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Synthesis, Characterization of New Azo Compounds and Their Biological Evaluation

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Abstract: *This paper presents the synthesis and characterization of new azo dyes derived from coumarin obtained. These dyes were obtained by the coupling of coumarin with the diazotized aromatic amines: 4-nitroaniline, 4-chloraniline,4-bromoaniline. There action yields were between 64%-79%. The proposed structures of the dyes were confirmed and characterized by using infrared spectroscopy IR and high performance liquid chromatography connected with mass spectrometry detector HPLC / MS detector. The obtained dyes showed an orange color and evaluated in vitro for their anticoagulants activities by measuring the prothrombin time (PT) and Thromboplastin time (APTT). PT and APTT assays were carried out citrated plasma of healthy volunteer donors with different concentration of the synthesized compound. It was noted that the azo 4-hydroxy coumarin containing Nitroaniline was posses high activity as anticoagulants compared to other prepared compounds.*

Keywords*:4-hydroxycomarin, azo dyes, aromatic amines, anticoagulants, APTT, PT.*

Introduction

Coumarin is an aromatic compound found throughout the vegetal reign and in some essential oils. Due to of its sweet flagrance, coumarin is used in perfumes, toiletries, tobacco products and previously as a food flavoring additive [1].Coumarin is currently being evaluated for the treatment of some types of cancer [2] and is used in the treatment of lymphedema [3]. Its use as a food additive was banned in the United States in 1954 because it was shown to be hepatotoxic in rats and dogs [4].

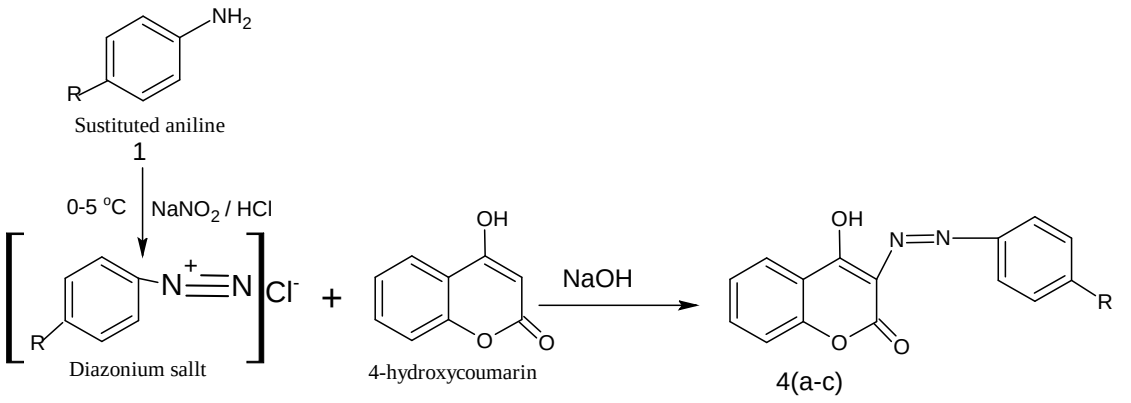
Out of the different classes of dyes, azo dyes constitutes one of the largest and important class of synthetic organic compounds containing an azo N=N group generally connected to aromatic rings. Mostly, synthesis of azo compounds involves diazotization of substituted primary aromatic amines followed by coupling with nucleophiles.They do not occur naturally but synthesized only through chemical synthesis [5] and have been extensively used in different applications such as dying textile fibres, colouring variety of materials, biomedicinal studies and in advanced organic synthesis as well as shows excellent antibacterial and pesticidal properties [6]. Azo compounds and its derivatives are also known for their use as antifungal,antidiabetics, antineoplastics, anti-inflammatory, antiseptic and other useful chemotheraptic agents [7-9]. A number of azo compounds particularly synthesized from *β*-naphthol, m-cresol, resorcinol, tyrosine, aspirin, paracetamoletc have been frequently reported and exhibited impressive biocidal effects. Since compounds with an azo moiety and 4-hydroxycoumarin moiety have been extensively used as dyes but their anticoagulant activity are less reported and hence in the present work, we have prepared three different substituted azo derivatives of 4-hydroxycoumarin namely 4(a-c), characterized and also screened for their anticoagulant activity at different concentrations from prepared compounds.

Materials and Methods

The chemicals used in the present studies were of synthetic grade, and some were sourced from Merck Company Ltd. The products were characterised by IR(JASCO FT/IR 4100 Spectrophotometer using KBr disc), UV (JASCO V-630 Spectropho-tometer), LC–MS (Shimadzu-Mass spectrophotometer). The melting points were determined by open capillary method. The purity of prepared compounds was checked by TLC using silica gel with appropriate solvents dioxane:methanol (1:1).

General method of synthesis of 4-hydroxy-3-(heteroaryl-2-yldiazenyl)-2H-chromen-2-one (4a, 4b, 4c)

Azo coumarin dyes were synthesized in two-step procedure, formation of diazonium salt, then coupling reaction with coumarin [10], as shown in Scheme 1, where Ar = substituted phenyl ring.



Scheme 1

3.1. Diazonium salt formation:

To a suspension prepared (0.01mole) from substituted aniline (4-nitroaniline, 4-bromoaniline, 4-chloroaniline) **(1)**, 12ml water and 12g ice, 6ml (0.052 mole) 32% hydrochloric acid was added under stirring at 8 ^{o}C, a solution of 3.5g (0.01 mole) 20% NaNO2. The reaction mass was stirred during 1 h at 0-7 ^{o}C with 10 mL concentrated sulfuric acid, till the end of the diazotization reaction. The solution of the obtained diazonium salt (**2**) was used in the coupling reaction.

