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Green Synthesis of 4-Nitro-4'-Methoxy Chalcone by Grinding Technique and its Antibacterial Activity

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Abstract

This research aims to synthesize 4-nitro-4'-methoxy chalcone and determine the potential of 4-nitro-4'-methoxy chalcone as an antibacterial against *Staphylococcus aureus* and *Escherichia coli* bacteria. The synthesis of 4-nitro-4'-methoxy chalcone was carried out using the grinding technique, an environmentally friendly green synthesis approach—characterization of the synthesized chalcone using FTIR, ^{1}H-NMR, and ^{13}C-NMR. The research demonstrated that 4-nitro-4'-methoxy chalcone could be synthesized via grinding techniques using 4-methoxyacetophenone, 4-nitrobenzaldehyde, and NaOH as catalysts. The synthetic process achieved higher yields and was environmentally friendly due to the absence of organic solvents. The resulting product was yellow crystalline with a yield of 54.42% and a melting point of 172°C–173°C. Antibacterial activity tests revealed that the synthesized chalcone exhibited greater inhibition against *E. coli* than *S. aureus*. This was evidenced by the clear zones formed (9.57 mm for *S. aureus* and 12.46 mm for *E. coli*), indicating that 4-nitro-4'-methoxy chalcone possesses strong antibacterial activity.

1. Introduction

Flavonoids are a group of polyphenols commonly found in plants, classified based on carbon substitution in the central aromatic group (C) into categories such as flavones, flavanols/catechins, anthocyanins, and chalcones [1]. Among these, chalcone is one of the least abundant due to the activity of the CSH enzyme, which converts chalcone into flavanones, making direct isolation from plants challenging [2]. Chalcone, also known as 1,3-diphenyl-1-propen-1-one or benzylidene acetophenone, is a precursor to flavonoids and isoflavonoids [3]. It acts as an open-chain intermediate in aurone synthesis and is typically found in conjugated forms in natural products [4]. Chalcone exhibits diverse pharmacological activities, influenced by substituents attached to its aromatic rings and carbonyl group. These substituents result in structural variations that give rise to various bioactivities, including anti-inflammatory, anticancer, antitumor, antimalarial, antioxidant, and antibacterial properties [5].

Global health challenges, particularly infections caused by antibiotic-resistant bacteria, remain a critical concern worldwide [6]. Chalcone and its derivatives hold significant potential as candidates for developing new antibacterial agents. The chalcone structure contains an α, β-unsaturated ketone group, also known as an ethylene keto group (-CO-CH=CH-), responsible for its antibacterial properties. Studies have shown that chalcones substituted with hydroxy groups on ring A (C2 and C4) and methoxy groups on ring B (C2 and C3) effectively inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* at a concentration of 62.5 ppm, while inhibition of *Bacillus subtilis* and *Escherichia carotovora* occurs at 125 ppm [7]. Furthermore, 2',4'-dihydroxychalcone and 2',4'-dihydroxy-3'-methoxychalcone exhibit strong activity against *S. aureus* [8]. Modifying chalcone molecules offers a promising route to enhance antibacterial properties, as homologous compounds often display equal or superior pharmacological effects. These structural changes can be achieved by varying the substituents in the aromatic systems of acetophenone and benzaldehyde.

Despite its numerous benefits, chalcone is challenging to obtain through direct plant isolation due to its minimal natural abundance compared to other flavonoid compounds. Isolation is hindered by high costs and time-consuming processes. A practical solution to these challenges is synthetic production, which offers several advantages, including the ability to produce chalcone in larger quantities, with higher purity, and in a more time- and cost-efficient manner. Furthermore, synthesis allows for modifications to the molecular structure of chalcone, enabling enhancements or alterations to its pharmacological activity [9].

Chalcone can be synthesized through cross-aldol condensation or the Claisen-Schmidt reaction [10]. The Claisen-Schmidt condensation involves reacting benzaldehyde or its derivatives with acetophenone or its derivatives, utilizing various catalysts such as acids, bases, sodium phosphate, or aluminum-magnesium hydroxide hydrate [11]. Among these, base catalysts are more effective than acid catalysts for synthesizing organic compounds via the Claisen-Schmidt condensation [12].

