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Synthesis, Characterisation and Cytotoxicity Activity of Thiazole Substitution of Coumarin Derivatives (Characterisation of Coumarin Derivatives)

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ABSTRACT: *Three types of coumarin derivatives were synthetised using the Knoevenagel condensation technique. All of these compounds have been characterised by the Fourier Transform Infrared (FTIR) and the Nuclear Magnetic Resonance (NMR) spectroscopy (^{1}H NMR, ^{13}C NMR) to evaluate their chemical properties. The first step of synthesis involved preparing 3-acetylcoumarin **1** followed by the synthetisation of hydrazine thiosemicarbazide coumarin derivative **2** by treating thiosemicarbazide in the presence of a catalytic amount of glacial acetic acid. The hydrazinyl thiazolyl of coumarin derivative **3** was prepared using the cyclisation of compound **2** with 3-bromoacetylcoumarin. The presence of the hydrazine and thiazole groups of the latter synthetised derivatives were confirmed using an NMR analysis that depicted the bond attachment of the imine and thiazole groups in the molecular structure of the first derivative **1**. An in-vitro cytotoxicity study of the synthetised coumarin derivatives were screened using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on Human Periodontal Ligament Fibroblast (HPDLF) cells. The cytotoxicity study demonstrates that the coumarin derivatives which contain the hydrazinyl thiazolyl group exhibit less toxicity values at a concentration of 5.51 mM for a half maximal inhibitory concentration (IC_{50}). The heterocyclic groups of the derivatives backbone will eventually contribute to promising biological properties of a biomaterial.*

Keywords: Synthesis, coumarin, thiazole, toxicity, hydrazinyl

1. INTRODUCTION

The biological activities of various compounds, either synthetic or natural, have been investigated extensively worldwide. The applications of these compounds in medical research have increased widely to improve the treatment methods of various diseases. Unfortunately, several of these compounds are not suitable for therapeutic use due to their toxicity level, carcinogenic effect and mutagenic property. As technology and knowledge advance, it is possible to synthetise an

active compound of the molecular structure with improved therapeutic activity and reduced toxicity.[1] Coumarin (2H-pyran-2-one) and its derivatives are well distributed in nature and exhibit a broad pharmacological profile, including anticancer[2] and the scavenging activity of superoxide anions generated by activated neutrophils.[3]

Coumarin is the simplest naturally occurring phenolic substance possessing fused benzene and α-pyrone rings, which are categorised as the basic molecules of several derivative families.[4] Coumarin exists in several forms due to the various substitutions possible in its structure, which modulates its biological activity.[1,5] Furthermore, coumarin is the parent molecule of warfarin, which acts as a vitamin K antagonist.[6] Warfarin is clinically used as an anticoagulant and widely used as a rodenticide, whose discovery was based on studies of bleeding cattle suffering from "sweet clover disease." Cattle bleeding is caused by bishydroxycoumarin (dicoumarol) formed from 4-hydroxycoumarin in spoiled hay.[7]

The thiazole ring of the coumarin derivatives is a heterocyclic structure that attracts most organic and medical chemists to synthesise these compounds. However, the nitrogen and sulphur elements in the heterocyclic structure are interesting due to their physicochemical properties that are relevant to the design of new drugs and new materials. Compounds containing the thiazole ring system are known to possess pharmacological properties, such as analgesic, antibacterial, anticonvulsant, antiparasitic, anti-inflammatory and herbicidal.[8–11] A few derivatives of thiazole are potent anti-HIV agents, and various therapeutic activities have been reported for pyrazoles.[12,13] A few of the coumarin's biological properties are believed to be related to their ability to act as phytoalexins. Typically, phytoalexins accumulate in leaves and fruits to inhibit the growth and spread of bacteria and act as orantimetabolites repellents against herbivorous insects, such as locusts. They are naturally synthetised by the plant to function as protective chemicals against traumatic injury, microorganism invasion, and insect damage. Because substitutions can occur at any of the six available sites of the basic coumarin nucleus, its structural diversity leads to multiple biological properties.[14]

