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Synthesis and Biological Evaluation of 6-Hydroxy-4-Methyl-5,7-(Bis-p-ChlorophenylAzo) Coumarin

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Abstract: 6-hydroxy-4-methyl-5,7-(bis-p-chlorophenylazo)coumarin has been synthesized and characterized by CHN elemental analysis, FTIR, ^{1}H-NMR-spectroscopy and mass-spectral data. Cytotoxic screening by MTT assay was carried out on the compound against breast cancer cells. The overall results from preliminary screening program revealed that the cell proliferation was highly inhibited by 6-hydroxy-4-methyl-5,7-(bis-p-chlorophenylazo) coumarin with the value of 2.81%, at concentration of 30 μg mL^{-1} compared with the untreated control cells and also possessed a good chelating activity with IC_{50} value 1.87 μg mL^{-1}. It is suggested that the cytotoxic activity is affected by hydroxyl and halogen groups as these groups have high electron affinity and high electronegativity.

Keywords: Bis-Coumarins, Cytotoxic Activity, Breast Cancer

Introduction

One of the member in benzopyrone family of compounds is known as “coumarin”. Here, a benzene ring is collectively formed by all the units and this ring is joined to a pyrone ring (Kostova, 2005). Many consequences on the different cellular systems are reported by the coumarins and divers biological attributes are also possessed by them. Researchers have comprehensively investigated their cytotoxic impacts among these attributes. Clinical studies and various in vitro and in vivo tests have exhibited their distinct ranges of effects on the tumours. Therefore, a consumable source of new anticancer agents is depicted by coumarins, due to which resistance phenomena and side-toxicity might be addressed in an effective way. A cluster of natural compounds found in diverse plant sources is incorporated in the coumarin substances. The hypotensive and spasmolytic actions are exhibited by all these compounds upon isolation of coumarins from the plants and this phenomenon has significant outcomes in physiology and plant biochemistry, which act as antioxidants, precursors/prototypes of toxic substances and enzyme interceptors. Moreover, growth regulators, photosynthesis process, plant growth hormones, defence against infection and the control of respiration are the activities performed by these compounds (Kostova *et al*., 2001a).

In biological systems, the unusual range of pharmacological and biochemical actions of these chemicals might be explained and justified by the prolonged relationship of plant coumarins with numerous animal species and other creatures throughout development. The antioxidant, spasmolytic, antibacterial, anticoagulant, antifungal and antiviral behaviours are possessed by coumarins and they are highly active biological materials. The antineoplastic action of coumarin derivatives has been witnessed in the literature to a great extent. Their role can be observed at different stages of cancer development; some of them have cytotoxic activity and the rest have cytostatic attributes (El-Ansary *et al*., 1987). In the cure of certain malignancies and lymphede-mas, coumarin is clinically administered and it is generally used as a healing agent. With diverse organic accomplishments, for instance, antitumoral action, the logically occurring substances are commonly known as 7-hydroxy- and 4-hydroxycoumarins. Several human tumour cell lines were employed to test the antitumor actions of coumarin and its recognized metabolite 7-hydroxy-coumarin (Stanchev *et al*., 2008). On the other hand, no attempt has been made in preparing 6-hydroxy-4-methyl-5,7-(bis-p-chlorophenylazo)coumarin according to the literature survey reports. It has been noticed that cytotoxic activity is possessed by coumarin and such attributes are also possessed by the coumarin derivatives as per the literature date. Along with cytotoxic screening, our synthesized compound is therefore taken into account.

Materials and Methods

The companies, such as Aldrich, Sigma and Merck were approached for the procurement of all starting materials. The organic compounds used were hydroquinone, ethyl acetoacetate, 4-chloroaniline, sulphuric acid, hydrochloric acid, sodium hydroxide, ethanol and sodium nitrite.

Synthesis of 6-Hydroxy-4-Methylcoumarin

In the presence of concentrated H_2SO_4, equivalent molar quantities of ethyl acetoacetate and hydroquinone were added to produce 6-hydroxy-4-methylcoumarin (Kalyanamaysen and Bagchi, 1959). A 100 mL beaker enclosed by salt ice bath was employed to place 43 mL concentrated H_2SO_4. Then, with continuous stirring, 25 mL of ethyl acetoacetate and 20 g of hydroquinone were dropped in prudently when the temperature fell below 10°C. Using an ice salt bath during the addition (5 h), the temperature was kept lower than 10°C. At room temperature, the reaction mixture was kept for almost 18 h and then dispensed into a mixture of water and crushed ice through active stirring. Suction filtration is the technique used to collect the precipitate and water was used to rinse this precipitate. A 300 mL of 5% NaOH solution was then used to liquefy the solid, filtered and dilute (1:10). With the help of active stirring, an amount of 110 mL of H_2SO_4 was added till the conversion of the solution into litmus. Through filtration at the pump, the group has accumulated the crude of 6-hydroxy-4-methylcoumarin, which was then washed with cold water and recrystallized from 25% ethanol.

