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GREEN SYNTHESIS AND CHARACTERIZATION OF 4'-HYDROXY-4-NITRO CHALCONE USING GRINDING TECHNIQUES

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ABSTRACT

The challenge of developing environmentally friendly synthesis methods for biologically active compounds remains a significant focus in green chemistry. This study aimed to synthesise 4'-hydroxy-4-nitro chalcone from 4-hydroxyacetophenone and 4-nitrobenzaldehyde using a grinding technique and then characterise the synthesised compounds. The 4'-hydroxy-4-nitro chalcone was synthesised via the Claisen-Schmidt grinding method for 45 minutes at room temperature. The grinding results were extracted with chloroform. The formed crystals were tested for purity by thin-layer chromatography (TLC), and further purification was carried out by recrystallisation. The synthesised chalcone was characterised using **FTIR, ^{1H}-NMR**, and **^{13}C-NMR** spectroscopy. The chalcone was also tested for antibacterial activity using the disc diffusion method against *Staphylococcus aureus* and *Escherichia coli*. The results showed that 4'-hydroxy-4-nitro chalcone could be successfully synthesised from 4-hydroxyacetophenone and 4-nitrobenzaldehyde using the grinding technique, yielding orangish-white crystals with a 70.63% yield and a melting point of 173°C. TLC confirmed purity and structural characterisation was achieved through **FTIR, ^{1}H-NMR** and **^{13}C-NMR** spectroscopy. Antibacterial testing revealed that the synthesised chalcone produced a clear zone diameter of 9.27 mm against *S. aureus* and 27.88 mm against *E. coli*. These results indicate the compound has relatively strong antibacterial activity against *E. coli* and relatively weak activity against *S. aureus*. This study demonstrates the effectiveness of the green synthesis method and provides valuable insights into the antibacterial properties of 4'-hydroxy-4-nitro chalcone.

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INTRODUCTION

Chalcone is an essential compound used as a starting material for synthesising heterocyclic compounds. Chalcone and its derivatives are classified as flavonoids with secondary metabolite types. It consists of two aryl rings (1,3-diaryl-2-propen-1-one) connected by an α-β unsaturated ketone [1]. Chalcone derivative compounds have the active group structure C=C and C=O conjugated with an aromatic ring, making them beneficial in pharmacology. Additionally, synthesised chalcone has the potential to be an antibacterial agent [2].

Chalcone can be found in various plants, such as Glycyrrhiza, Angelica,

Ruscus, and Piper (betel) species, which have long been used for medicinal purposes in Asia, Africa, and South America. Its benefits as an anticancer and antioxidant agent have gained attention recently due to its excellent pharmacological activities, such as anti-inflammatory, antifungal, antiviral, antibacterial, antioxidant, and antineoplastic properties [3].

The quantity of chalcones in nature is minimal compared to other flavonoids. Chalcones are classified as minor flavonoids, present in small amounts and with slight structural variations [4]. Despite their well-researched benefits, the natural distribution of chalcones is limited, and the isolation process is lengthy and not proportional to the required amount [2].

The limited natural occurrence of chalcones and the complexity of their isolation due to the enzyme chalcone synthase (CSH) necessitates synthetic approaches to obtain sufficient quantities for pharmacological research [5]. The CSH enzyme converts chalcone into flavanones. Chalcone, a bioactive flavonoid, has pharmacological potential as an antidiabetic, anticancer, anti-inflammatory, antimicrobial, antioxidant, antiparasitic, psychoactive, and neuroprotective agent [2], [6]. This compound comprises one unsaturated α,β carbon atom and two aromatic rings. Although chalcone has many health benefits, it is difficult to isolate from plants, necessitating laboratory synthesis. Chalcone synthesis is based on the Claisen-Schmidt condensation reaction between benzaldehyde (and its derivatives) and acetophenone (and its derivatives) with the help of alkaline or acid catalysts [7]-[9]

Chalcone synthesis can be achieved through Claisen-Schmidt condensation, which involves the reaction between aromatic aldehydes and ketones to form α-β unsaturated aromatic ketones [10]. Using a catalyst, substituted aromatic aldehydes and acetophenone derivatives can react efficiently. The Claisen-Schmidt reaction catalysed by NaOH yields better results (93-98%) compared to catalysts like KOH, NaOAc, and NH_4OAc (81-85%) [11].