Biocompatibility studies of coumarin derivatives are necessary to determine their therapeutic effect and toxicity level. The measurement of the toxicity for the coumarin derivatives in a normal fibroblast in-vitro will determine the toxic level of the compounds that can be applied in the human body. Coumarins with derivatives containing ortho-dihydroxy substituents typically exhibit a cytotoxicity effect in-vitro on the structure-activity relationship. The addition of the cathecolic group to the basic chemical structure increases the toxicity activity of the coumarins. However, a different toxicity value of the coumarin can be

related to the presence and the position of the hydroxyl group in its structures.[1,15] Therefore, the chemical structure of the coumarin could be modified to reduce the toxicity level that affects the human body.

The study was conducted with two primary objectives. The first objective was to synthetise the hydrazinyl thiazolyl coumarin derivatives, and the second objective was to evaluate the cytotoxicity study of the derivatives on a normal fibroblast cell line. In this study, derivative **1** was prepared by Knoevenagel condensation technique and derivative **2** was synthetised using Schiff base reaction. Whilst, the third derivative was prepared by cyclocondensation of thiosemicarbazone from substituted aldehyde with 3-bromoacetylcoumarin (Figure 1). The synthetisation of derivatives **1** and **2** followed the procedures provided by Arshad et al.[16]

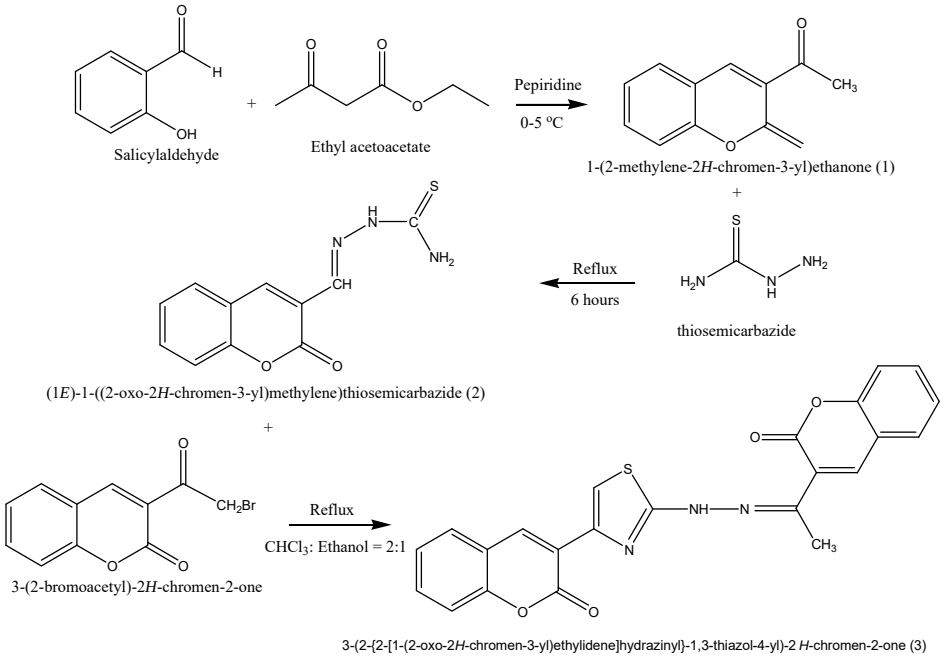

Figure 1: Synthesis of coumarin derivatives (1,2,3).

The identification of the molecular structure of compound **3** was confirmed using the FTIR and NMR (^{1}H NMR, ^{13}C NMR) analysis. Using the cytotoxicity analysis, the samples were characterised through an IC_{50} evaluation using an MTT assay to obtain the toxic level of compounds towards the normal cells.