Synthesiskofk6-Hydroxy-4-Methyl-5,7-(Bis-P-Chloro Phenylazo) Coumarin

An ice salt bath was employed for the purpose of cooling a well-stirred solution of 20 mL of 2M HCl and p-chloro aniline (0.02 mole in 40 mL ethanol) and this solution was then diazotized with aqueous sodium nitrite solution (20 mL, 0.01 mole). The cooled (0-5°C) diazonium solution was added gradually to a mixed solution of 0.01 mole 6-hydroxy-4-methylcoumarin in 100 mL ethanol comprising of sodium hydroxide (10g). Then, at room temperature, the reaction mixture was stirred for an hour and subsequently it was acidified with diluted HCl (100 mL, 2.5M) so that the mixture could be propelled and the reaction mixture could be balanced properly (Ibrahim and Abdel-Latif, 2013).

Cytotoxic Activities of 6-Hydroxy-4-Methyl-5, 7-(bis-p-Chlorophenylazo) Coumarin

The MTT assessment was performed to study the cytotoxic activity (Kovala-Demertzi *et al*., 2006; Mosmann, 1983) through which this compound would be evaluated on viability breast cancer cell MCF-7. In 96-well plate (200 µL/well), the cells were coated with density of 2×10^5 per millilitre, then controlled with several concentrations (0.936, 0.468, 1.875, 3.75, 7.5, 15 and 30 µM/L) of the compound, then incubated for 72 h at 37°C where control breast cells did not have the compound. The PBS buffer was used to wash the cells and for inducing the reaction at 37°C for 4 h, addition of MTT solution (0.5 mg mL^{-1}) was performed to the cells. With the addition of 100 µL dimethyl sulfoxide (DMSO), the purple MTT formazan crystals were dissipated. The ELISA reader was used to measure the absorbance at 570 nm.

Results

6-hydroxy-4-methyl-5,7-(bis-p-chlorophenylazo)coumarin ($C_{22}H_{14}N_4O_3Cl_2$. Elemental analysis: Calc. (%): C, 58.27; H, 3.09; N, 12.36. Found: C, 52.09; H, 3.68; N, 12.64. Colour: Dark yellow; m.p. 282-283°C; yield 95 %. FT-IR and ^{1}H NMR spectral data (Table 1 and 2) were utilized to determine the molecular structure of the compound. Thus, the structure of 6-hydroxy-4-methyl-5,7-(bis-p-chlorophenyl azo)coumarin is proposed as in Fig. 1. The compound possessed cytotoxicity against MCF7cells with an IC_{50} value of 1.87 µg mL^{-1}.

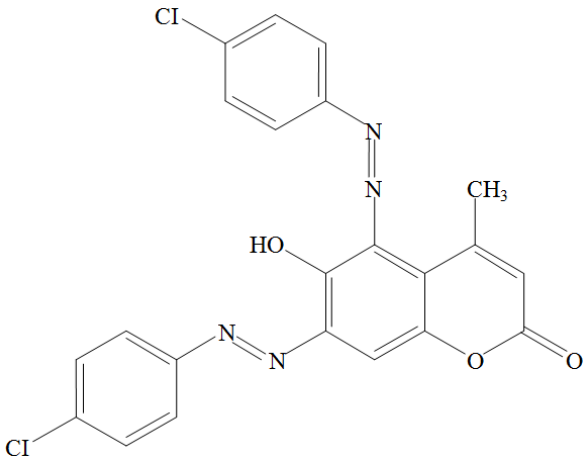

Fig. 1. Structural formulae of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo) coumarin

Table 1. IR Frequencies and band assignments

L (cm^{-1})	Band assignment
3350	ν_{OH}
3100	$\nu_{C\text{-}H\ aromatic}$
1600	$\nu_{C=O}$
1550	$\nu_{C=C}$
1448	$\nu_{N=N}$
1250	$\nu_{C\text{-}N}$

Table 2. ^{1}H NMR spectral data

Chemical shift (δ) ppm	Assignment
9.82	OH
7.88	Aromatic C-H protons
7.16	Pyrone ring C-H
3.75	Chloride C-H
1.83	CH_3 pyrone ring
1.28	$CDCl_3$

