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Original Article

Effect of Carvacrol in Attenuating the TNF-α Induced Sarcopenia

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ABSTRACT

Background: Sarcopenia is a condition characterized by muscle loss, which is accompanied by inflammation, oxidative stress, and mitochondrial dysfunction. The current treatment of sarcopenia is insufficient in handling the issues it faces, which may need alternative therapy, especially from natural sources. Carvacrol is a natural compound that has shown antioxidant and anti-inflammatory properties previously, making it an ideal choice for evaluating its potential in sarcopenia treatment. **Objectives:** The objective of this study is to evaluate the effect of Carvacrol in attenuating the TNF-α induced sarcopenia using an *in vitro* model. **Materials and Methods:** TNF-α pretreated rat myoblast cells L6 were treated with different concentrations (0.75-12.5 µg/mL) of carvacrol to determine the cytoprotective effect. Later, TNF-α pretreated L6 cells were treated with the IC_{50} concentration of carvacrol, and the level of Lactate Dehydrogenase (LDH) leakage, antioxidant enzyme activities (catalase and SOD), ATPase activities (Na^+/K^+, Ca^{2+}, Mg^{2+}), Mitochondrial Membrane Potential (MMP), and gene expression of SIRT1 and AMPK using real-time PCR were evaluated. **Results:** The IC_{50} of carvacrol in L6 cells was obtained as 60.87 µg/mL. The study demonstrated that Carvacrol was able to protect against TNF-α-induced cell toxicity at a dose of 6.25 µg/mL, reducing cell rounding and blebbing. The TNF-α induced LDH leakage was 300 units, which was reduced by carvacrol to 100±11.25 units. The antioxidant enzymes, such as catalase and SOD, were significantly restored to normal levels by carvacrol. In addition, the ATPase levels were also reduced to 3±0.01 mg/dL for Na^+/K^+, 2.5±0.01 mg/dL for Ca^{2+}, and 3.5±0.02 mg/dL for Mg^{2+}. Carvacrol reduced the mitochondrial depolarization in the treated cells from 31.65±4.25% to 19.0±2.6% significantly. It also upregulated the gene expression levels of SIRT1 (1.6-fold) and AMPK (2-fold) compared to TNF-α-induced downregulation. **Conclusion:** The results demonstrate that carvacrol has the potential to treat the sarcopenia condition. It has recovered sarcopenia-related muscle toxicity by influencing the antioxidant system, mitochondrial function, and upregulation of SIRT1 and AMPK genes. These findings suggest carvacrol's potential as a therapeutic agent for sarcopenia, warranting further preclinical and clinical studies.

Keywords: Sarcopenia, Carvacrol, Oxidative Stress, Mitochondria, TNF-α.

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INTRODUCTION

Sarcopenia is a condition which has accompanied by progressive loss of muscle mass and its function. This condition is more prevalent in the elderly population, which can gradually lead to frailty and negatively impact the quality of life. As per the epidemiological data, this condition affects 10-16% of people who are aged between 60 and 70 years. Studies also indicate that the prevalence is a little higher in the diabetic population, which can go up to 18%.[1] Untreated and uncontrolled sarcopenia is associated with frailty, mobility limitations, and increased mortality risk.[2] Studies so far have shown that there are multi-factorial conditions such as elevated TNF-α levels, chronic inflammation, elevated oxidative stress, mitochondrial dysfunction and impaired protein synthesis associated with sarcopenia.[3] Management of sarcopenia-associated symptoms can be managed by targeted nutritional supplements to help muscle mass, strengthen the muscle with resistance exercises, and anabolic steroids-related pharmacological management of pain.[4] However, despite all these management efforts, to date, there is no satisfactory drug developed for sarcopenia, which emphasises the need for more research in novel drug discovery.

Natural products have been trusted for many centuries and by many populations due to their time-tested and safe nature. Hence, potential plant products can be considered promising leads for drug discovery against sarcopenia. Compared to other synthetic origin drugs, natural products have shown significant

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antioxidant, anti-inflammatory and cytoprotective properties, which makes them the first choice for scientists for drug discovery against chronic conditions that are induced by oxidative stress and inflammation.[5,6] Carvacrol is a monoterpenoid phenol that is found in the essential oils of oregano and thyme. It has recently become increasingly common since it is a potent antioxidant, anti-inflammatory, and mitochondrial-protective substance.[7,8] Previous studies have demonstrated that carvacrol has the ability to modulate oxidative stress, stabilize cellular membranes, and regulate key signalling pathways, such as AMP-Activated Protein Kinase (AMPK) and Sirtuin 1 (SIRT1), which are critical in muscle health.[9,10] These characteristics make carvacrol a potential therapeutic agent for combating sarcopenia by addressing its inflammatory and oxidative underpinnings.

This study explored the potential of carvacrol, a naturally occurring compound, to counteract TNF-α-induced muscle atrophy in L6 rat skeletal muscle cells, an *in vitro* model relevant to sarcopenia research. TNF-α, an inflammatory cytokine, increases with age and drives muscle atrophy through multiple pathways.[11] Elevated TNF-α levels promote protein degradation, suppress protein synthesis, and hinder muscle cell growth and differentiation in skeletal muscle.[12] A key outcome of these processes is the activation of the ubiquitin-proteasome system, which degrades structural muscle proteins, weakening muscle fibers and upregulating genes associated with muscle loss.[13] Additionally, TNF-α induces oxidative stress and mitochondrial damage, reducing cellular energy availability and promoting apoptosis, both of which exacerbate muscle loss in age-related sarcopenia.