A shift in organic compound synthesis methods is underway, moving away from conventional approaches that rely on solvents and heat—practices that pose environmental risks—toward greener synthesis techniques. Green synthesis offers a sustainable solution by minimizing hazardous chemicals and reducing waste generation. One such method, the grinding technique, aligns with the principles of green synthesis as it eliminates the need for solvents. In addition to reducing waste, this technique is straightforward, cost-effective, and environmentally friendly, with lower operational expenses [13].

The grinding technique was demonstrated by Susanti V. H. *et al.* [14], who synthesized 2',6'-dihydroxy-3,4-dimethoxy chalcone from a mixture of 2,6-dihydroxy acetophenone and 3,4-dimethoxy benzaldehyde using a NaOH catalyst. This method produced a high yield (70%) after purification through column chromatography and recrystallization. In contrast, conventional synthesis methods require organic solvents and high concentrations of bases, take significantly longer (24 hours), and yield a lower product amount (65%) [14].

Rehana *et al.* [15] synthesized 3,4,4'-Trimethoxychalcone via the Claisen-Schmidt condensation reaction using 4-methoxyacetophenone and 3,4-dimethoxybenzaldehyde as key reactants, with NaOH as the catalyst. The yields varied based on the NaOH concentrations of 2, 4, and 8 mmol, producing 60.06%, 62.60%, and 80.19%, respectively. However, yield declined when NaOH concentration exceeded 8 mmol, with yields decreasing to 78.43%, 77.09%, and 75.50% for 12, 16, and 20 mmol, respectively. Previous studies indicated that amino groups in the chalcone structure enhance biological activity, particularly when positioned meta, as they improve interaction with aromatic rings and facilitate cell penetration. Additionally, chalcones with methoxy groups also demonstrated excellent biological activity [8].

Different reagents and substituted groups in chalcone synthesis studies result in structural variations in the chalcone products. This diversity is crucial for developing new antibacterial agents with enhanced potency and reduced toxicity and understanding the relationship between compound structure and biological activity. To further explore this potential, this research aims to synthesize chalcone from 4-nitrobenzaldehyde and 4-methoxyacetophenone using the grinding technique and evaluate its antibacterial properties.

2. Experimental

2.1. Materials and Equipment

All solvents and chemicals used were of reagent grade, including 4-nitrobenzaldehyde, 4-methoxyacetophenone, ethanol, sodium hydroxide, hydrochloric acid, chloroform, n-hexane, ethyl acetate, DMSO, agar medium, anhydrous sodium sulfate, Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), and bacterial isolates of *Escherichia coli* (*E. coli*) ATCC 25922 and *Staphylococcus aureus* (*S. aureus*) ATCC 25922. The tools and instruments employed in the study included Whatman paper, TLC plates, Pyrex laboratory glassware, a magnetic stirrer, mortar and pestle, desiccator, magnetic stirring plate, UV lamp (254 nm), Fourier Transform infrared spectrometer (FTIR; Shimadzu), and Nuclear magnetic resonance spectrometer (NMR) for ^{1}H (JEOL-MY500) and ^{13}C (125 MHz, JEOL-MY500).

2.2. Procedures

2.2.1. Synthesis of Chalcone

Ten mmol (1.50 g) of 4-methoxyacetophenone, 10 mmol (1.51 g) of 4-nitrobenzaldehyde, and 20 mmol (0.8 g) of NaOH were ground together in a mortar and pestle for 45 minutes at room temperature using unidirectional movements. The resulting mixture was gradually diluted with cold distilled water until it could be easily transferred from the mortar. The solution was then acidified with cold 10% HCl to a pH of 1 to 2. The precipitate formed was collected using Whatman filter paper and placed in a desiccator to dry. Once dried, the precipitate was weighed, and its purity was assessed using Thin Layer Chromatography (TLC).