Discussion

Infrared (IR) Spectral Analysis

OH Band

The band near 3350 cm^{-1} was actually the infrared absorption band developing from the OH valence vibration, which seemed to be linked with the hydroxyl group (Aschkinasi, 1995). In the range of 3339-3200 cm^{-1}, broad absorption bands are likely to get upsurge from the hydrogen bonds. As far as the broad shape is concerned, the OH based molecule would join with various polymeric kinds, where, the molecules would be engaged in hydrogen bonding. The broad band spotted is therefore a composite one having various sharper bands. A broad band at 3350 cm^{-1} is shown by the IR spectrum of the compound under examination (Fig. 2).

C=C-Vibration

Researchers have indicated that the ring breathing vibrations have a tendency to absorb near 1450, 1500, 1580 and 1600 cm^{-1} in mono- and di-substituted benzenes (Imen *et al*., 2012). Nevertheless, this set of bands is condensed to two bands in benzene itself, only for the reason that the same frequency is detected by the two half ring stretching vibration. Therefore, this gets degenerated, whereas it can be the cause for the emergence of two bands in the spectrum near 1600 and 1500 cm^{-1} comparable to the C=C vibration. This band is displayed at 1550 cm^{-1} by the IR spectrum of the existing compound.

C-H-Vibration

In the region 3100 cm^{-1}, the C-H aromatic stretching absorption is exhibited by the compound and it is hence distinguished from saturated compound. Owing to the (C-H) stretching modes of alkanes, the region having values 3000-2820 cm^{-1} usually contains the bands (Tyagi *et al*., 2005).

C-N Group

Absorption near 1250 cm^{-1} can be given to assignment intended for C-N band (Thompson *et al*., 1948). Quoted the range of 1325-1280 cm^{-1} when the carbon is unsaturated, other bands likely to emerge are C-H and O-H in-plane deformation modes, ϕ O-H stretching across this region. Therefore, 1250 cm^{-1} would be the value of the band appearing in the IR spectrum of the compound. According to Abdel-Latifa *et al*. (2013) strong band seen at 1448 cm^{-1} would be the value of the band appearing in the IR spectrum of the compound.

C=O Band

The range 1635-1650 cm^{-1} have detected the band having value $\upsilon_{C=O.}$ (McMurry, 2011). Moreover, the range 1674-1650 cm^{-1} had detected the value, C=O (Abdel-Latif *et al*., 2003). The expanding frequency of the C=O group would accept the strong band appearing at 1654 cm^{-1} (Kostova *et al*., 2001b). Additionally, the band containing the range 1707-1713 cm^{-1} was allocated to $\upsilon_{C=O}$ (Brett *et al*., 2000). Hence, 1600 cm^{-1} FTIR spectral data would be displayed by the band appearing in the IR spectrum of the compound.

Nuclear Magnetic Resonance (^1H NMR) Spectral Analysis

The ^1H NMR ranges of the synthesized compound in TMS as an internal standard and $CDCl_3$ as solvent. Table 2 illustrates the chemical shift values of the multifaceted protons in the tested compound. A sharp signal at 9.82 ppm is presented by the ^1H NMR spectra of the compound in $CDCl_3$. The proton of the OH group subsequently receives this signal for onwards demonstration of another signal at 1.28 ppm which is then allocated to the solvent's proton. The protons of the aromatic ring in the ^1H NMR spectra are likely to receive the signals detected at 8.00-6.68 ppm (Francis, 1950). Moreover, the proton of the pyrone ring is likely to obtain the signal, which was noticed at 7.16 ppm (Fig. 3).

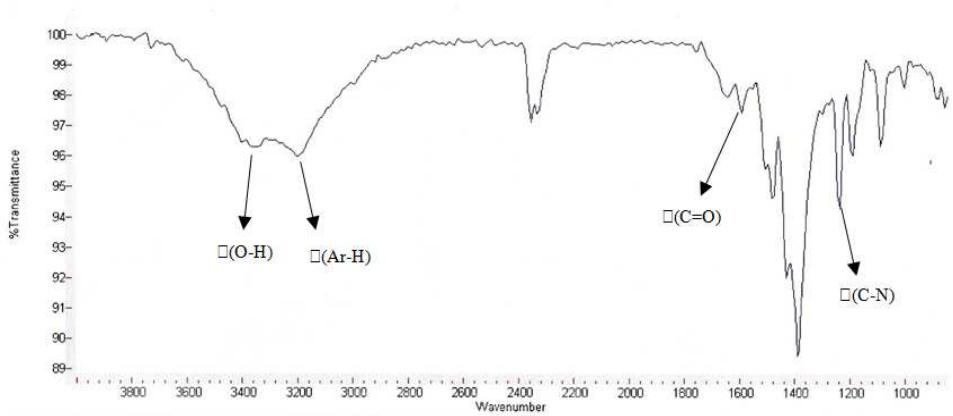

Fig. 2. Infrared spectrum of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo)coumarin