Chalcone was synthesised by reacting 2,4-dihydroxyacetophenone with 3,4-dimethoxybenzaldehyde using NaOH as a catalyst, employing both grinding and conventional techniques. The grinding technique yielded 2',4'-dihydroxy-3,4-dimethoxychalcone at 84%, while the traditional method using ethanol solvent resulted in a lower yield of 70% [12].

In another study, 14 chalcones were synthesised via Claisen-Schmidt condensation between aryl methyl ketone and aldehydes in the presence of NaOH or H_3BO_3. The chalcones were tested for in vitro antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*). The results showed moderate to good activity against Gram-positive bacteria, while *E. coli* was resistant. Structure-activity relationship (SAR) analysis indicated that an electron-withdrawing group (NO_2) on ring A and an electron donor group on ring B enhance antibacterial activity. Hydroxyl and alkyl substituents on ring B at positions 3 and 4 also positively influence activity [13].

Research also synthesised 1-4(E)-4'-hydroxy-4-methoxychalcone using KOH as a catalyst at concentrations of 30%, 40%, 50%, and 60%, achieving yields of 82%, 90%, 96%, and 97%, respectively. This indicates that 50% and 60% KOH concentrations produce similar high yields [14].

The functional groups in chalcone significantly affect the compound's toxicity. Chalcones with methoxy groups on aromatic rings A and B have high toxicity and potential as anticancer agents [15]. Studies on chalcone derivatives for anticancer activity against breast (T47D) and colon (WiDr) cancer cells showed that compounds with methoxy groups, such as (E)-4'-hydroxy-3,4-dimethoxychalcone, have greater inhibitory effects than other chalcone variants [14]. Further research on chalcones with various methoxy group substitutions is needed.

Several techniques for synthesising chalcone in the laboratory include conventional solvents, solvent-free synthesis such as grinding, and microwave irradiation [16]. The grinding technique is more environmentally friendly since it does not use organic solvents, reducing waste discharged into the environment. Advantages of the grinding technique include a shorter reaction time and more straightforward product processing. Recent studies have focused on green synthesis methods for chalcones, using techniques like grinding and microwave irradiation [17], [18]. Despite these advancements, further research is needed to explore the full pharmacological potential of chalcone derivatives [20].

Based on previous research, further structural modifications of chalcone are necessary to enhance its variety and benefits. This study focuses on synthesising 4'-hydroxy-4-nitro chalcone using a green grinding technique, which has yet to be widely applied to this compound. The goal is to achieve high yields with minimal environmental impact. The green grinding technique offers an innovative approach by reducing the need for organic solvents and decreasing waste, making the process more sustainable and eco-friendly. The research aims to explore the efficiency and practicality of this method, potentially leading to improved synthesis techniques for chalcone derivatives with significant pharmacological properties [21].

4'-hydroxy-4-nitrochalcone will be synthesised from 4-nitrobenzaldehyde and 4-methoxyacetophenone using a KOH catalyst with the grinding technique. The synthesis results will then be purified using recrystallisation techniques and identified using thin-layer chromatography (TLC). The compounds will be characterised using FTIR, ^{1}H-NMR and ^{13}C-NMR. The synthesised 4'-hydroxy-4-nitrochalcone will also be tested for antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, providing insights into its practical applications as an antibacterial agent [19].

Given the pharmacological significance of chalcones and the challenges in their natural isolation, synthetic methods offer a viable alternative. This study focuses on the green synthesis of 4'-hydroxy-4-nitro chalcone, characterised by its potential antibacterial properties.

METHODS

1. Chemicals and Instrumentation

The materials used included 4-hydroxybenzaldehyde, 4-hydroxyacetophenone, NaOH, HCl, distilled water, ethyl acetate, ethanol, n-hexane, chloroform, anhydrous sodium sulfate, Whatman paper, and TLC plates, all of analytical grade quality (E-Merck Germany). The instruments used were laboratory glassware (Pyrex), a magnetic stirrer, a desiccator, a magnetic stirring plate, a UV lamp (254 nm), a proton nuclear magnetic resonance spectrometer ^{1}H-NMR, JEOL-MY500), and a carbon nuclear magnetic resonance spectrometer ^{13}C-NMR, 125 MHz, JEOL-MY500).

