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# Synthesis of Dihydromyricetin-Nickel Complex and Preliminary Investigation of Its Cytotoxicity: Postprint

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## Abstract

This study investigated the feed safety of dihydromyricetin-nickel complex (DMY-Ni) as a feed additive based on its synthesis. DMY-Ni was synthesized from dihydromyricetin (DMY) and nickel acetate via a heating reflux method and characterized by UV and infrared spectroscopy. The MTT assay was employed to investigate the effects of DMY and DMY-Ni on the proliferation of mouse normal hepatocyte AML12 cells at different concentrations (10, 20, 40, 80, and 160 g/mL) and treatment durations (12, 24, 36, and 48 h). The results showed that DMY-Ni could be formed through the complexation of DMY with nickel ions. After treatment of AML12 cells with 10, 20, and 40 g/mL DMY or DMY-Ni for 48 h, cell viability showed no significant difference compared with the blank control group ($P>0.05$). After treatment with 80 g/mL and higher concentrations of DMY-Ni for 48 h, AML12 cell viability was significantly lower than that of the blank control group ($P<0.01$ or $P<0.001$), and after treatment with 160 g/mL DMY for 48 h, AML12 cell viability was significantly lower than that of the blank control group ($P<0.01$). The viability of AML12 cells gradually decreased with increasing concentrations of DMY or DMY-Ni and prolonged treatment time. The half-maximal inhibitory concentrations (IC50) of DMY and DMY-Ni against AML12 cells were 285.1 and 222.84 g/mL, respectively. It was concluded that both DMY and DMY-Ni exhibited relatively low toxicity to AML12 cells, and the toxicity of DMY-Ni to AML12 cells was slightly higher than that of DMY.

## Full Text

# Synthesis of Dihydromyricetin-Nickel Complex and Preliminary Study on Its Cytotoxicity

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#### Abstract

This experiment synthesized dihydromyricetin-nickel complex (DMY-Ni) and investigated its feeding safety as a feed additive. DMY-Ni was prepared by heating reflux method using dihydromyricetin (DMY) and nickel acetate as raw materials, and characterized by ultraviolet-visible (UV-Vis) and infrared (IR) spectroscopy. The effects of DMY and DMY-Ni on the proliferation of mouse normal liver parenchymal cells AML12 were studied using the MTT assay at different concentrations (10, 20, 40, 80, and 160 μg/mL) and treatment durations (12, 24, 36, and 48 h). The results confirmed successful synthesis of DMY-Ni. After 48 h treatment, AML12 cell survival rates exposed to 10, 20, and 40 μg/mL of either DMY or DMY-Ni showed no significant difference compared with the blank control group ($P>0.05$). However, treatment with 80 μg/mL and above of DMY-Ni for 48 h significantly reduced AML12 cell survival ($P<0.01$ or $P<0.001$), while 160 μg/mL DMY also significantly decreased survival ($P<0.01$). Cell survival gradually decreased with increasing concentration and prolonged treatment time for both compounds. The half-maximal inhibitory concentrations (IC ) of DMY and DMY-Ni on AML12 cells were 285.1 μg/mL and 222.84 μg/mL, respectively. These findings indicate that both DMY and DMY-Ni exhibit relatively low toxicity to AML12 cells, with DMY-Ni showing slightly increased cytotoxicity compared to DMY alone.

**Keywords:** feed additives; dihydromyricetin-nickel complex; synthesis; cytotoxicity

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## Introduction

With the progress of human society, the aquaculture industry has rapidly developed toward intensification and large-scale operations. To achieve high productivity, prevent livestock and poultry diseases, and improve feed conversion efficiency, feed additives have emerged as essential components in modern animal production. These additives can enhance animal performance, strengthen organism resistance, and ultimately increase economic benefits for producers.

However, safety and toxicological evaluation of feed additives is particularly crucial as it directly affects human health.

Dihydromyricetin (DMY) is a flavonol compound with the chemical name 3,5,7,3' ,4' ,5' -hexahydroxyflavanone. Its molecular structure is shown in [Figure 1: see original paper]. The molecule exhibits high delocalization and forms a large -bond conjugated system, conferring strong chelating capacity. Due to its diverse physiological activities including antimicrobial, antitumor, hepatoprotective, and antioxidant effects, DMY has been widely applied as a novel feed additive in recent years. Nickel (Ni) is an essential trace element for organisms, primarily functioning to activate various enzymes and possessing unique coordination and catalytic electron transfer properties. Nickel deficiency can lead to growth retardation and reduced reproductive capacity, while excess nickel may cause degenerative changes in the heart, brain, lungs, liver, and kidneys.

Research has demonstrated that DMY metal complexes exhibit significantly enhanced biological activity compared to DMY alone, while simultaneously supplementing essential trace elements required for livestock growth. Consequently, dihydromyricetin-nickel complex (DMY-Ni) has become a research focus. However, most current studies on DMY-Ni have focused on preparation processes, antioxidant properties, and antimicrobial activity, with limited data on stability and safety. Since the late 1980s, cell toxicity detection methods have been established to simulate biological environments in vitro and assess drug-induced growth inhibition and toxic effects, with the MTT assay being the most commonly used due to its simplicity and intuitive results. This study synthesized DMY-Ni from DMY and nickel acetate, and investigated the effects of DMY and DMY-Ni on cell proliferation using mouse liver parenchymal cells, aiming to provide guidance for the safe and rational application of DMY-Ni as a feed additive.