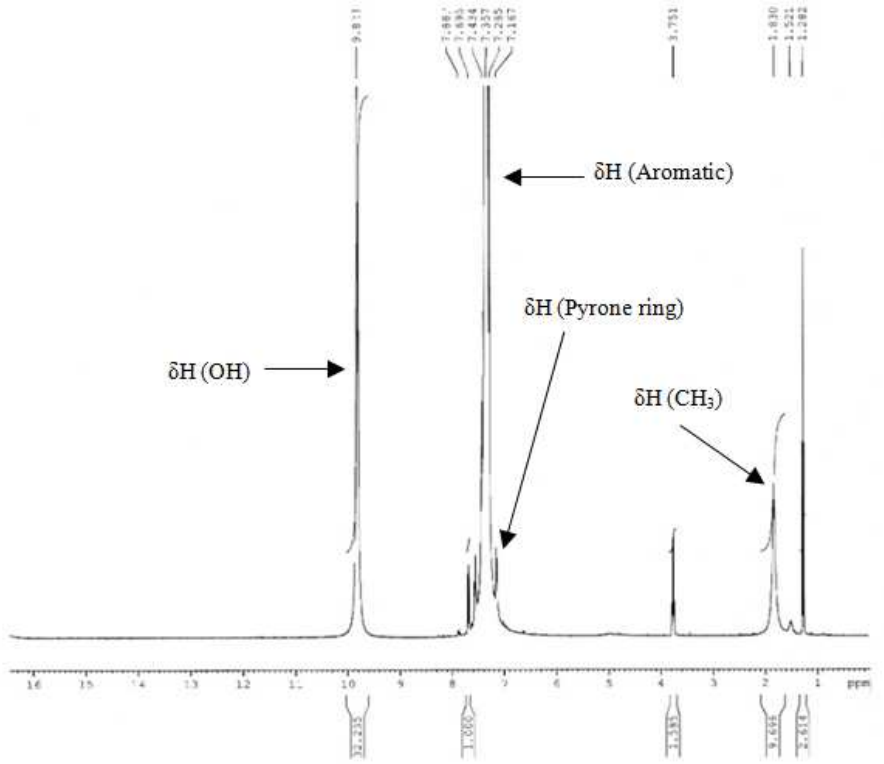

Fig. 3. ^{1}H NMR spectra of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo)coumarin

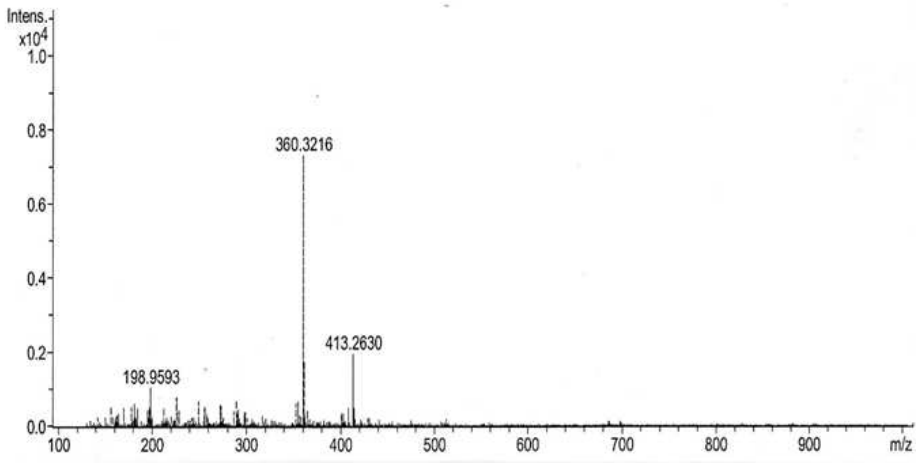

Fig. 4. The mass fragmentation pattern of of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenyl azo) coumarin

Mass Studies

The mass spectra of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo)coumarin has been studied. In the spectrum of the compound (Fig. 4) the molecular ion peak M^{+} is observed at m\z 413.