We selected L6 cells for this study due to their ability to mimic skeletal muscle cell behavior, including growth, differentiation, and inflammatory responses. When exposed to TNF-α, these cells exhibit hallmark features of sarcopenia, such as reduced muscle fiber synthesis, elevated expression of atrophy-related genes, and increased oxidative stress.[14] These characteristics make L6 cells an ideal model for evaluating carvacrol's effects on sarcopenia. This research aims to elucidate carvacrol's therapeutic potential as a candidate for mitigating sarcopenia, laying the groundwork for future preclinical and clinical studies to develop targeted interventions for this debilitating condition.

MATERIALS AND METHODS

Cell culture

L6 (Rat Myoblast) cells were purchased from ATCC and cultured in RPMI media supplemented with 10% FBS and 1% Antibiotics in a CO_2 incubator. Cells were grown to confluence for further testing.

MTT assay

A basic cell toxicity profile was conducted to optimize the dose selection for the cytotoxicity assay. In brief, 10,000 cells/well were seeded into 96-well plates, and they were allowed to attach overnight to form a monolayer at 37°C in a 5% CO_2 incubator. The next day, the medium was replaced with different concentrations of carvacrol dissolved in RPMI media and incubated for 24 hr. At the end of the experiment, the plates were removed from the incubator, and using a phase contrast microscope, the cells were captured to analyze the morphological changes. Subsequently, 10 µL of MTT solution (5 mg/mL in sterile PBS) was added to each well, and plates were incubated for another 4 hr at 37°C in the dark. The medium was aspirated, and 100 µL of DMSO was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a microplate reader (BioTek Synergy HT). The percentage viability of the cells with treatment and control was calculated using the following formula:

$$\% \text{ of viability} = \frac{\text{Mean Intensity of Samples} \times 100}{\text{Mean Intensity of the control}}$$

Cytoprotection assay against TNF-α induced toxicity

The cytoprotective effect of carvacrol was evaluated using the MTT assay on the L6 cells following the induction of cytotoxicity with TNF-α.[15] Briefly, 10000 cells per well were plated into 96-well culture plates and allowed to adhere overnight in a CO_2 incubator. The next day, the cells were treated with TNF-α (10 ng/mL) and incubated for 1 hr, then freshly prepared crvacrol samples in different concentrations (12.5 µg/mL, 6.25 µg/mL, 3.1 µg/mL, 1.5 µg/mL, and 0.75 µg/mL of DMEM) were added. The plates were kept again in the CO_2 incubator for 24 hr. In separate wells, untreated control cells and TNF-α (10 ng/mL) alone-treated wells were also maintained. At the end of treatment, plates were photographed under a phase contrast microscope to check the morphological changes. MTT assay has been performed as described in the earlier assay.

Lactate Dehydrogenase (LDH) assay

LDH assay was used to detect the level of TNF-α-induced cytotoxicity and the protective effect of carvacrol. Initially, cells were cultured and grown in culture plates, as described earlier. The cells were treated with TNF-α (10 ng/mL) for 1 hr to induce toxicity, followed by the addition of 6.25 µg/mL carvacrol. Different flasks without treatment and only TNF-α-treated cells were kept as controls. The cells were allowed to grow for 24 hr, and the supernatant from each flask was collected and the LDH was measured using a commercial kit. Briefly, A 50 µL supernatant sample was mixed with 1 mL working reagent from the kit, and the decrease in Optical Density (OD) was recorded at 340 nm after 1 min of incubation using a spectrophotometer. LDH activity was calculated using the formula:

$$\text{LDH activity (U/mL)} = ((\Delta OD/\text{min}) \times 3333)$$

Catalase and SOD enzyme activity assay

To evaluate the antioxidant potential of carvacrol against TNF-α-induced toxicity, we investigated its effects on catalase enzyme activity. Cells were exposed to TNF-α (10 ng/mL) for 1 hr to induce toxicity, followed by treatment with carvacrol at a concentration of 6.25 µg/mL. Control groups included untreated cells and cells treated solely with TNF-α. After 24 hr of incubation, the cells were harvested by trypsinization and collected in an Eppendorf tube. The samples were centrifuged at 5,000 rpm for 15 min, and the supernatant was removed. The resulting cell pellets were resuspended in lysis buffer (0.1 M Tris, 0.2 M EDTA, 2 M NaCl, 0.5% Triton) and incubated at 4°C for 20 min. The cell lysate was then used to assess the activities of catalase and Superoxide Dismutase (SOD) enzymes.

For catalase enzyme measurement, 0.5 mL of lysate was mixed with 1.2 mL of 0.01 M phosphate buffer at pH 7, and the reaction was initiated by adding 1 mL of 0.2 mM hydrogen peroxide. The decreases in absorbance of the reaction mixture were recorded at 240 nm for 3 min at 30-sec intervals. An enzyme blank was run concurrently, substituting hydrogen peroxide with 1 mL of distilled water. Catalase activity was calculated using the formula:

$$\text{Units/mL}=((\Delta A/\text{min\_blank} - \Delta A/\text{min\_sample})\times d\times 1)/(V\times 0.043)$$

where d is the dilution factor and V is the volume of the sample. The activity was expressed as moles of hydrogen peroxide decomposed per min per mg of protein.