2. EXPERIMENTAL

2.1 Preparation of (2-{2-[1-(2-oxo-2H-chromen-3-yl)ethylidene]hydrazinyl}-1,3-thiazol-4-yl)-2H-chromen-2-one (3)

A mixture of derivative **2** (0.41 mmol) and 3-bromoacetylcoumarin (0.49 mmol) in hot ethanol was refluxed at 85°C. Then, distilled water was poured onto the resulting product, followed by the addition of a large amount of chloroform. A sufficient amount of potassium carbonate was added to neutralise the basified solution. The solution mixture was stirred until two different layers were obtained, and the mixture was then extracted to obtain an organic phase. Magnesium sulphate was mixed into a solution of the organic phase to absorb humidity. The reflux process was allowed to continue until the complete reaction was obtained. To achieve a successful reaction, the synthesis was monitored using thin layer chromatography (TLC). The TLC plate was cut to the required dimensions (5 cm × 2.5 cm). The reaction mixture and starting materials were spotted on the TLC plates and then developed in a solvent system of petroleum ether: ethyl acetate (70%:30%). The TLC plate was visualised under ultra violet radiation at wavelengths of 254/365 nm. The crude sample was filtered and concentrated using a rotary evaporator. Then, the sample was allowed to dry at room temperature for several days.

2.2 In-vitro Cytotoxicity Study

The cytotoxicity analysis was performed on human periodontal ligament fibroblast (HPdLF) cells. The cell line was cultured and maintained for several passages to obtain stable and healthy cells for analysis application. Stock solutions were prepared for each of the sample types, i.e., **1**, **2** and **3,** by dissolving 10% of dimethyl sulphoxide (DMSO) of total volume for solubility purposes and creating a concentration of 10 mM. The stock solutions were incubated for 72 h at 37°C to obtain the maximum solubility of the sample extraction. Serial dilutions were performed to obtain five different sub-stocks with concentrations of 5.0, 2.5, 1.25, 0.625 and 0.3125 mM. The diluted solutions were filtered with a 0.2 μm syringe filter for the sterilisation process.

The monolayer of the HPDLF cell culture was trypsinised, and the cell count obtained was approximately 2–3 × 10^6 ml^{-1} using an alpha MEM as a medium containing 10% foetal bovine serum. The seed cells, at a concentration of 1 × 10^4 cells ml^{-1} in a 100 μl culture medium, and the plates were incubated at 37°C in a 5% CO_2 incubator. After 24 h of cell incubation, the supernatant of 100 μl was discarded and replaced by previously prepared diluted sample solutions at different concentrations. Cells without treatment (positive control) and a blank medium (negative control) were prepared in the same plate. The samples and

controls were prepared in triplicate. All of the plates were then incubated for 72 h at 37°C in a 5% CO_2 incubator. After 72 h, 10 µl of the MTT solution was added to all of the wells and incubated for 4 h in a 5% CO_2 incubator. The supernatant of each well was discarded, and DMSO was added to dissolve the purple formazan crystals. The absorbance of each well was studied using a microplate reader at 570 nm. The percentage of cell viability can be calculated using the formula as follows:

$$\frac{\text{Absorbance value at test compound (OD)}}{\text{Absorbance value of control (OD)}} \times 100\ \%$$

