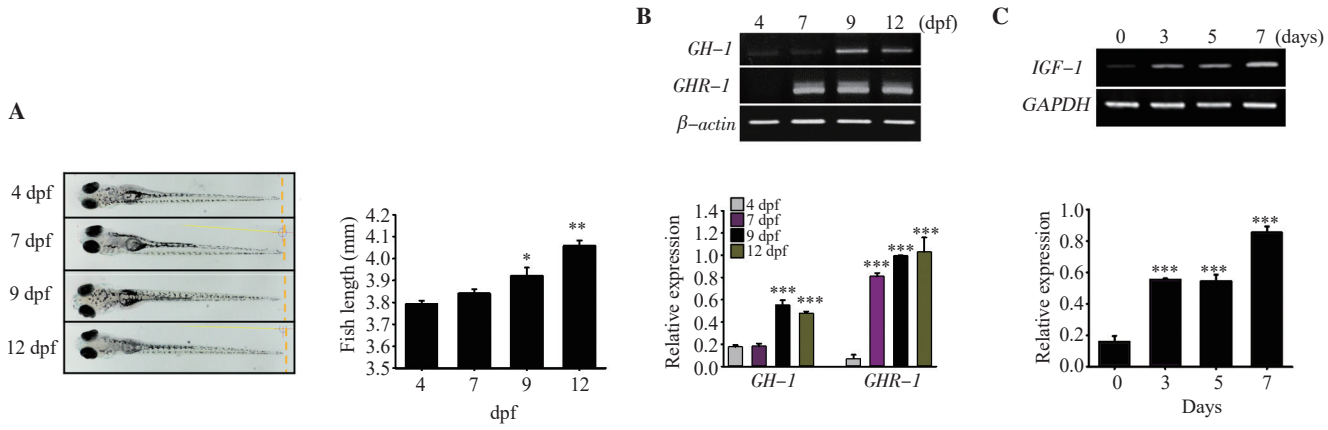

Figure 3. AEPB promotes a time-dependent growth rate and growth-enhancing gene expression. (A) Zebrafish larvae (n=20) were treated with 200 μg/mL of AEPB at 3 dpf, and the lateral length was measured at 4, 7, 9, and 12 dpf. The graph represents the average length. (B) Total RNA was extracted from zebrafish larvae at the indicated dpf, and RT-PCR was performed to assess the expression of *GH–1* and *GHR–1*. *β-Actin* was used as a loading control. (C) MC3T3-E1 cells were treated with 20 μg/mL of AEPB. RT-PCR was performed to detect *IGF–1*. *GAPDH* was used as a loading control. The relative density of each gene was calculated using ImageJ software. Significant differences among the groups were determined using one-way ANOVA followed by the Bonferroni method. The values represent mean±SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs.* untreated group.

zebrafish larvae and preosteoblast MC3T3-E1 cells. In zebrafish larvae, treatment with the aqueous extract of *P. brevitarsis* larvae resulted in a significant increase in growth rate, with the maximum effect observed at 12 dpf [(4.06 ± 0.03) mm] (P<0.05) (Figure 3A). Furthermore, the aqueous extract of *P. brevitarsis* larvae upregulated *GH-1* expression in zebrafish larvae, starting at 9 days of development (P<0.001) (Figure 3B). The expression of *GHR-1* also showed a remarkable approximately 11-fold increase since 7 dpf compared to the levels observed at 4 dpf. In preosteoblast MC3T3-E1 cells, the aqueous extract influenced *IGF-1* expression in a time-dependent manner, which was significantly increased from day 3 to day 7 (P<0.001) (Figure 3C). These findings suggest that the aqueous extract of *P. brevitarsis* larvae exerts time-dependent effects on growth rate and expression of growth-enhancing genes in zebrafish larvae and preosteoblast cells. The observed increase in growth rate and the upregulation of *IGF-1* indicate that the aqueous extract of *P. brevitarsis* larvae has the potential to enhance growth over time in these experimental models.