For the Superoxide Dismutase (SOD) enzyme activity assay, 50 µL aliquot of cell lysate was added to a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 45 µM methionine, 5.3 mM riboflavin, 84 µM potassium ferricyanide, and 0.1 M Nitroblue Tetrazolium (NBT). The mixture was incubated at 25°C for 10 min, and the absorbance was measured at 600 nm using a spectrophotometer. SOD activity was determined by the inhibition of NBT reduction, with higher SOD activity correlating with reduced absorbance. The calculation used the formula:

$$\text{Percentage of Inhibition} = ((\text{Control-Test})/\text{Control}) \text{ X } 100;$$
$$\text{Enzyme Units} = \text{Percentage Inhibition}/ 50.$$

ATPase assay

Catalase enzyme activity has been analyzed to determine the antioxidant potential of carvacrol in protecting against TNF-α-induced toxicity. The cells were treated with TNF-α (10 ng/mL) for 1 hr to induce toxicity, followed by the addition of 6.25 µg/mL carvacrol. Different flasks without treatment and only TNF-α-treated cells were kept as controls. The cells were allowed to grow for 24 hr, and then they were trypsinized and collected into an Eppendorf tube. It was centrifuged at 5000 rpm for 15 min, and the supernatant was discarded. The cell pellets were added with lysis buffer (0.1M Tris, 0.2M EDTA, 2M NaCl, 0.5% Triton) and incubated for 20 min at 4°C. The cell lysate thus obtained was used to measure ATPase activity".

"To measure Na^+/K^+-ATPase activity, we adapted a protocol based on established enzymatic assay methods. The reaction mixture contained 1 mL of 90 mM Tris-HCl buffer (pH 7.5), 0.2 mL each of 50 mM magnesium sulfate, 50 mM potassium chloride, 600 mM sodium chloride, 1 mM EDTA, 40 mM ATP, and cell lysate. This mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 1 mL of 10% Trichloroacetic Acid (TCA), followed by thorough mixing and centrifugation. The inorganic phosphorus released was then quantified. For calcium ATPase activity, we modified an established enzymatic assay protocol. The reaction mixture consisted of 0.1 mL each of 125 mM Tris-HCl buffer (pH 7.5, assumed based on standard protocols), 50 mM calcium chloride, 10 mM ATP, and cell lysate. After incubation at 37°C for 15 min, the reaction was terminated with 1 mL of 10% TCA, followed by centrifugation, and the liberated inorganic phosphorus was measured. Similarly, Mg^{2+}-ATPase activity was assessed using a modified protocol. The reaction mixture included 0.1 mL each of 375 mM Tris-HCl buffer (pH 7.5), 25 mM magnesium chloride, 10 mM ATP, and cell lysate. Following a 15-min incubation at 37°C, the reaction was halted with 1 mL of 10% TCA, centrifuged, and the released inorganic phosphorus was quantified".

Inorganic phosphorus was quantified using the Erba Phosphorus Kit (120226). Duplicate test tubes were labeled as blank, standard, control, TNFα, and TNFα + Carvacrol. Blank tubes received 1000 µL reagent and 20 µL distilled water; standard tubes got 1000 µL reagent and 20 µL standard; sample tubes had 1000 µL reagent and 20 µL sample. Tubes were mixed and incubated at 37°C for 5 min, and absorbance was measured at 340 nm against the blank. Calculations have been made by the formula:

$$\text{Phosphorus (mg/dL)}=(\text{Absorbance of test/Absorbance of standard}) \times \text{Concentration of standard (5 mg/dL)}$$

Mitochondrial membrane potential analysis

Catalase enzyme activity has been analyzed to determine the mitochondrial protective function of carvacrol against TNF-α-induced toxicity. The cells were treated with TNF-α (10 ng/mL) for 1 hr to induce toxicity, followed by adding 6.25 µg/mL carvacrol. Different flasks without treatment and only TNF-α-treated cells were kept as controls. At the end of the experiment, the cells were trypsinized and collected for flow cytometry. Briefly, followed by centrifugation, the cells were added with 1X assay buffer and 95 µL of mitopotentail working solution (Muse™ MitoPotential Dye 1:1000 in 1X assay buffer). The cells were mixed gently using pipetting and kept in the incubator for 20 min for staining; subsequently, 5 µL of Muse MitoPotential 7-AAD was added, mixed by pipetting or vortexing for 3-5 sec, and incubated for five additional min, after which samples were loaded onto a flow

cytometer (Millipore, USA) for event acquisition following gating and comparison with controls.

Gene expression study by Real-time PCR

To determine the gene expressed while carvacrol acts to protect against TNF-α-induced toxicity, real-time PCR was carried out. The cells were treated with TNF-α (10 ng/mL) for 1 hr to induce toxicity, followed by the addition of 6.25 µg/mL carvacrol. Different flasks without treatment and only TNF-α-treated cells were kept as controls. The cells were allowed to grow for 24 hr. Following a 24-hr incubation period, the cells were harvested from the culture flask for RNA extraction. RNA isolation was performed using an RNA isolation reagent (Invitrogen, catalog number 10296010). In brief, 1 mL of TRIzol reagent was added to the flask and incubated for 5 min. The cell lysate was then transferred to a sterile Eppendorf tube, mixed with 200 µL of chloroform, and maintained at room temperature. The mixture was centrifuged at 14,000 rpm for 15 min at 4°C. The upper aqueous phase was carefully transferred to a new tube, and 500 µL of 100% isopropanol was added. After incubation at room temperature, the sample was centrifuged again at 14,000 rpm for 15 min at 4°C. The supernatant was removed, and the resulting RNA pellet was washed with 200 µL of ethanol. A final centrifugation at 14,000 rpm for 15 min at 4°C was performed using a refrigerated centrifuge. The RNA pellet was air-dried and resuspended in TE buffer for subsequent cDNA synthesis.

Prior to the cDNA synthesis, the purity and integrity of the RNA that had been obtained were analyzed. Template cDNA was prepared using a cDNA kit iscript cDNA Synthesis kit (Biorad, Cat# 1708891 master premix for first-strand cDNA synthesis). In a nuclease-free tube, 4 µL of 5x iScript reaction buffer, 1 µL of iScript Reverse Transcriptase, and 0.5 µg of total RNA template were combined. Sterile distilled water was added to achieve a final reaction volume of 20 µL. The mixture was gently mixed by pipetting. cDNA synthesis was performed using an Eppendorf Master Cycler with the following protocol: an initial priming step at 25°C for 5 min, cDNA synthesis at 46°C for 20 min, and reverse transcriptase inactivation at 95°C for 1 min. Each step was conducted for a single cycle.