3. RESULTS AND DISCUSSION

3.1 Chemical Analysis

3-acetylcoumarin,**1** was synthetised in a cold reaction as a first compound for the derivative function to prepare a heterocyclic substitute for the coumarin derivatives **2**,**3**. The yield of the dried product **1** was calculated to be 24.41%, and the melting point was obtained to be 110°C. The FTIR spectra of derivative **1** revealed the presence of lactone (C=O) functional group at peaks 1677 cm^{-1} and 3029 cm^{-1} (C-H) in the aromatic ring. The formation of the imine group (C=N) was identified for the imine group of coumarin derivatives **2** and **3** exerted at peaks 1600 cm^{-1} and 1606 cm^{-1}, respectively (Figure 2). The presence of the hydrazine (N-H) functional group could be observed at 3275 cm^{-1}, and the appearance of the thiol group (C-S) at 1100 cm^{-1} indicated the presence of the thiazole structure (Table 1). Further evidence of the structural identification was depicted in the ^{1}HNMR and the ^{13}C NMR of the coumarin derivatives. The ^{1}HNMR spectra of derivative **1** demonstrates two multiplets and one singlet at chemical shifts of 7.3 ppm (m, 2H), 7.6 ppm (m, 2H) and 8.4 ppm (s, 1H), which indicated the presence of aromatic protons. Another singlet (s, 3H) at a chemical shift 2.7 ppm was observed for the aliphatic protons. The ^{13}C NMR analysis further confirmed the structural identification, and resonances were observed at a chemical shift between 155.2 ppm–195.3 ppm (C=O). The presence of the aromatic carbons was confirmed at 116.5–147.4 ppm, and a peak at 3.5 ppm indicated the presence of the aliphatic carbons. The formation of the hydrazine group of coumarin derivative **2** indicated a few changes to the chemical formulation of the derivative. The presence of methine protons (N=H) at a chemical shift of 8.1 ppm marked the success of obtaining the second derivative of the coumarin. The methyl (CH_3) protons were observed as a singlet at 2.6 ppm while the aromatic protons were observed at 7.3 ppm and 7.6 ppm (m, 2H).

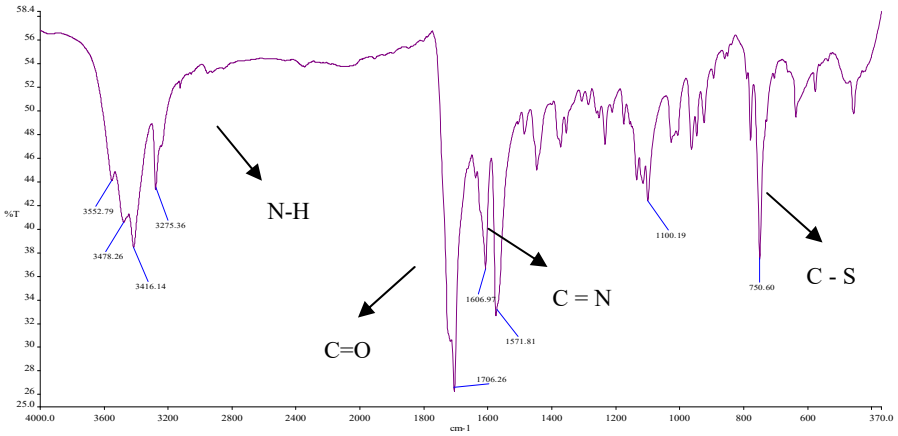

Figure 2: FTIR spectra of compound **3**.

The ^{13}C NMR spectra for the second derivative indicated that lactone (C=O) and imine (C=N) functional groups were confirmed at 163 ppm and 153 ppm, respectively. The proton NMR spectra indicated the appearance of thiazole methine protons at a peak of 8.5 ppm (Figure 3), which depicted the formation of the derivative bonds upon condensation to the hydrazine thiosemicarbazide derivative of coumarin **3**. Furthermore, the analysis of ^{13}C NMR confirmed the presence of the thiazole (2-C) structure at 167 ppm (Figure 4).