Evaluation of Cytotoxicity Activity against Breast Cancer Cell using MTT Assay

In MCF7 breast cancer cells, this compound shows good cytotoxic activity at 30 μg mL^{-1} along with values 2.81% (Fig. 5). Substantially cytotoxic values have been reported by some coumarin derivatives containing halogen groups (Kulkarni *et al*., 2006). Thus, it can be concluded that the key role in the anti-breast cancer activity of this compound class is performed by the presence of p-chloro substituents. In addition, the compound is assumed to be the most active on MCF7 cells by 1.87μg mL^{-1} as per the findings of the IC_{50} concentration (Fig. 6).

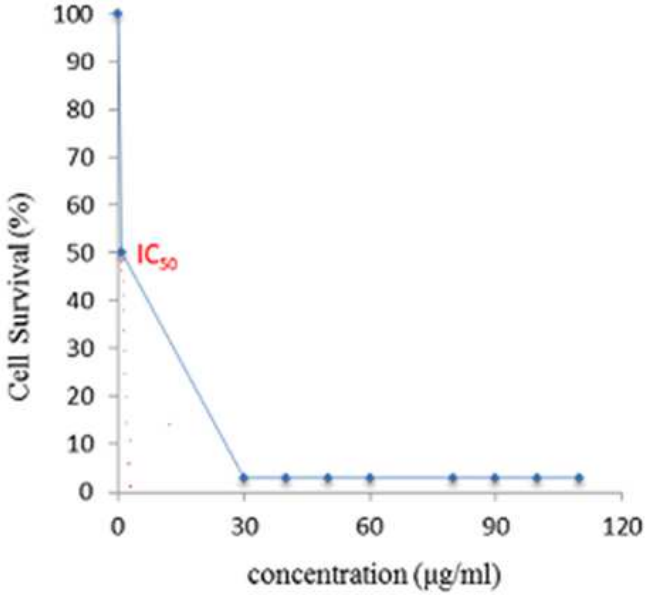

Fig. 5. IC50 value of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo)coumarin on MCF7 cells

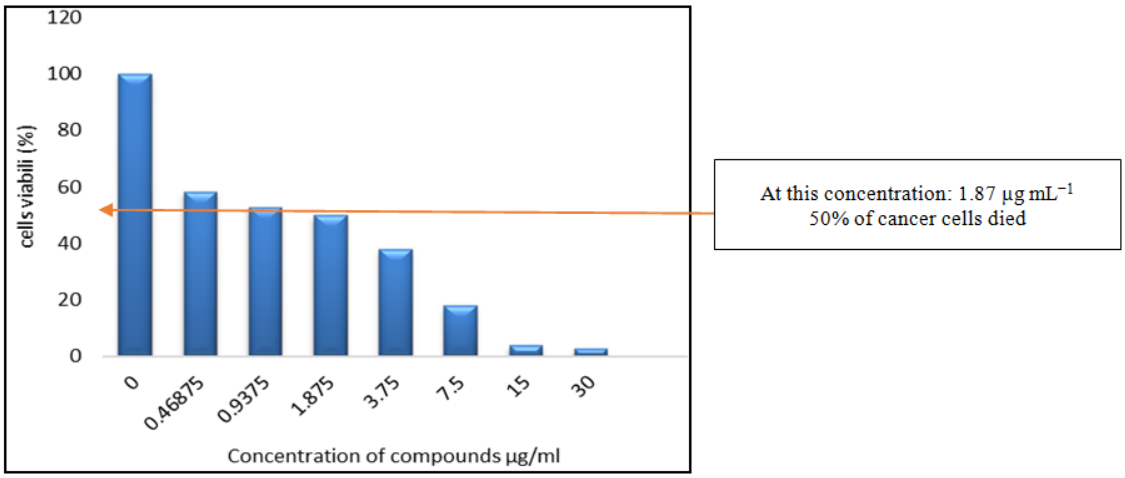

Fig. 6. Effect of 6-hydroxy-4-methyl-5,7-(bis p-chlorophenylazo) coumarin on cell viability of cancer cells

Conclusion

From CHN elemental analysis, mass spectral data, FTIR and ^{1}H NMR spectroscopy analysis, the new compound was successfully synthesized. The overall results from cytotoxic activity revealed that the cell proliferation was highly inhibited by the compound with the value of 2.81%, at concentration of 30 µg mL, as compared with the untreated control cells. Therefore, it is suggested that this compound exhibits good cytotoxic activity. The chloro-substituents and hydroxyl group play important roles in the anti-breast cancer activity of this compound class.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of

the other authors have read and approved the manuscript and no ethical issues involved.

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