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed to analyze gene expression using SYBR Green Master Mix (G BIOSCIENCES, Product code-786-5062) on a Lightcycler 96 system (Roche). Reactions were conducted in triplicate, and data were evaluated using the ΔΔCt method via Light Cycler 96 SW 1.1 Software. The protocol included an initial activation at 95°C for 2 min, followed by 40 cycles of three-step cycling: denaturation at 95°C for 10 sec, annealing at 58°C for 1 min, and extension at 72°C for 1 min per kilobase. PCR cycling concluded with indefinite holding at 4°C. Primer sequences for target genes were as follows: AMPK forward (5'-GCTGAGGAACTGGCGGGCG-3', Tm 65.3°C) and reverse (5'-GGGAATTAGGTCATAGCAGC-3', Tm 57.3°C); SIRT1 forward (5'-CGGCTACCGAGGTCCATATAC-3', Tm 61.8°C) and reverse (5'-CAGCTCAGGTGGAGGAATTGT-3', Tm 59.8°C); GAPDH forward (5'-AATGCATCCTGCACCACCAACTGC-3', Tm 64.4°C) and reverse (5'-GGAGGCCATGTAGGCCATGAGGTC-3', Tm 67.8°C). The finished PCR product was also run in an electrophoresis tank, and the gel was visualized.

Statistical analysis

All the experiments were performed in triplicate. The statistical analysis was run using GraphPad Prism software, version 6.0. Significance was measured using one-way ANOVA and Duncan's multiple-range test. The statistical significance was set at $*p<0.05$ compared to the control group.

RESULTS

Carvacrol-induced cytoprotection in L6 cells

To know the range of cytotoxicity produced by Carvacrol, we first used the MTT assay to measure the effect of Carvacrol on L6 (rat myoblast) cells. We photographed the cells to observe whether the cell morphology had changed. As shown in Figure 1, the highest concentration used in this study significantly lowers the cell number with visible morphological changes in the cells. The assay has determined that the IC_{50} produced by Carvacrol in the myoblast cells is 60.87 µg/mL. Following the cytotoxicity assay, we have tested the effect of a sub-cytotoxic dose of carvacrol in protecting against the toxicity produced by TNF-α. Cells were pre-treated with 10 ng/mL of TNF-α, and the cells were treated with Carvacrol from 0.75 -12.5 µg/mL (Figure 2). After 24 hr, it was observed that carvacrol at the selected doses was able to protect against the toxicity of TNF-α till 6.25 µg/mL. Photographs taken during the study revealed that TNF-α induced cell toxicity in the cells with rounding and blebbing, which was well protected in the carvacrol treatment.

Carvacrol reduced the LDH leakage in L6 cells induced by TNF-α

We have conducted an LDH assay to obtain comparative data on cellular toxicity. LDH is an enzyme found in the cytoplasm of nearly all living cells, and its release into the culture medium indicates cell membrane damage or cell lysis, which are hallmarks of cytotoxicity or cell death. As shown in Figure 3A, the control group showed the very minimum amount of LDH leakage. Meanwhile, the TNF-α alone group showed a significant increase in LDH release, with an average LDH activity of approximately 350 units. Cells treated with a combination of TNF-α and Carvacrol displayed a reduced LDH release compared to the TNF-α alone group, with an average LDH activity of approximately 100±11.25 units. This value is significantly lower than that of the TNF-α group, but still higher than that of the control group. This indicates