[Figure 1: see original paper]

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## Materials and Methods

### 1.1 Materials and Reagents

DMY (purity 98%) was purchased from Xi' an Siji Biological Technology Co., Ltd. Anhydrous ethanol, nickel acetate, and anhydrous sodium acetate were obtained from Sinopharm Chemical Reagent Co., Ltd. (all analytical grade). Distilled water was from Guangzhou Watsons. Mouse liver parenchymal cells AML12 were from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. DMEM (high glucose) medium and fetal bovine serum were from Gibco (USA). Penicillin-streptomycin antibiotic solution was from Fisher Scientific (USA). Trypsin was from BioBasieUnit (USA). MTT was from Sigma (USA). Dimethyl sulfoxide was from Shanghai Sangon Biotech Co., Ltd.

### 1.2 Instruments

Main instruments included: DZF-6050 vacuum drying oven (Shanghai Yiheng Scientific Instruments), UV-2450 UV-Vis spectrophotometer (Shimadzu, Japan), NICOLET-380 Fourier transform infrared spectrometer (Bruker, Germany), CO-150 INNOVA CO incubator (NBS, USA), SpectraMax Paradigm microplate reader (Molecular Devices, Australia), and high-speed centrifuge (Shanghai Anting Scientific Instrument Factory).

### 1.3 Synthesis of DMY-Ni

Accurately weighed 0.640 g (2 mmol) of DMY was placed in a 250 mL three-neck round-bottom flask with a magnetic stirrer, and 100 mL of anhydrous ethanol was added. After complete dissolution with heating and stirring, a certain amount of anhydrous sodium acetate was added to adjust pH to approximately 7.5. After continued stirring for 0.5 h, 0.498 g (2 mmol) of nickel acetate (same molar amount) was added. The mixture was stirred and refluxed at 70 °C for 6 h. After cooling to room temperature, the product was filtered under reduced pressure, washed alternately with distilled water and anhydrous ethanol three times, and vacuum-dried at 40 °C for 10 h to obtain a yellow-brown solid powder.

### 1.4 Characterization of DMY-Ni

**UV-Vis Spectroscopy:** DMY and the synthesized complex were first dissolved in a small amount of dimethyl sulfoxide, then diluted with water to the required concentration. Using water as reference, UV-Vis absorption spectra were scanned in the range of 200-600 nm.

**IR Spectroscopy:** DMY and the complex were scanned by IR spectroscopy using the potassium bromide (KBr) pellet method.

### 1.5 Cytotoxicity Assay

Logarithmic growth phase AML12 cells were adjusted to 5×10 cells/mL using DMEM (high glucose) medium and seeded into 96-well plates at 100 µL/well. Phosphate-buffered saline (PBS) was added to the edge wells. Cells were cultured at 37 °C in a 5% CO incubator. After cell adhesion, the supernatant was discarded, and DMY or DMY-Ni were prepared at designated concentration gradients using complete medium. Each well received 100 µL of medium containing DMY or DMY-Ni, followed by incubation for 12, 24, 36, or 48 h. Final concentrations of DMY or DMY-Ni were 10, 20, 40, 80, and 160 µg/mL, with six replicate wells per concentration. Blank control and zero adjustment groups were also established.

After the designated treatment duration, the supernatant was discarded, and 100 µL of 0.5 mg/mL MTT solution was added to each well. After 4 h incubation, the supernatant was removed, 150 µL of dimethyl sulfoxide was added, and the plates were left for 10 min. Absorbance (OD) values at 490 nm were measured

using a microplate reader. The experiment was repeated three times, and cell proliferation rate was calculated using the formula:

Cell proliferation rate (%) = (Average OD of treatment group / Average OD of blank control group) × 100

### 1.6 Statistical Analysis

Data were analyzed using one-way ANOVA in GraphPad Prism 7.0 software. $P<0.05$ was considered statistically significant.

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## Results

### 2.1 Characterization Results

**2.1.1 UV-Vis Spectroscopy** As shown in [Figure 2: see original paper], DMY exhibited a strong absorption peak at 292 nm. After complexation with nickel ions, the absorption peak shifted to 305 nm, demonstrating a significant redshift compared to DMY. This indicates that the DMY structure was altered and a new product was formed.