2.2.2. Antibacterial Activity

The antibacterial properties of the synthesized chalcone were tested using the channel paper circle spread method against *E. coli* and *S. aureus*. Bacterial cultures were prepared by mixing with a sterile NaCl solution to achieve an approximately 0.5 CFU/mL turbidity. For the assay, 20 mL of MHA was poured into Petri dishes, and the bacterial suspension was evenly spread over the solidified agar. A 20 µL drop of chalcone solution (250 µg/mL) was applied to a channel paper circle (Whatman) and placed on the agar surface. Tetracycline (5 µL/disc) was the positive control, while DMSO was the negative control. The plates were incubated at 37°C for 24 hours. All tests were performed in triplicate, and antibacterial activity was determined by the clear inhibition zone around the paper circle, with diameters exceeding 2 mm [16].

3. Results and Discussion

3.1. Synthesis of 4-Nitro-4'-Methoxy Chalcone

4-Nitro-4'-methoxy chalcone was synthesized by grinding using 4-methoxyacetophenone and 4- nitrobenzaldehyde. The reaction was carried out for 45 minutes at room temperature, with the mechanism shown in Figure 1. The synthesis employed NaOH as a base catalyst, with a molar ratio of acetophenone, benzaldehyde, and NaOH of 1:1:2. The reaction yielded an orange precipitate (3.15 g, 111.31%). TLC analysis indicated the product was impure and required recrystallization. After recrystallization, the chalcone was confirmed to be pure, as evidenced by a single spot on the TLC plate using various eluents. The final product yielded 1.54 g (54.42%) and a melting point of 172–173°C.

3.2. Characterization of the Synthesized Chalcone

FTIR spectroscopy was used to identify the functional groups of the synthesized chalcone by analyzing the characteristic absorption bands, enabling confirmation of the compound's structure. The FTIR spectra of the synthesized chalcone are shown in Figure 2.

The FTIR spectrum of the synthesized chalcone shows the presence of aromatic C–H bonds at 3110.35 cm^{-1} and aliphatic C–H bonds at 2976.28 cm^{-1}, both with weak intensity. An absorption band at 1613.52 cm^{-1} with weak intensity corresponds to aliphatic C=C, while a strong absorption at 1598.09 cm^{-1} indicates aromatic C=C. A sharp absorption at 1658.85 cm^{-1} confirms the presence of a carbonyl group (C=O). According to Ramaganthan *et al.* [17], aromatic C–H bonds typically show absorption at 2938 cm^{-1}, aliphatic C–H bonds at 2896 cm^{-1}, and C=O groups within the range of 1722–1606 cm^{-1}.