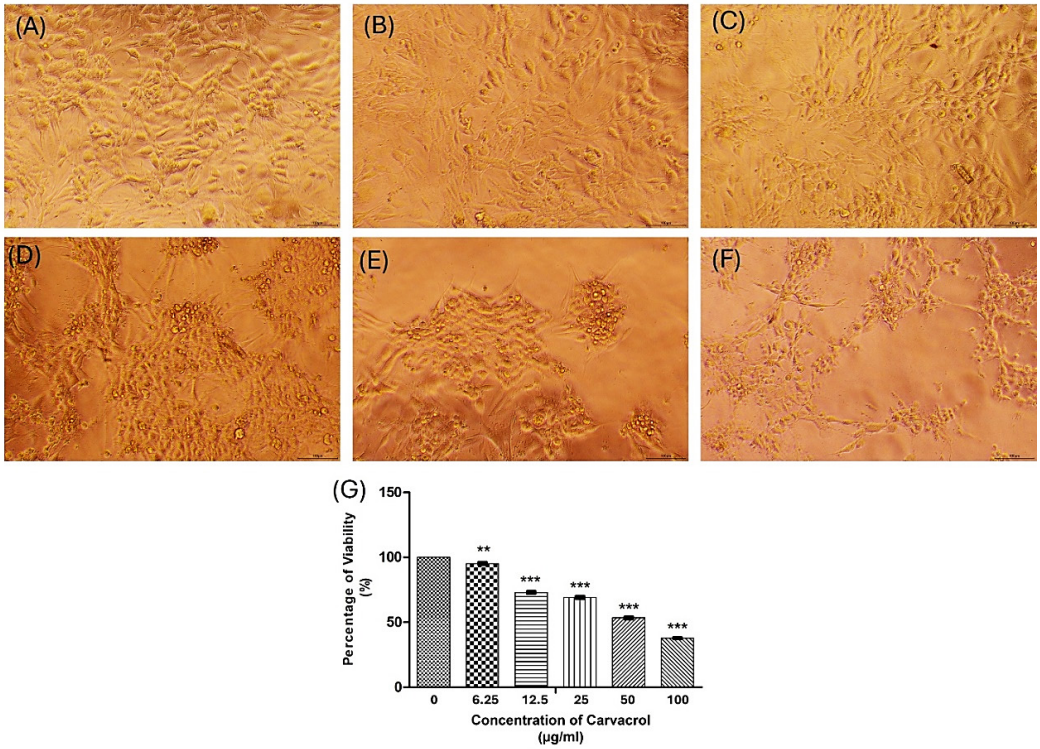

Figure 1: L6 (Rat Myoblast) cells were treated with Carvacrol at different concentrations and the cytotoxicity was examined using an MTT assay. Cell morphology changes were photographed at the end of treatment, Control (A) Carvacrol treatment 6.25-100 µg/mL (B-F), respectively. The effect of Carvacrol in the cells was measured in triplicates and quantified and represented in Figure (G). Results were presented as Mean±SE. *** p<0.001 compared to the control group, ** p<0.05 compared to control group.

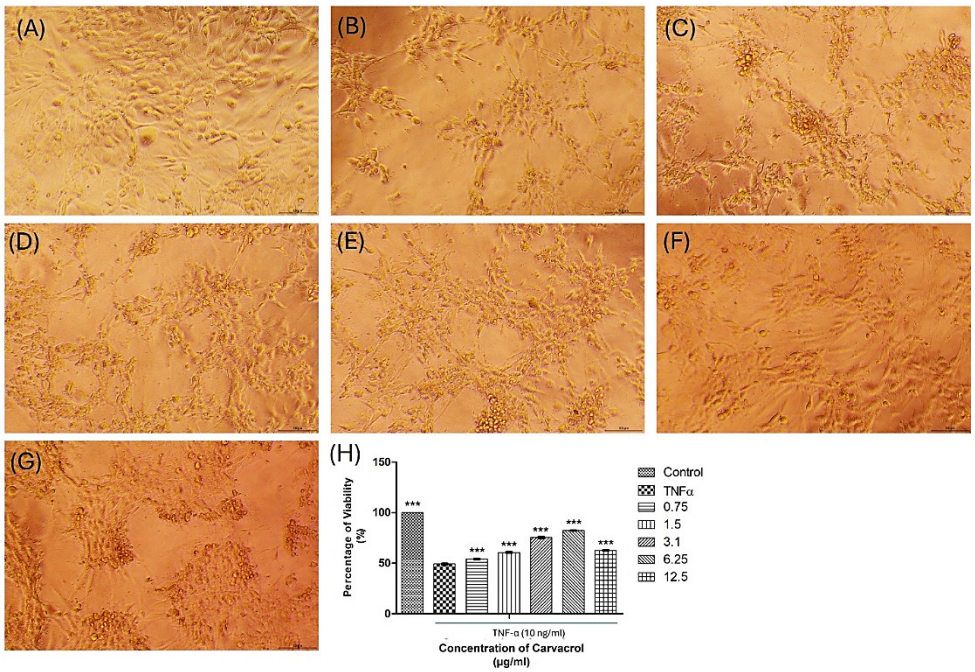

Figure 2: L6 (Rat Myoblast) cells were pretreated with TNF-α at 10 ng/mL, and treated with carvacrol at different concentrations. Figure (A) represents the control cells, and Figure (B) represents the TNF-α 10 ng/mL alone treatment. (C-G) represents conditioning with TNF-α and treatment with carvacrol 0.75-12.5 µg/mL, respectively. The protective effect of Carvacrol in the cells was measured in triplicate, quantified, and represented in Figure (H). Results were presented as Mean±SE. ***p<0.001 compared to the control group, **p<0.05 compared to control group.

that Carvacrol partially mitigated the cytotoxic effects of TNF-α, reducing cell membrane damage and cell death.

Carvacrol restored the reduced antioxidant enzymes

To know the effect of carvacrol in protecting the TNF-α induced toxicity via anti-oxidant mechanism, we have investigated the catalase and SOD enzyme activities. As shown in Figures 3B and C, both enzymes demonstrated distinct changes in protein expression across the treatment groups. In the catalase assay (Figure 3B), the control group exhibited the highest catalase protein expression at approximately 0.8 units/mg. The TNF-α-treated group showed a significant reduction in catalase expression, which was partially restored by carvacrol. Even though it remained below the control level, the difference between the TNF-α and TNF-α + Carvacrol groups was statistically significant (*p<0.05). Similarly, in the SOD assay (Figure 3C), the control group displayed the highest SOD protein expression at approximately 0.20 enzyme protein units/mg. The TNF-α treated group exhibited a decrease in SOD expression, which was also restored by carvacrol significantly.

Carvacrol regulated the ATPase activities

As shown in Figure 3, carvacrol restored Na_ K, Ca^{2+}ATPase, and Mg^{2+}, which were reduced by TNF-α pretreatment. TNF-α pretreatment reduced Na^+/K^+ ATPase activity compared to the control (from 3.5±0.02 mg/dL to 2±0.001 mg/dL) (Figure 3D). In the case of Ca^{2+} ATPase, it was reduced from 3.0±0.02 to 1.5±0.001 mg/dL (Figure 3E). In the case of Mg^{2+} ATPase, I reduced from 4±0.02 to 2±0.001 mg/dL (Figure 3F). Carvacrol restored the reduced enzyme level to 3±0.01, 2.5±0.01, and 3.5±0.02 for Na^+/K^+, Ca^{2+}, and Mg^{2+}, respectively.