3.4. Aqueous extract of P. brevitarsis larvae enhances mTOR expression in MC3T3-E1 cells

To investigate the effect of the aqueous extract of *P. brevitarsis* larvae on mTOR expression in MC3T3-E1 cells, the cells were treated with various concentrations of the extract (0-20 μg/mL) for 7 d, and mTOR expression was analyzed using Western blotting. The results showed that mTOR expression gradually increased with increasing concentrations of the aqueous extract of *P. brevitarsis* larvae (Figure 4).

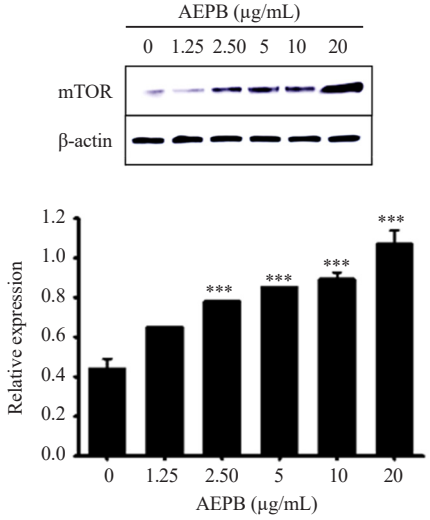

Figure 4. AEPB enhances mammalian target of rapamycin (mTOR) expression in MC3T3-E1 preosteoblast cells. MC3T3-E1 cells were treated with various concentrations of AEPB (0-20 μg/mL) for 7 days, and mTOR expression was analyzed by Western blotting. β-Actin was used as a loading control. The relative density of mTOR expression was quantified using ImageJ software. Significant differences among the groups were determined using one-way ANOVA followed by the Bonferroni method. The values represent mean±SEM of three independent experiments. $^{***}P < 0.001$ *vs.* untreated cells.

3.5. Rapamycin inhibits the aqueous extract of P. brevitarsis larvae-induced growth mediated by the mTOR signaling pathway

We examined the impact of mTOR on growth induced by the aqueous extract of *P. brevitarsis* larvae and observed that the extract resulted in a significant increase in the lateral length of zebrafish larvae at 12 dpf, indicating its growth-stimulating effect. To further verify its underlying mechanism, we evaluate the effect of rapamycin, an mTOR inhibitor, on extract-induced growth. The co-administration of rapamycin effectively suppressed the growth-promoting effect of the aqueous extract of *P. brevitarsis* larvae (P<0.001) (Figure 5A). Treatment with rapamycin alone did not significantly alter the growth rate of zebrafish larvae compared to untreated larvae. Additionally, rapamycin abolished the upregulating effect of the extract on the expression of *GH-1*, *IGF-1*, *GHR-1*, and *CCKA* (P<0.001) (Figure 5B). In MC3T3-E1 cells, the aqueous extract of *P. brevitarsis* larvae induced an increase in *IGF-1* expression, which was markedly suppressed by rapamycin treatment (P<0.001) (Figure 5C). These findings highlight the crucial role of the mTOR signaling pathway in mediating the effect of the aqueous extract of *P. brevitarsis* larvae on growth-promoting genes and *IGF-1* expression. Inhibition of mTOR activation by rapamycin effectively counteracts the growth-enhancing effects of the aqueous extract of *P. brevitarsis* larvae.