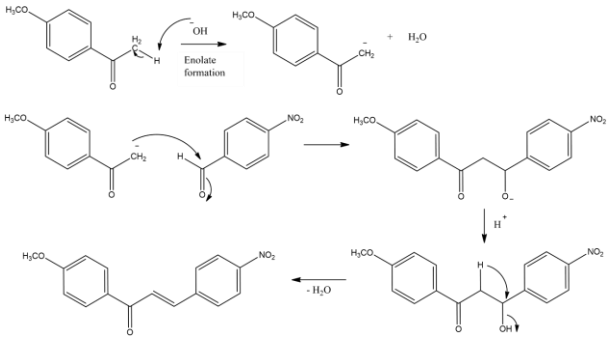

Figure 1. Reaction mechanism for the synthesis of 4- nitro-4'-methoxy chalcone

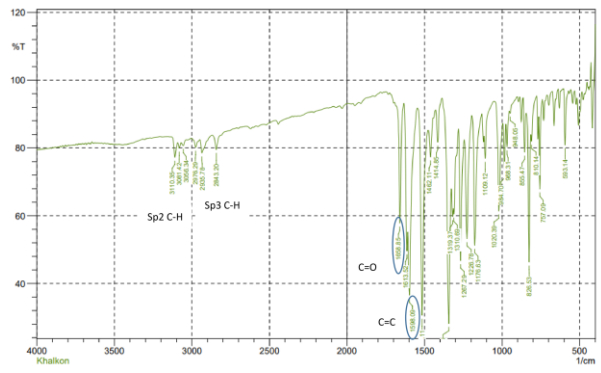

Figure 2. FTIR spectrum of synthesized chalcone

The presence of the methoxy group is confirmed by strong absorption bands at 1267.29 cm^{-1} and 1176.63 cm^{-1}, while the nitro group (NO_2) is identified by absorptions at 1516.11 cm^{-1} and 1343.48 cm^{-1}. Dhaliwal *et al.* [18] reported the methoxy group at absorptions of 1273 cm^{-1} and 1033 cm^{-1}, and the N=O group at 1519 cm^{-1} for the compound 1- (4'-nitrophenyl)-3-(3'-4'-dimethoxyphenyl)-2-propen-1-one. Research by Susanti V. H. and Mulyani [19] on the characterization of methoxy chalcone compounds using FTIR identified the (C=O) group at 1646 cm^{-1} and the aromatic (C=C) group at 1581 cm^{-1} and 1553 cm^{-1} [20]. The functional groups detected in the FTIR spectra are consistent with those present in the structure of 4-nitro-4'-methoxy chalcone.

Characterization using ^{1}H-NMR spectroscopy was performed to determine the position of the protons in 4-nitro-4'-methoxy chalcone. The ^{1}H-NMR spectrum of the synthesized chalcone is shown in Figure 3. The signal at a chemical shift of 3.91 ppm with a singlet peak (A) corresponds to proton absorption from the CH_3 group. The chemical shift at 7.01 ppm (B, 2H, d, J = 8.75 Hz) indicates the presence of equivalent protons in the C-3' and C-5' positions of the aromatic ring as a doublet peak due to coupling with a neighboring proton (either the proton at C-2' or C-6'). The signal at 7.78 ppm (D, 2H, d, J = 8.9 Hz) represents symmetrical protons at the C-2 and C-6 positions of the aromatic ring, also as a doublet peak. The chemical shift at 8.06 ppm (F) indicates the presence of symmetric protons in the aromatic groups at the C-2' and C-6' positions. Finally, the absorption at 8.28 ppm reveals the presence of symmetric protons in the C-3 and C-5 positions of the aromatic ring, showing a doublet peak.

The doublet peak at a chemical shift of 7.66 ppm (C) (2H, d, J = 15.7 Hz) corresponds to the absorption of C-α protons, while the C-β protons appear at 7.82 ppm (E). The H-Cα proton exhibits a doublet peak due to coupling with the neighboring H-Cβ proton. The chemical shifts of the C-α and C-β protons are higher than those typically observed for C=C alkenes due to the conjugation between the carbonyl (C=O) and aromatic systems. This conjugation reduces electron density around the protons, making them more deshielded. The C-α proton is more shielded than the C-β proton due to the resonance effect of the carbonyl group, which protects the C-α proton from deshielding [15].

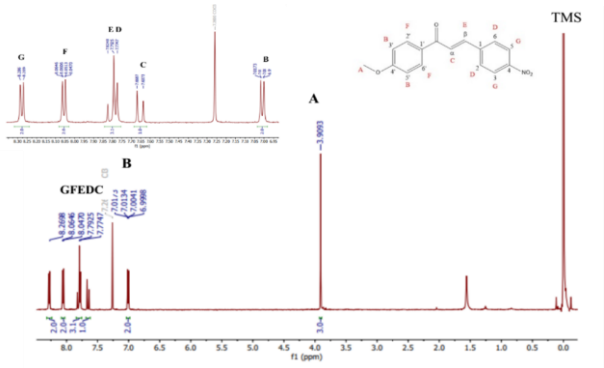

Figure 3. ^{1}H-NMR spectrum of 4-nitro-4'-methoxy chalcone

The significant chemical shift of the C-β proton is attributed to the nitro group in the ortho position, which facilitates the delocalization of π electrons, thereby reducing electron density. The C-α proton signal exhibits a coupling constant of 15.7 Hz. Based on this coupling constant, it can be inferred that the synthesized chalcone has a trans configuration. This is consistent with chalcone compounds, where the trans isomer typically shows a coupling constant (J) in the range of 15.3–15.9 Hz.