Carvacrol protected the L6 cells from toxicity via the prevention of Mitochondrial potential loss

MMP assay was carried out to determine the effect of carvacrol in protecting the MMP from initiation by TNF-α. As shown in Figure 4A, the cell population profile showed 96.15% of cells as live, with only 0.00% depolarized/dead, which corresponds to a high MMP. Upon treatment with TNF-α, a marked decline in depolarized cells was observed in Figure 4B. Pretreatment with carvacrol in the TNF-α + carvacrol group restored or protected the cells from MMP loss (Figure 4C). The data have been quantified and shown in Figure 4D. As per the Figure, the depolarized cell pollution was 31.65±4.25%, which was significantly reduced to 19.0±2.6% (p<0.05). Effects of Carvacrol on TNF-α-Induced changes in SIRT1, and AMPK gene expression in L6 Cells

The gene expression analysis of SIRT1 and AMPK in L6 cells has been conducted, and the results are shown in Figure 5. SIRT1 expression was normalized to a baseline of 1-fold. Treatment with TNF-α significantly reduced SIRT1 expression to approximately -0.2-fold, indicating a strong downregulation; meanwhile, Carvacrol showed an upregulation of 1.6-fold. In the case of AMPK (Figure 5B), from the one-fold control baseline, TNF-α treatment led to a substantial decrease in AMPK expression, reaching -1.2 fold. Upon Carvacrol pre-treatment, the APMPK gene expression increased by 2-fold. Discussion

This study demonstrates that carvacrol exerts a significant protective effect against TNF-α-induced toxicity in L6 rat myoblast cells. The obtained results suggest that carvacrol can be considered as a compound for further drug discovery against sarcopenia, which is a condition of progressive muscle loss driven by inflammation, oxidative stress, and mitochondrial dysfunction. Our results show that carvacrol mitigates cytotoxicity, reduces

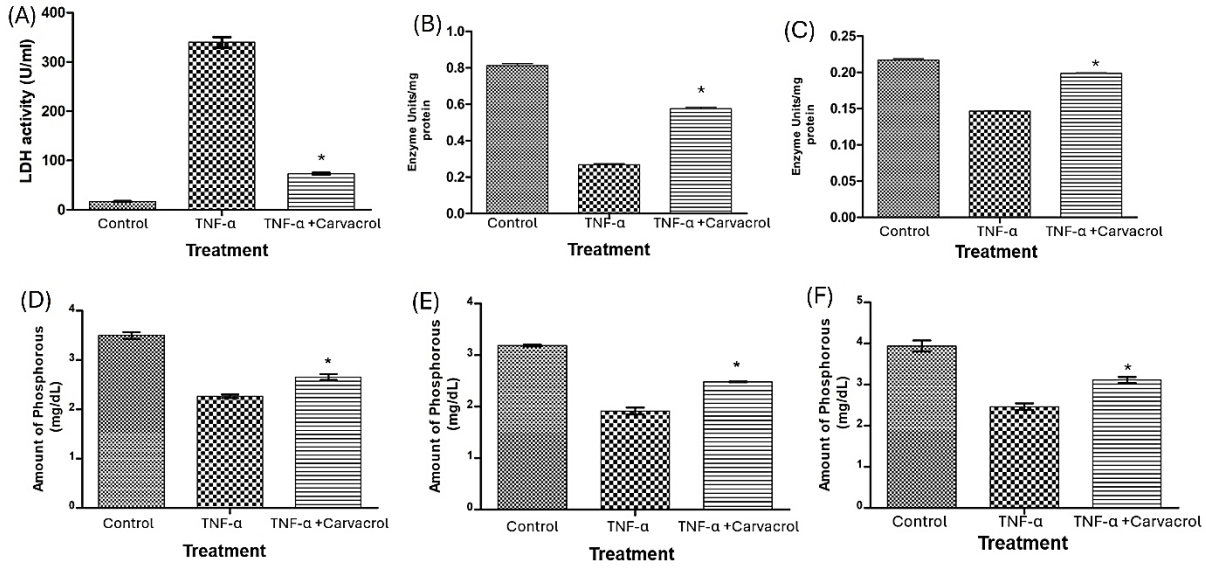

Figure 3: Effect of Carvacrol in inducing protective effect in L6 cells measured by LDH assay (A); catalase enzyme assay (B); SOD assay (C); Na^+, K^+ ATPase (D); Ca^{2+}ATPase (E) and Mg^{2+}ATPase (F). Results were presented as Mean±SE. *p<0.05 compared to the TNF-α group.

Lactate Dehydrogenase (LDH) leakage, restores antioxidant enzyme activities (catalase and SOD), preserves ATPase functions (Na^+/K^+, Ca^{2+}, and Mg^{2+}), maintains Mitochondrial Membrane Potential (MMP), and upregulates SIRT1 and AMPK gene expression. These findings collectively suggest that carvacrol targets key pathological mechanisms of sarcopenia, offering a promising approach to counteract muscle cell damage.

The cytotoxicity assay (MTT) revealed that carvacrol has the ability to protect the L6 cells from the toxicity of TNF-α. This indicates its capacity to preserve cellular morphology and viability. The cytotoxicity assay revealed a significant reduction of rounded and blebbing cells at an IC_{50} of 60 µg/mL. This suggests that the action of carvacrol is within the safe dose. The results we have received are consistent with the other results obtained in prior studies. Additionally, we have successfully demonstrated that carvacrol protection can be obtained at sub-cytotoxic doses. This cytoprotection is closely linked to reduced LDH leakage with carvacrol treatment, indicating stabilized cell membrane integrity. Carvacrol's ability to stabilize membranes may assist in preventing muscle cell loss since sarcopenia is characterized by TNF-α-driven membrane damage and cell death.[16]