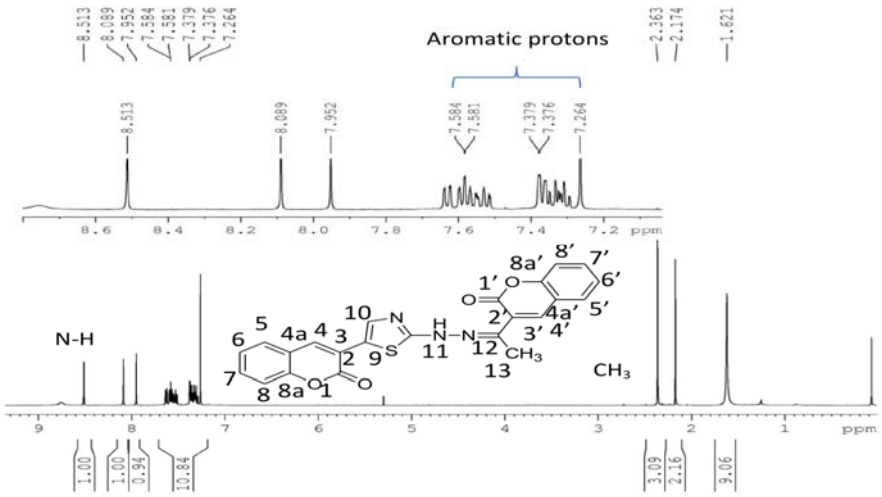

Figure 3: ^{1}H NMR spectroscopy of (3).

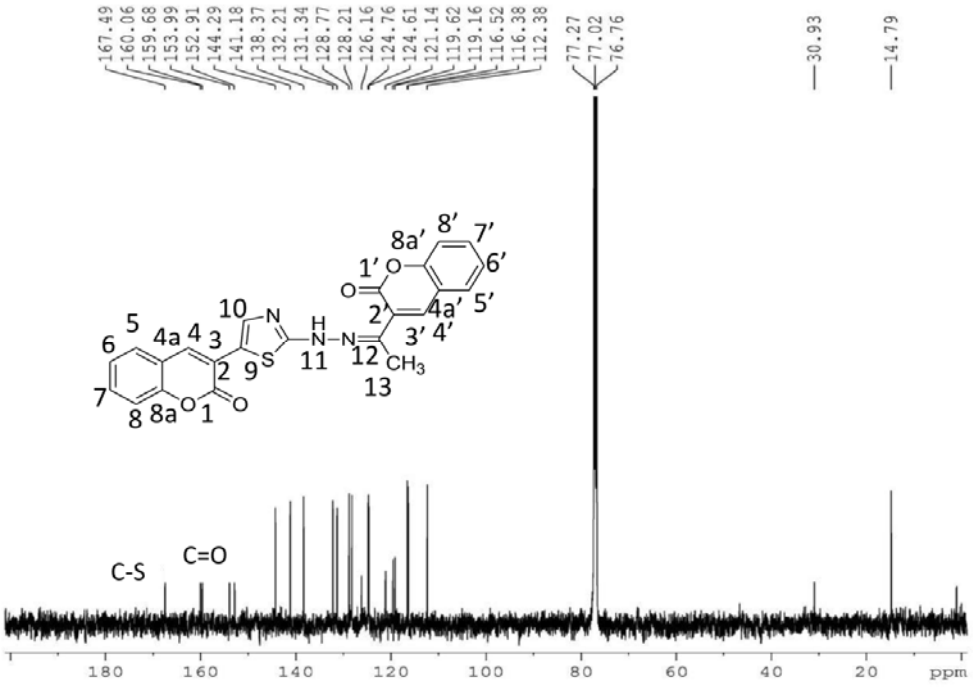

Figure 4: ^{13}CNMR spectroscopy of (3).

3.2 In-vitro Cytotoxicity Analysis

The determination of the cytotoxicity level of the material that has been developed was the primary study, prior to it being used as a biomaterial. The MTT assay[17] was performed to evaluate the cytotoxicity value for the synthetised coumarin derivatives. The results of the cytotoxicity (Figure 5) indicate that compound **1** was observed to be more toxic based on the high percentage of death cells (less than 20%) at a concentration greater than 2 mM. Furthermore, compound **2** was shown to be less toxic than compound **3** due to the higher percentage of live cells at a concentration of 10 mM.