[Figure 2: see original paper]

**2.1.2 IR Spectroscopy** [Figure 3: see original paper] shows that the absorption at 3407.78 cm $^{1}$ corresponds to hydroxyl group stretching vibration on the benzene ring, indicating that hydroxyl groups remained in the complex. DMY showed a strong absorption peak at 1642.37 cm $^{1}$ from the carbonyl group at position 4. During complex formation, coupling between the carbonyl group at position 4 and the hydroxyl group at position 5 weakened the C=O bond, shifting the absorption peak to 1598.43 cm $^{1}$ (a 43.94 cm $^{1}$ shift to lower wavenumber), confirming carbonyl group participation in the reaction. The benzene ring skeleton absorption peaks at 1400-1500 cm $^{1}$ showed minimal changes, indicating that the benzene ring structure was preserved in both DMY and the complex. The ether bond absorption peaks at 1050-1150 cm $^{1}$ remained essentially unchanged, suggesting that the C-ring ether bond did not undergo ring opening during complex formation. However, in the low-frequency fingerprint region, a new absorption peak appeared at 651.87 cm $^{1}$ in the complex compared to DMY, indicating interaction between DMY and nickel ions with formation of Ni-O bonds in DMY-Ni.

[Figure 3: see original paper]

### 2.2 Effects of DMY and DMY-Ni on AML12 Cell Proliferation

As shown in [Figure 4: see original paper]-A, after 12 and 24 h treatment, AML12 cell survival rates remained essentially unchanged with increasing DMY

concentration. After 36 and 48 h treatment, survival rates decreased with increasing DMY concentration, though the inhibitory effect was not pronounced. At the same concentration, survival rates decreased with prolonged treatment duration.

[Figure 4: see original paper]-B shows that after 12 and 24 h treatment, AML12 cell survival rates remained essentially unchanged with increasing DMY-Ni concentration, consistent with DMY treatment. However, after 36 and 48 h treatment, survival rates remained stable at low DMY-Ni concentrations but decreased significantly at high concentrations, with a more pronounced decline than observed with DMY under the same conditions. At the same concentration, survival rates also showed time-dependent decreases.

[Figure 4: see original paper]

[Figure 5: see original paper] illustrates the effects of DMY and DMY-Ni on AML12 cell proliferation after 48 h treatment. At 160 μg/mL, DMY showed significant inhibitory effects, with cell survival significantly lower than the blank control group (P<0.01). At concentrations below 160 μg/mL, survival rates remained essentially unchanged with no significant difference from the control (P>0.05). DMY-Ni at 80-160 μg/mL exhibited certain toxic effects, with survival rates significantly different from the control (P<0.01 or P<0.001). Below 80 μg/mL, DMY-Ni showed no significant effect on survival (P>0.05). Using GraphPad Prism 7.0, the IC values were calculated as 285.1 μg/mL for DMY and 222.84 μg/mL for DMY-Ni. The lower IC for DMY-Ni indicates slightly increased cytotoxicity compared to DMY.

Compared with the blank control group, significant differences are expressed as: *, P<0.05;* ***, P<0.01;*** , P<0.001.

[Figure 5: see original paper]

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## Discussion

The successful formation of DMY-Ni complex was confirmed by the redshift in UV-Vis absorption spectra and the appearance of new characteristic absorption peaks in the IR fingerprint region corresponding to Ni-O bonds.

Dosage is a fundamental concept in toxicology research. As a potential feed additive, understanding the dose-response relationship of DMY-Ni is crucial. Studies have shown good correlation between in vitro cytotoxicity of chemical substances and both animal mortality rates and human lethal blood concentrations. Since chemical-induced damage and death ultimately manifest at the cellular level, in vitro cytotoxicity studies can predict in vivo acute toxicity.

Previous research has demonstrated the low toxicity of DMY at the cellular level. Zhou et al. reported an IC of 324.8 μg/mL for DMY on L-02 cells after 48 h treatment. Shu found that DMY showed no significant inhibitory effects on two

types of normal human immortalized liver cells. Su et al. administered DMY at 5.0 g/kg BW to Wistar rats via oral gavage (maximum concentration and volume) with no animal deaths or histopathological changes observed, further confirming DMY' s low toxicity. However, no explicit safety dosage for DMY has been established.

The current study demonstrates that DMY-Ni complexation enhances biological activities such as antioxidant and antimicrobial properties. Regarding safety, our MTT assay revealed an IC of 285.1 μg/mL for DMY on AML12 cells. Although different cell lines exhibit varying tolerances to the same drug, our results are consistent with previous reports confirming DMY' s low toxicity. The IC of DMY-Ni on AML12 cells was 222.84 μg/mL, which, while lower than that of DMY, represents only a modest increase in toxicity.

The increased toxicity of DMY-Ni may be attributed to synergistic effects between the metal ion and DMY ligand, which has been reported to enhance antitumor activity in other DMY metal complexes. This synergistic interaction likely contributes to the observed increase in cytotoxicity to normal mouse liver parenchymal AML12 cells compared to DMY alone.

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## Conclusions

1. DMY-Ni complex was successfully synthesized using DMY and nickel acetate as raw materials.
2. Both DMY and DMY-Ni exhibited dose-dependent inhibitory effects on AML12 cells, with toxicity increasing with prolonged treatment duration.
3. DMY showed low toxicity to AML12 cells, while DMY-Ni demonstrated slightly increased cytotoxicity compared to DMY, though still within the low toxicity range.

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