The restoration of antioxidant enzyme activities such as catalase and SOD and ATPase functions (Na^+/K^+, Ca^{2+}, and Mg^{2+}) by carvacrol further highlights its role in counteracting oxidative stress and metabolic dysfunction, both central to sarcopenia.[17,18] SOD is an enzyme that protects the cell from damage caused by endogenous or exogenous free radicals. SOD can regulate oxidative stress, inflammation and lipid metabolism.[19,20] Thus, it helps prevent lipid peroxidation and inflammation.[21] Catalase is an antioxidant enzyme almost present in all living organisms involved with the removal of toxic substabce from the body.[22] TNF-α caused a significant drop in catalase and SOD expression, which indicates how oxidative stress impairs muscle function.[23] Carvacrol has been shown to partially restore these enzymes but not to control levels. This suggests that carvacrol exhibits a higher antioxidant capacity, a finding also observed in other studies involving carvacrol.[24,25]

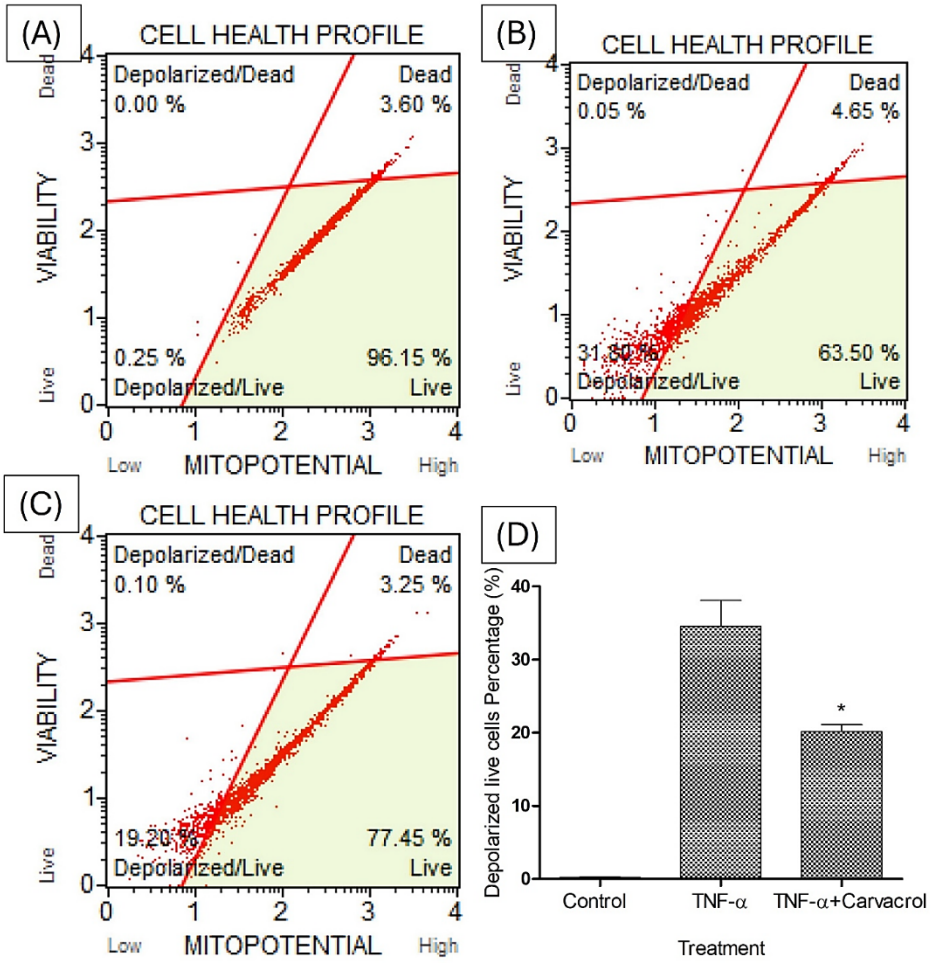

Figure 4: The effects of carvacrol on the MMP of L6 cells treated for 24 hr. (A) represents the control group; (B) represents the TNF-α treated group, and (C) represents the group that was pretreated with TNF-α and carvacrol. The results were quantified in (D). All values represent the average of triplicated values±SE. *p<0.05 compared to the TNF-α group.

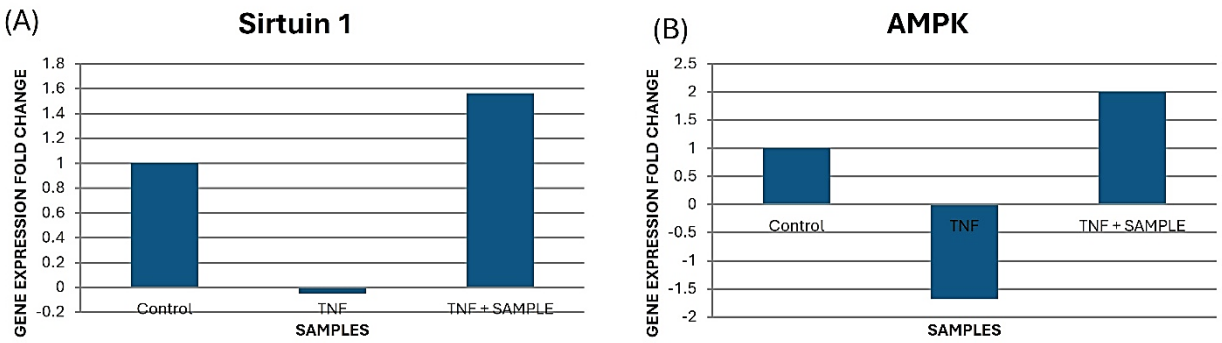

Figure 5: Graphical representation depicting the expression pattern of (A) AMPK and (B) SIRT1.