2. Synthesis of Chalcone Using the Grinding Technique

The grinding technique was chosen because it reduces reaction time and eliminates the need for organic solvents, making it a greener alternative to traditional synthesis methods [18]. Chalcone was synthesised by grinding 4-hydroxybenzaldehyde (1.22 g, ten mmol) with 4-hydroxyacetophenone (1.36 g, 10 mmol) at room temperature in a mortar and pestle for 30 minutes. Thin-layer chromatography (TLC) monitored the reaction's progress. The reaction mixture was diluted with cold water and neutralised with 10% HCl (v/v, cold).

3. Purification and characterization

Purification was performed by recrystallisation, followed by determining the melting point. The synthesised chalcone was characterised using ^{1}H- and ^{13}C-NMR spectrometers.

4. Antibacterial activity

The antibacterial potential of the synthesised chalcone was determined using the filter paper disc diffusion method [2]. The chalcone was tested against *Escherichia coli* and *Staphylococcus aureus*. The bacterial culture was mixed with a sterile NaCl solution to achieve a turbidity of around 0.5 CFU/mL (colony-forming units per millilitre). Petri dishes containing 20 mL MH agar were used as the bacterial test medium. The inoculum was spread onto the surface of the solid media. Whatman disc filter paper was dripped with 20 µL of the synthesised chalcone and then placed on the plate's surface. Tetracycline (5 µL/disc) was used as a positive control, while DMSO was a negative control. The bacteria-inoculated plates were incubated at 37°C for 24 hours. The inhibition zone diameter indicated positive inhibition if a clear zone of more than 2 mm formed around the filter paper [20].

RESULTS AND DISCUSSION

The synthesis of 4'-hydroxy-4-nitro chalcone was conducted by grinding for 45 minutes, yielding a yellowish-black paste. Some of the paste adhered and solidified in the mortar and pestle, requiring removal with a spatula and adding 5 mL of cold distilled water to form an orange solution, which was stirred without heating. Cold 10% HCl was then added, ensuring the solution's pH was checked with pH paper, changing the colour from orange to white. Filtration using Whatman paper produced a cloudy white precipitate and a clear filtrate. The precipitate was dried in a desiccator for 2-3 days,

producing 2.29 grams of greenish-yellow powder.

Recrystallisation produced a cloudy yellow solution. Greenish-yellow powder formed after 8-10 days, as shown in **Figure 1.** The mass obtained after recrystallisation was 0.95 grams. The significant difference in mass compared to before recrystallisation was noted. The recrystallised 4'-hydroxy-4-nitro chalcone was tested for purity using thin-layer chromatography (TLC). According to theory, the weight of 4'-hydroxy-4-nitro chalcone should be 1.345 grams, yielding 70.63%.

Purity testing of the recrystallised product was performed using TLC with hexane acetate (6:4) as the eluent. The results showed three distinct spots, indicating different components.

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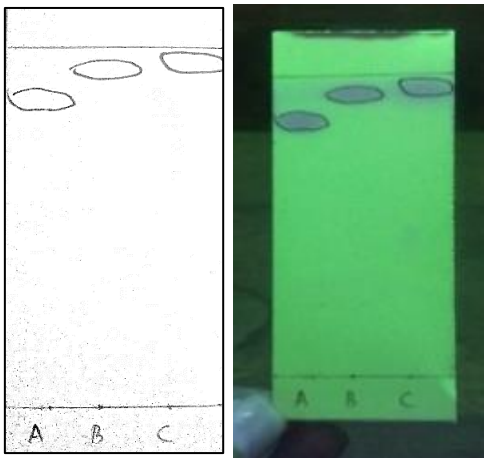

Figure 1. TLC results for the synthesised compound

The spots in **Figure 1** correspond to 4-hydroxyacetophenone, 4-nitrobenzaldehyde, and 4'-hydroxy-4-nitro chalcone. The sample spot distance was 6.5 cm, with the eluent distance being 7 cm. The Rf value was calculated to be 0.92. The sample spot was tailless and separate from the reactant spots, indicating the purity of the synthesised chalcone. A second recrystallisation process was conducted to purify the crystals further.