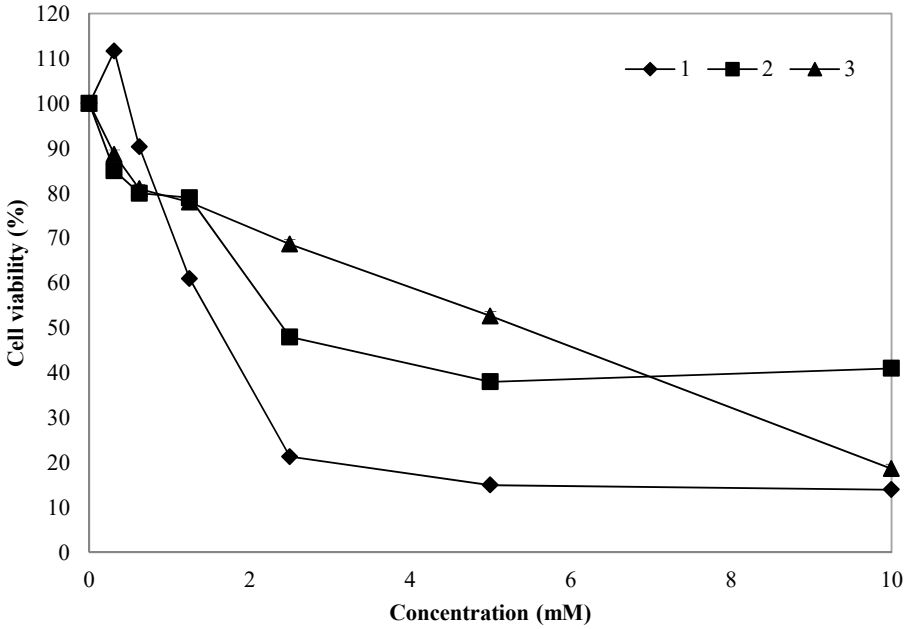

Figure 5: Cytotoxicity effect by MTT assay against HPDLF cell line.

Table 1: List of functional groups with respect to wavelength of compound **3**.

Functional group	Wavelength (cm^{-1})
N-H	3275
C=O	1677
C=N	1606
C-S	1100

However, Table 2 exhibits the half percentage of death cells (IC_{50}) of compound **3** that obtained higher values than the rest at 5.51 mM. However, compounds **1** and **3** have a small difference in value, thus compound **2** was considered to be less toxic (2.47 mM). Based on the results, the compound with the heterocyclic group, i.e., **3**, consisting of a thiazole structure was revealed to be less toxic towards normal cells. Synthetised organic compounds without a substituent at the C-4 position of the thiazole ring were observed to be the least potent inhibitor of cancer cells.[18]

Table 2: The value of inhibition concentration at 50% cell death (IC_{50}) of different compound of coumarin derivatives.

Compound	IC_{50} (mM)
1	1.68
2	2.47
3	5.51

Additionally, a study[19] has reported on the synthetisation of certain new thiazole derivatives bearing a coumarin nucleus and their cytotoxicity activity on human keratinocyte cells. The study depicts the advantage of the thiazole group as a substitute derivative in the molecular structure of coumarin to reduce toxicity. Kaushik et al. also reported that a synthetised compound with the thiazole group exhibits less toxicity to the HEK (normal) cells with 75%–97% viability at 60 μg ml^{-1} concentration for all time intervals, which further increased by decreasing the concentration of the compounds.[20]

4. CONCLUSION

The synthetised coumarin derivatives displayed a perfect match to the structural identification studies using the NMR and FTIR spectroscopy. The thiazole derivatives of the synthetised coumarins **2** and **3** exhibited a lower toxic value to normal cells compared to that of the parent derivative **1**. The presence of heterocyclic rings in their structure possessed remarkable biological features that could be widely used in the medical field. Furthermore, the substituted thiazole rings at carbon-3 of the coumarin derivatives exhibited potential pharmacological and biochemical characteristics.[16] Additionally, the substitution of a derivative compound, such as the thiazole group, allows their biological properties to be modified based on the medical application.

5. ACKNOWLEDGEMENT

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