The increase in ATPase activity further demonstrates that carvacrol helps maintain ion balance and regulate energy metabolism, both of which are crucial for muscle contraction and mitochondrial function.[26] Cellular levels of Na^+ and K^+ are essential for the force generation in skeletal muscle. ATPase corresponding to this is an essential enzyme for the balance of such ions.[27] As we get older, the regulation of such ions and the maintenance of the membrane potential become weaker. As a consequence, sustained repeated muscle contractions will become challenging to perform. Ca^{2+} is essential for muscle contraction and proper energy release. The impaired regulation of intracellular Ca^{2+} levels due to the Ca^{2+} disruption and reduced mitochondrial Ca^{2+} uptake as we age seriously disrupts repeated muscle contraction and leads to disability.[28] Similarly, the lower abundance of Mg^{2+} in skeletal muscle decreases muscle strength, leading to sarcopenia.[29] In the current research. The results we got during the antioxidant enzyme assay and the ATPase assay results are linked because oxidative stress inhibits the generation of ATP, which in turn leads to a slowdown in ATPase activity and worsens muscle function.

Mitochondrial dysfunction is a key mechanism in sarcopenia.[30] In our research, carvacrol was able to preserve the MMP by reducing the number of depolarized cells. Mitochondrial dysfunction through TNF-α is a key mechanism of sarcopenia, in which TNF-α produces increased Reactive Oxygen Species (ROS) and eventually leads to apoptosis and energy deficiency.[31] Carvacrol likely reduces ROS generation and mitochondrial permeability transition by stabilizing the Mitochondrial Membrane Potential (MMP). These results align with other published studies in which carvacrol has been shown to exert a protective function against oxidative stress.[32] This protection of mitochondria is directly linked to the rise in gene expression of SIRT1 and AMPK. SIRT1 is a metabolic regulator involved in various cellular processes such as DNA repair, cell regulation, and cellular metabolism. It has been shown to attenuate the stress to protect the cells.[33] AMPK is an energy sensor protein kinase; its upregulation is beneficial in preventing skeletal muscle atrophy. Many natural compounds, such as ampelopsin and quercetin, has been earlier exhibited attenuation of muscle atrophy by the regulation of AMPK.[34,35] Studies have shown that regulating AMPK could be beneficial in oxidative stress and mitochondrial dysfunction prevention in skeletal muscle to prevent sarcopenia.[35] Earlier, we have seen that TNF-α reduced the level of SIRT1 and AMPK, which may contribute to muscle atrophy. Carvacrol's ability to restore both gene expressions suggests that the mechanism by which it exerts protection in L6 cells is linked to mitochondrial function, antioxidant defense, and metabolic regulation.

While our *in vitro* model provides valuable insights into the cellular mechanisms underlying sarcopenia, it has inherent limitations that warrant consideration. Notably, *in vitro* systems cannot fully replicate systemic factors, such as immune responses, hormonal influences, or inter-tissue interactions, which play critical roles in sarcopenia's progression *in vivo*. These factors may modulate the disease's pathophysiology in ways not conducted by our model. Future studies incorporating *in vivo* models or advanced co-culture systems could help address these limitations and provide a more comprehensive understanding of sarcopenia's systemic context.

CONCLUSION

In conclusion, preliminary evidence suggests that carvacrol protects L6 rat myoblast cells from TNF-α-induced toxicity by enhancing cell viability, membrane stability, antioxidant restoration and ATPase activities, preserving MMP, and upregulating SIRT1 and AMPK expression. These interconnected effects highlight its potential as a therapeutic agent for sarcopenia, warranting further investigation to translate these findings into clinical applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TNF-α: Tumor Necrosis Factor-alpha; **LDH:** Lactate Dehydrogenase; **SOD:** Superoxide Dismutase; **ATPase:** Adenosine Triphosphatase; **Na^+/K^+:** Sodium/Potassium; **Ca^{2+}:** Calcium; **Mg^{2+}:** Magnesium; MMP: Mitochondrial Membrane Potential; SIRT1: Sirtuin 1; **AMPK - AMP:** activated Protein Kinase; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **IC_{50}:** Half Maximal Inhibitory Concentration; **RPMI:** Roswell Park Memorial Institute (cell culture medium); **FBS:** Fetal Bovine Serum; **CO_2:** Carbon Dioxide; **DMSO:** Dimethyl Sulfoxide; **PBS:** Phosphate-Buffered Saline; **OD:** Optical Density; **qRT-PCR:** Quantitative Reverse Transcription Polymerase Chain Reaction; **ΔΔCt:** Delta-Delta Cycle Threshold (method for gene expression analysis); **Tris:** Tris(Hydroxymethyl)Aminomethane; **EDTA:** Ethylenediaminetetraacetic Acid; **NBT:** Nitroblue Tetrazolium; **TCA:** Trichloroacetic Acid; **7-AAD:** 7-Aminoactinomycin D; **ANOVA:** Analysis Of Variance.

AUTHOR CONTRIBUTIONS

Conception and design: AMA and RIN; Acquisition of data: AMA and RIN; Writing of the first draft: AMA and RIN; Critical revision of the draft: AMA and RIN.

SUMMARY

Sarcopenia, characterized by muscle loss, inflammation, oxidative stress, and mitochondrial dysfunction, lacks effective treatments. This study evaluates carvacrol, a natural compound with antioxidant and anti-inflammatory properties, for its potential in attenuating TNF-α-induced sarcopenia in L6 rat myoblast cells. Carvacrol, at 6.25 µg/mL, reduced TNF-α-induced cytotoxicity, LDH leakage, and mitochondrial depolarization while restoring antioxidant enzyme (catalase, SOD) and ATPase (Na^+/K^+, Ca^{2+}, Mg^{2+}) activities. It also upregulated SIRT1 and AMPK gene expression. These findings suggest carvacrol's therapeutic potential for sarcopenia by mitigating muscle toxicity, oxidative stress, and mitochondrial dysfunction, warranting further preclinical and clinical studies.

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