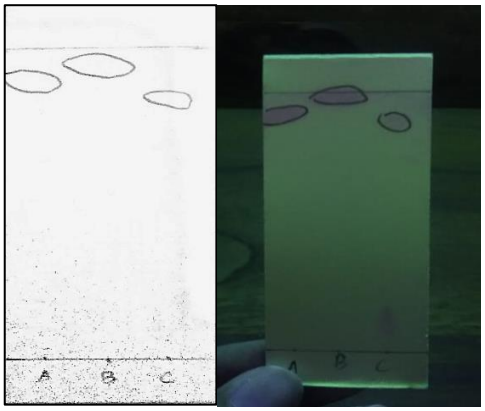

Figure 2. TLC results in the synthesised compound after recrystallisation

The spots in **Figure 2** correspond to 4-hydroxyacetophenone, 4-nitrobenzaldehyde, and 4'-hydroxy-4-nitro chalcone after the first recrystallisation. The distance of the sample spot was 6 cm, while the eluent travelled a distance of 7 cm. The calculated Rf value was 0.85. The distinct separation of the sample spot from the reactant spots indicates the high purity of the synthesised chalcone. The clear, tailless spots suggest effective separation and purification, reinforcing the successful synthesis process. The yellow stain in the image corresponds to the chalcone, confirming the compound's identity and purity. Thin-layer chromatography (TLC) is a widely accepted method for assessing the purity and composition of synthesised compounds, providing visual evidence of successful separation [22].

The Rf value, being close to 1, suggests a non-polar nature of the compound in the chosen solvent system, which is consistent with the expected behaviour of chalcones in

TLC analysis. The distinct spots and their separation from the reactants also validate the effectiveness of the recrystallisation process in purifying the compound. Such results are essential for confirming synthesised compounds' structural integrity and purity, which are crucial for subsequent applications and studies [23].

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1. Characterization of synthesised chalcone using Melting Point Test

The melting point is the temperature at which a solid substance transitions to a liquid, providing crucial insights into the purity and stability of a compound. Impurities typically lower and broaden the melting point range of a substance, making a sharp and definitive melting point indicative of high purity. The synthesised 4'-hydroxy-4-nitro chalcone was subjected to a melting point test to determine its thermal properties. The compound exhibited a sharp and clear melting point at 173°C. This specific temperature suggests that the synthesised chalcone is of high purity, as impurities would generally cause a lower and less defined melting point. The narrow melting point range also indicates good stability of the chalcone, affirming the success of the synthesis and purification processes. These results are consistent with previous studies that have used melting point determination to assess synthesised compounds' purity [25], [26].

The high melting point and stability of the synthesised 4'-hydroxy-4-nitro chalcone imply that the compound is well-suited for further pharmacological applications. In addition, a sharp melting point is often associated with well-ordered crystalline structures, which can be advantageous in drug formulation and other applications [27]. This chalcone's successful synthesis and purification provide a foundation for further research into its potential uses in various fields, including pharmaceuticals and materials science.

2. Characterization with FTIR

The synthesised compound was characterised to identify the functional groups present and provide detailed structural information using Fourier Transform Infrared (FTIR) spectroscopy. FTIR is a vital analytical technique for determining the vibrational modes of different functional groups within a molecule. Key absorption peaks were identified and associated with corresponding functional groups in the chalcone by analysing the specific wavenumber values. This process provided a comprehensive understanding of the compound’s structure.

The specific wavenumber values for key absorption peaks and associate them with corresponding functional groups of the chalcone, the carbonyl group (C=O), hydroxy group (OH), aromatic group, and alkene group (C=C) (**Figure 3**).