4. Discussion

Our investigation shows the notable influence of the aqueous extract of *P. brevitarsis* larvae on the growth rate of zebrafish larvae and the expression of growth-enhancing genes. Moreover, we observed a significant increase in mTOR expression following treatment with the aqueous extract of *P. brevitarsis* larvae, indicating its role as a key regulator of cell growth and proliferation. Importantly, our findings highlight the growth-promoting effects of the aqueous extract of *P. brevitarsis* larvae, as evidenced by the inhibition of growth enhancement with rapamycin. These findings provide valuable insights into the potential application of the aqueous extract of *P. brevitarsis* larvae as a growth supplement and emphasize the importance of considering both morphometric and molecular markers when studying growth. Traditional approaches to assessing growth have relied on morphometric markers such as weight and body size; however, incorporating molecular markers that elucidate the underlying mechanisms is crucial for a comprehensive understanding of growth processes[33,34]. Previous research showed that the aqueous extract of *P. brevitarsis* larvae stimulates osteogenesis, suggesting its potential as a growth-enhancing agent[31]. Nevertheless, the comprehensive effects of the aqueous extract of *P. brevitarsis* larvae on growth have not been thoroughly

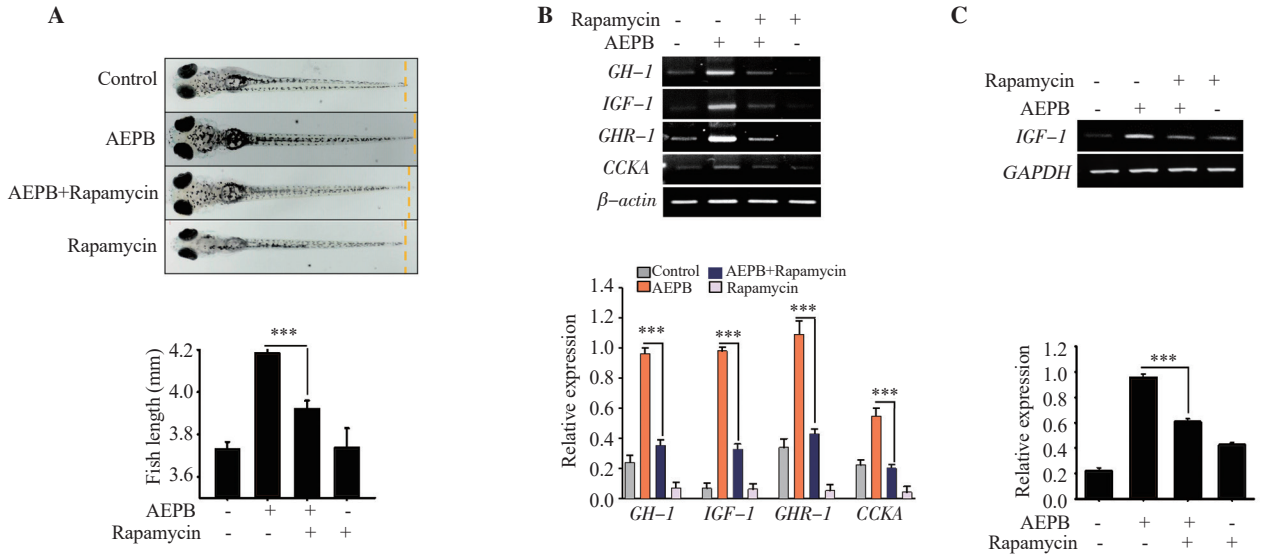

Figure 5. Rapamycin inhibits AEPB-induced linear growth and growth-promoting gene expression. (A) Zebrafish larvae at 3 dpf were pretreated with rapamycin (20 μM) for 2 h before treatment with 200 μg/mL of AEPB. The average zebrafish length was measured at 12 dpf. (B) Total RNA was extracted at 12 dpf, and RT-PCR was performed to detect the expression of *GH–1*, *IGF–1*, *GHR–1*, and *CCKA*. The relative density of gene expression was calculated using ImageJ, with β-actin as the loading control. (C) MC3T3-E1 cells were pretreated with 20 μM rapamycin for 2 h and stimulated with 20 μg/mL of AEPB for 7 days. Total RNA was extracted, and RT-PCR was performed to determine *IGF–1*. The relative densities of gene expressions were calculated using ImageJ, with *GAPDH* as the loading control. Significant differences among the groups were determined using one-way ANOVA followed by the Bonferroni method. The values represent mean±SEM of three independent experiments. $^{***}P < 0.001$ *vs.* untreated zebrafish larvae or cells.

investigated. In this study, we employed both morphometric and molecular markers to validate the growth-enhancing potential of the aqueous extract of *P. brevitarsis* larvae as a supplement, in order to contribute to a more comprehensive understanding of growth processes.