The characterization result of ^{13}C-NMR is presented in Figure 4. The first chemical shift observed is the carbon of the methoxy group, which appears at 55.72 ppm. The methoxy group has a higher electron density, which results in shielding, causing it to appear in regions closer to TMS. The absorption bands for aromatic C=C and alkene C=C typically appear in the δ 100–160 ppm range [13]. This is consistent with the following chemical shifts: 114.22 ppm (C-3' and C-5'), 125.82 ppm (C-3 and C-5), 128.98 ppm (C-2 and C-6), 130.65 ppm (C-1', C-2' and C- 6'), 131.15 ppm (C-1), 141.47 ppm (C-4), and 164.05 ppm (C-4').

The signal for aromatic C=C in ring A was observed at chemical shifts of 114.22 ppm (C-3' and C-5') and 130.65 ppm (C-2' and C-6'). The carbon signals from C-3' and C-5' are more shielded than those from C-2' and C-6' because the electron-donating methoxy group (-OCH_3) at the ortho position influences the carbons at C-3' and C- 5', placing them in a more electron-rich environment. In contrast, C-2' and C-6' are in the meta position relative to the -OCH_3 group, making them less shielded. The carbon signals from ring B were detected at 125.82 ppm (C-3 and C-5) and 128.98 ppm (C-2 and C-4). The signals from C-3 and C-5 are more shielded than those from C-2 and C-6.

The signal at 130.65 ppm corresponds to the absorption of C-1', which is more shielded than C-1 (131.15 ppm) because the methoxy group increases the electron density around C-1'. In contrast, the nitro group reduces the electron density around C-1, leading to less shielding. The signal at 141.67 ppm corresponds to the absorption of C-4, which is more shielded than C-4' (164.05 ppm) due to the conjugation of the carbonyl group and double bonds, which introduces a deshielding effect on C-4'.

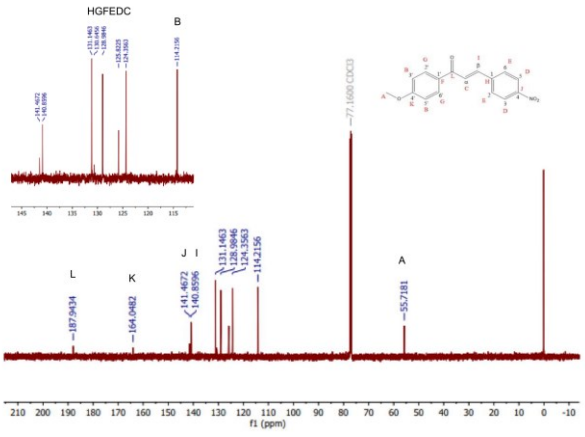

Figure 4. ^{13}C-NMR spectrum of 4-nitro-4'-methoxy chalcone

The C-α signal appears at 124.36 ppm, while the C-β signal is at 140.86 ppm. The C-α signal is more shielded than C-β because, although C-α is closer to the carbonyl group, conjugation between the carbonyl group and the π system of the aromatic ring facilitates electron delocalization, reducing the deshielding effect on C-α. In contrast, the C-β signal is more deshielded due to the combined effects of conjugation and interaction with the aromatic ring. The C-α signal appears at 118.7 ppm, the C- β at 141.8 ppm, and the C-carbonyl at 197.7 ppm. The signal at 187.94 ppm corresponds to the C=O group, as carbonyl group signals are typically observed at chemical shifts above 160 ppm [18].

3.3. Antibacterial Activity of Synthesized Chalcone

The antibacterial activity of 4-nitro-4'-methoxy chalcone against *S. aureus* and *E. coli* is shown in Table 1. The zone of inhibition observed in the antibacterial activity test is depicted in Figure 5. The results of the antibacterial activity determination indicate that the synthesized chalcone exhibits slightly stronger inhibition against the Gram-negative bacterium *E. coli* than the Gram-positive bacterium *S. aureus*. Chloramphenicol was used as the positive control, while DMSO was the negative control. According to Davis and Stout [20], the zone of inhibition (ZOI) is classified into four categories based on the diameter of the ZOI: >20 mm (very strong), 10-20 mm (strong), 5-10 mm (medium), and <5 mm (no response). The clear zones (inhibition zones) observed were 9.57 mm for *S. aureus* and 12.46 mm for *E. coli*, indicating that 4-nitro-4'-methoxy chalcone effectively inhibits bacterial growth with strong activity.