The synthesised 4'-hydroxy-4-nitro chalcone was characterised using FTIR, and the results are presented as a spectrum-like graph in **Figure 4.**

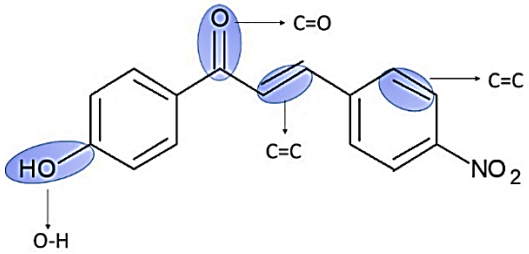

Figure 3. Function group of 4'-hydroxy-4-nitro chalcone

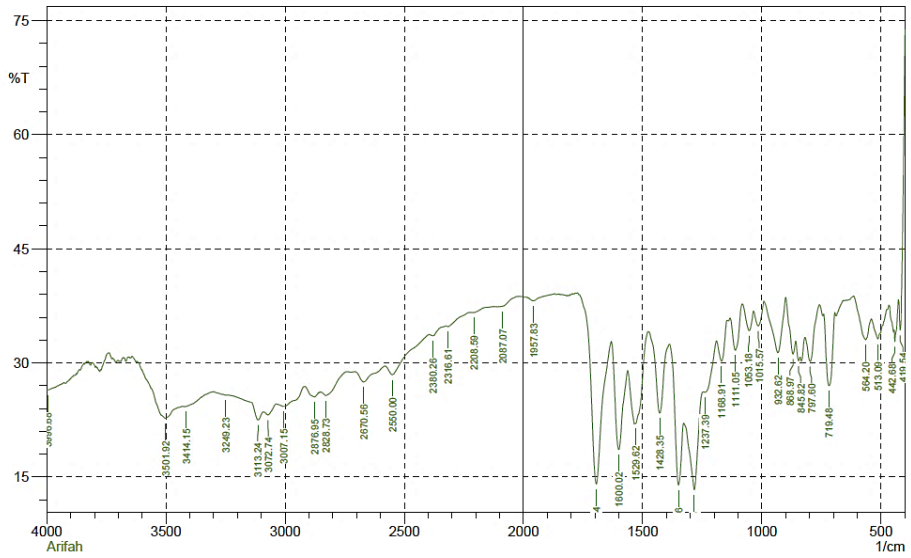

Figure 4. FTIR spectrum of 4'-hydroxy-4-nitro chalcone

The FTIR spectrum of the synthesised 4'-hydroxy-4-nitro chalcone provides detailed information about its functional groups, utilising Hooke's law to estimate vibrational frequencies. This law states that the greater the force constant (f), the higher the vibration frequency, and the greater the energy distance between vibrational quantum levels. In the FTIR spectrum, a weak peak at 3501.92 cm^{-1} indicates the presence of an O-H (hydroxy) group. A medium peak at 3113.24 cm^{-1} corresponds to the Csp2 alkene group, suggesting the presence of unsaturated bonds. A weak peak at 3072.74 cm^{-1} denotes aromatic C-H stretching vibrations, confirming the aromatic nature of the compound.

A sharp peak at 1694.54 cm^{-1} indicates the carbonyl (C=O) group, which is characteristic of α,β-unsaturated carbonyl compounds, aligning with previous research that states these groups typically appear as sharp bands in the 1640-1700 cm^{-1} region [27]. Furthermore, a sharp peak at 1600.02 cm^{-1} signifies the presence of the C=C group, while a medium peak at 1529.62 cm^{-1} indicates an aromatic C=C group. Lastly, the peak at 1349.26 cm^{-1} suggests the presence of the NO_2 group. These specific wavenumber values and their corresponding functional groups validate the successful synthesis and structural integrity of the 4'-hydroxy-4-nitro chalcone. The FTIR spectrum confirms that the synthesised compound contains all the expected functional groups, verifying its purity

and the effectiveness of the synthesis process [28], [29]

3. Characterization by ^{1}H-NMR

Analysis of the synthesised 4'-hydroxy-4-nitro chalcone using ^{1}H-NMR aims to determine the location of hydrogen protons in the molecule. The ^{1}H-NMR spectrometer measures the resonance of protons, differentiating results based on the chemical environment of each proton. The chemical shift, symbolised by δ (delta), is measured in units of parts per million (ppm). This value indicates the shift in proton resonance relative to tetramethylsilane (TMS) in ppm, with the chemical shift value for ^{1}H-NMR typically ranging from 0 to 12 ppm. The ^{1}H-NMR characterisation results for the synthesised chalcone are shown in **Figure 5.**