Growth regulation in animals and humans involves a complex interplay of signals mediated by the hypothalamic-pituitary-somatotroph axis[35]. Additionally, various gastrointestinal and adipogenic hormones contribute to feeding regulation, hypothalamic development, and peripheral activation[36,37]. The GH-IGF-1 axis, which plays a crucial role in both linear and vertical growth[38–40], is particularly important in the diagnosis and treatment of growth disorders[41]. Nutraceuticals or nutritional supplements are sometimes utilized to stimulate the GH-IGF-1 axis, to stimulate rapid growth and increased height. In our study, we observed that the aqueous extract of *P. brevitarsis* larvae promoted lateral growth in zebrafish larvae and induced growth-promoting genes. However, it is imperative to note that excessive or imbalanced supplement intake may potentially lead to feedback inhibition of the GH-IGF-1 axis[42,43]. Therefore, it is crucial to conduct separate investigations to comprehensively examine any potential negative impact of high doses of the aqueous extract of *P. brevitarsis* larvae on the regulation of the GH-IGF-1 axis. Such studies will contribute to a better understanding of the overall effects and safety considerations associated with the aqueous extract of *P. brevitarsis* larvae.

The growth-promoting effects of the aqueous extract of *P. brevitarsis* larvae are mediated by the mTOR pathway, which is a crucial component of the PI3K-AKT-mTOR axis involved in regulating cellular growth and aging. This pathway stimulates ribosome biogenesis and translation through the activation of IGF-1R[17,18]. The inhibitory effect of the mTOR inhibitor rapamycin on the aqueous extract of *P. brevitarsis* larvae-induced growth suggests that the extract may be a promising nutritional supplement for enhancing growth. However, further research is necessary to identify the specific active compounds in the aqueous extract of *P. brevitarsis* larvae that contribute to these effects. It is also necessary to elucidate the transcription factors involved in mTOR regulation and understand the post-translational modifications that modulate mTOR. It is also worthwhile to consider the potential inhibitory effects of excessive intake of the aqueous extract of *P. brevitarsis* larvae on the GH-IGF-1-mTOR axis, as overnutrition and obesity can disrupt this axis, leading to GH deficiency[44]. Moreover, given the association between mTOR activation and complex pathological processes and diseases such as cancer development and aging[45], it is crucial to thoroughly investigate the role of mTOR in growth regulation and to ensure the safe and effective use of the aqueous extract of *P. brevitarsis* larvae as a growth supplement.

In conclusion, this study shows that the aqueous extract of *P. brevitarsis* larvae has growth-promoting effects in zebrafish larvae through its regulation of the GH-IGF-1-mTOR axis. These results suggest that the aqueous extract of *P. brevitarsis* larvae holds promise as a nutritional supplement for growth. However, further research

is needed to evaluate the safety and efficacy of the aqueous extract of *P. brevitarsis* larvae, identify the specific active compounds responsible for its effects, and investigate the long-term implications of intake of the aqueous extract of *P. brevitarsis*. Understanding the transcription factors involved in mTOR regulation and exploring the potential impact of mTOR activation on aging and disease are also critical areas for future investigation. Overall, this study contributes to our understanding of growth regulation and highlights the potential of nutraceuticals as growth supplements in both animals and humans.

Conflict of interest statement

The authors declare no potential conflicts of interest.

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Authors' contributions

JACCJ and GYK designed the work and contributed to the concept. JACCJ, KTL, and YHC performed experiments and data validation. JACCJ wrote original draft, and YHC and GYK performed the critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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