Table 1. Inhibition zone diameter of the synthesized chalcone

Bacteria	Compound	Inhibition zone (mm)
S. aureus ATCC 25923	4-nitro-4'-methoxy chalcone	9.57
	Chloramphenicol	27.23
	DMSO	0
E. coli ATCC 25922	4-nitro-4'-methoxy chalcone	12.46
	Chloramphenicol	34.95
	DMSO	0

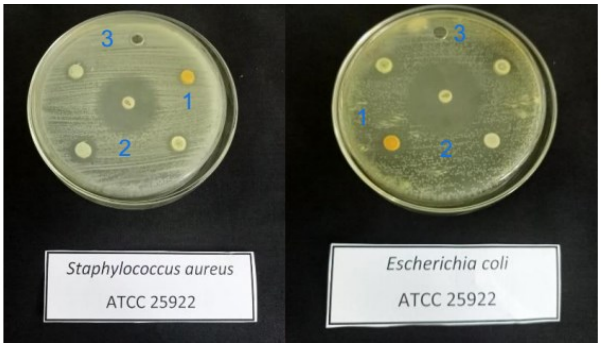

Figure 5. Zone of inhibition in *S. aureus* and *E. coli* (1 = Chalcone, 2 = Chloramphenicol, 3 = DMSO)

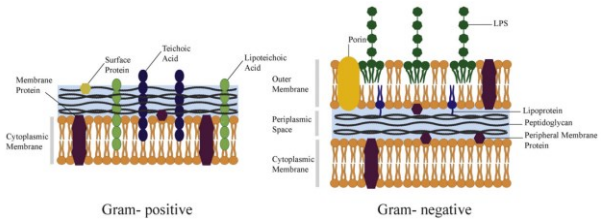

Figure 6. Comparison of Gram-positive (left) and Gram-negative (right) bacterial cell membranes [19]

The molecular components and membrane morphology of Gram-positive bacteria fundamentally differ from those of Gram-negative bacteria (Figure 6). Gram-negative bacteria are surrounded by both a cytoplasmic membrane and an outer membrane. The outer monolayer of the membrane contains lipopolysaccharide, a lipid component unique to Gram-negative bacteria. In contrast, Gram-positive bacteria lack an additional outer membrane layer but have a cell wall surrounding the cytoplasmic membrane. This cell wall is primarily composed of peptidoglycan, which is much thicker in Gram-positive bacteria than in Gram-negative bacteria. Additionally, Gram-positive bacteria's cell walls contain teichoic acids. The main lipid component of the inner monolayer of the outer membrane in Gram-negative bacteria and both monolayers of the cell membrane in both types of bacteria is phospholipid [19].

Research by Susanti V. H. and Mulyani [19] on the antibacterial activity of methoxy chalcone compounds, specifically 4-bromo-4'-methoxy chalcone and 4- hydroxy-4'-methoxy chalcone, against *S. aureus* and *E. coli*, demonstrates that the synthesized chalcone exhibits more significant inhibition against Gram-negative bacteria than Gram-positive bacteria. In this study, the methoxy chalcone was more effective in inhibiting *E. coli* compared to *S. aureus*, with a zone of inhibition of 12.46 mm for *E. coli* and 9.57 mm for *S. aureus* [21].

4. Conclusion

The research results demonstrate that 4-nitro-4'-methoxy chalcone can be synthesized from 4- methoxyacetophenone, 4-nitrobenzaldehyde, and NaOH base catalysts using a grinding technique. The resulting product is a yellow crystalline solid with a yield of 54.42% and a melting point of 172-173°C. Based on the inhibition zones, the antibacterial activity analysis shows that 4-nitro-4'-methoxy chalcone exhibits strong antibacterial potential against *S. aureus* and *E. coli*.

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