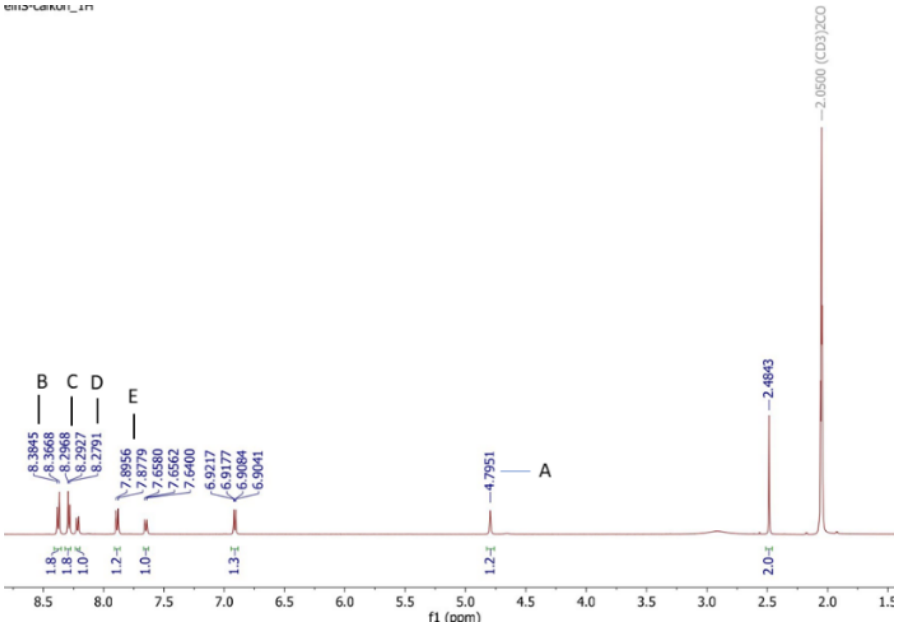

Figure 5. ^{1}H-NMR Spectra Results

The spectrum reveals four distinct proton signals. Each type of proton is given an atomic code, as seen in **Figure 6.**

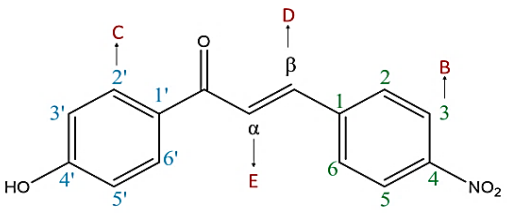

Figure 6. Proton signal from the 4'-hydroxy-4-nitro chalcone

The absorption at the chemical shift (δ) of 4.7951 ppm indicates the presence of aromatic protons, specifically the H-OH proton. This high chemical shift value is due to the magnetic field induction effect from the electronegative oxygen atom, where the electron cloud circulation aligns with the external magnetic field, enhancing the magnetic field induction effect. The chemical shift in O-H is influenced by the electronegativity of atoms like N and O and by the anisotropy of chemical bonds, especially in compounds with alkene, alkyne, and aromatic groups [30].

A chemical shift (δ) of 8.3731 ppm corresponds to alkene protons H-3 and H-5 absorption. Another absorption appears at a chemical shift (δ) of 8.2879 ppm, indicating aromatic protons H-2' and H-6'. These similar chemical shift values are due to the protons being in the same chemical environment, causing overlapping spectra. The chemical shift (δ) of 8.2157 ppm signifies the absorption of aromatic protons, specifically H-β. A chemical shift (δ) of 7.8868 ppm suggests the absorption of H-α alkene protons [31].

Interpretation of the spectral data confirms the presence of proton signals from the 4'-hydroxy-4-nitro chalcone. However, some spectra still need to be identified due to their chemical shift values not corresponding to protons in the synthesised compound. This discrepancy might be due to impurities or incomplete reactions [27].

4. ^{13}C-NMR characterisation

^{13}C-NMR aims to determine the arrangement or number of carbon atoms in the synthesised product compound [32]. The 13C-NMR spectrum of the 4'-hydroxy-4-nitro chalcone, shown in Figure 7, reveals 11 distinct carbon atom signals. The chemical shift values range from 115.91 ppm to 196.26 ppm. Each carbon atom in the 4'-hydroxy-4-nitro chalcone is assigned an atomic code, as illustrated in **Figure 7.**

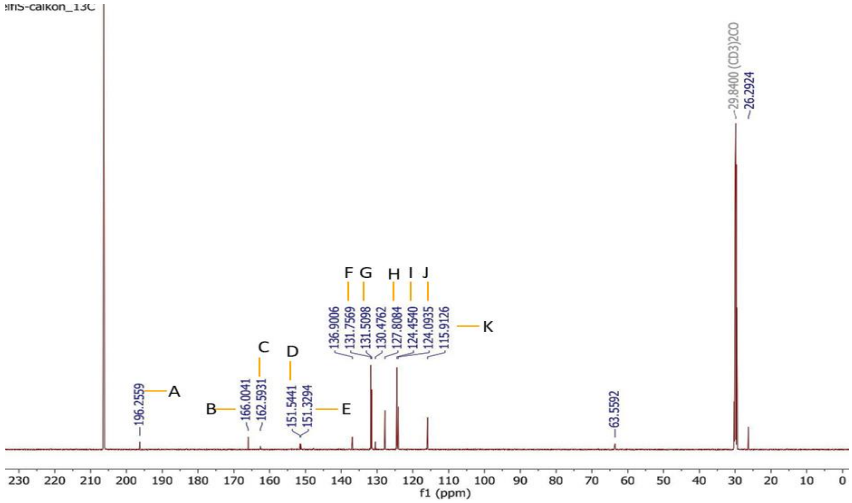

Figure 7. ^{13}C-NMR Spectra Results

The spectrum reveals four distinct proton signals. Each type of proton is given an atomic code, as seen in **Figure 8.**

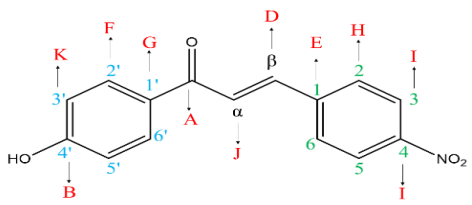

Figure 8. Proton signal from 4'-hydroxy-4-nitro chalcone

The ^{13}C-NMR spectrum shows 11 distinct carbon atom signals. The chemical shift at 115.91 ppm corresponds to the carbon atoms in the aromatic ring, specifically C-3' and C-5'. The signal at 124.45 ppm corresponds to the C-3 and C-5 carbon atoms. Meanwhile, the chemical shift at 127.81 ppm indicates the C-2 and C-6 carbon atoms in the aromatic ring. The signal at

131.51 ppm represents the carbon atom C-1' in the aromatic ring. The chemical shift at 131.76 ppm corresponds to the C-6' and C-2' carbon atoms. The signal at 151.33 ppm is associated with the C-1 carbon atom.

The chemical shift at 124.09 ppm indicates the alpha C-α carbon atom, while the shift at 151.54 ppm corresponds to the C-β carbon atom, which has a double bond with C-α (alkene). The C-β peak appears further downfield than the C-α atom, consistent with previous research stating that the absorption of Cβ in chalcone compounds occurs at a greater chemical shift than Cα [32]. The chemical shift at 162.59 ppm is associated with the C-4 carbon atom in the aromatic ring. The shift at 166.00 ppm corresponds to the C-4' carbon atom in the aromatic ring. The highest signal, at 196.26 ppm, indicates the presence of a carbonyl group (C=O) in the chalcone. The carbonyl group has the most significant chemical shift value due to the influence of electronegative atoms, such as oxygen bonds [33].

The ^{13}C-NMR data confirm the structure of the synthesised 4'-hydroxy-4-nitro chalcone and validate its purity and successful synthesis. The consistent chemical shift values observed in the spectrum align with theoretical predictions and previous research, reinforcing the reliability of the synthesis method used [34]

CONCLUSION

The Claisen-Schmidt method with grinding techniques successfully synthesised 4'-hydroxy-4-nitro chalcone from 4-hydroxybenzaldehyde and 4-hydroxyacetophenone. The synthesised chalcone compound yielded white-orange crystals with a yield of 66.67% and a melting point of 85-88°C. The 4'-hydroxy-4-nitro chalcone exhibited weak antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Future research should focus on synthesising chalcones using acetophenone and benzaldehyde derivatives with different functional groups to enhance antibacterial activity.

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