3.2. Coupling reaction:

0.01mole of the coupling component, coumarin, was dissolved in a sodium hydroxide solution (10 g NaOH in 100mL water) and then it was cooled up to 5-9^{o}C. The previously prepared diazonium salt solution was drop wisely added to a basic solution of unsubstituted coumarin (**3**) during 15-30 minutes at 0-7 ^{o}C,under a stirring and by adjusting of the pH (8.5-9) with 15% NaOH solution.Then the obtained reaction mass was filtered.The precipitate was washed with20% HCl solution to remove the

traces of unreacted amines and then with waterup to neutral pH. Then the precipitate was dried and recrystallized from ethanol:water (1:10).

3.3. Purification:

The melting point of the synthesized dyes was recorded with a Boetiusmicro apparatus [11, 12]. The purity of the obtained dyes were verified by TLCusing Silica Gel F254 plates and dioxane:methanol (1:1) as mobile phase.For purification the compounds were recrystallized from their diluted HClsolutions [13].

Anticoagulant assay

4.1. Preparation of Pool of Plasma and Red Blood Cell (RBC) Suspension:

Blood samples were collected from healthy volunteers, using a disposable polypropylene syringe, and then anti-coagulated using 3.8% tri-sodium citrate in a polypropylene container (9 parts of blood to 1 part of tri-sodium citrate solution). It was immediately centrifuged at 4000 × g for 15 min and plasma was separated and pooled. The freshly prepared plasma was stored at 4 °C until its use[14].

4.2.Prothrombin Time PT Test:

The action in extrinsic pathway was evaluated by PT test, as previously described in literature with a few modifications [15,16]. The test was carried out using commercial reagent kits (DiagnosticaStago, France). Plasma (90 µL) was mixed with 10 µL of samples (10,20,30,40µg/mL) in saline containing a DMSO concentration of 2% (v/v) and incubated at 37°C for 5 minat 37°C. Then, 200 µL of PT assay reagent (rabbit brain extract and calcium chloride) pre-warmed at 37°C for 10 min.Thetube was shaken to mix the contents and it was tilted gently back and forth and the stopwatch was stopped as soon as the clot formation began. Plasma alone was used as control (absence of anticoagulant activity).Heparin 10µg/mL (Chandra Bhaga, Pharmapvt. Ltd, Mumbai, India) was used as positive control, and DMSO 2%was used in place of the extracts for the negative control.

4.3. Activated Partial Thromboplastin Time APTT Test:

The action in intrinsic and common pathways was evaluated by APTT test, as previously described in literature, with a few modifications [16-18]. The test was carried out, using commercial reagent kits kits (DiagnosticaStago, France). Plasma (90 µL) was mixed with 10 µL of samples (10, 20, 30, 40 µg/mL) in saline containing a DMSO concentration of 2% (v/v) and incubated at 37°C for 5 min at 37°C, before the addition of pre-warmedAPTT reagent (rabbit brain extract and ellagic acid) and incubation at 37°C for 2 min. Pre-warmed 37°C,25 mM calcium chloride was then added .

The tube was shaken to mix the contents and it was tilted gently back and forth and the stopwatch was stopped as soon as the clot formation began. Plasma alone was used as control (absence of anticoagulant activity Heparin 10µg/mL(Chandra Bhaga, Pharmapvt. Ltd, Mumbai, India) was used as positive control, and Normal saline water 0.9% was used in place of the extracts for the negative control.

Results and Discussion

The 3 new azocoumarin dyes were synthesized by diazotization of different aromatic amines: 4-nitroaniline, 4-chloraniline, 4-bromoanilinewith the formation of diazonium salts, followed by coupling with the unsubstituted coumarin according to Scheme 1. In this scheme Ar is a substituted phenyl ring, to synthesize azocoumarin dyes (4a-4c),The dyes were obtained according to the procedure described in the experimental part with the reaction conditions given in Table 1, for an amount of (0.01mole) coumarin as coupling agent. The coupling reaction temperature was between 5-9 ^{o}C. The yield, the color and the coupling time are also shown in Table 1. The main physical and chemical data were determined and they are shown in Table 2. They confirmed the structures of the new synthesized dyes according to the standard procedures.

Table(1): Dyes, Compounds names, percentage yields of synthesized azo compounds 4(a-c)

Dye	Compounds name	Coupling time (min)	Yields (%)	Dye Color
4a	4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one	20	73	Orange-red
Ab	4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one	15	79	Orange-Brown
Ac	4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one	30	64	Orange-yellow

Table (2): Physical data of the synthesized azocompounds4(a-c)

Dye	Chemical Formula	M.P °C	Rf
4a	$C_{15}H_9O_5N_3$	268-270	0.52
ab	$C_{15}H_9O_3N_2Cl$	165-167	0.68
ac	$C_{15}H_9O_3N_2Br$	149-151	0.71

5.1. Characterization of synthesized azo compounds:

4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one(4a)
IR (KBr)cm^{-1} 3225 (OH str.), 3980 (Ar-H), 1710 (C=O str. of lactone), 1460(-N=N-), 1621 (C=C str. coumarin), (1550, 1360) (NO2 str.), 835 (Ar-CH def); m/z: 310 (100.0%)**.**

4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one (4b)
IR (KBr)cm^{-1} 3230 (OH str.), 2985 (Ar-H), 1720 (C=O str. of lactone), 1455(-N=N-), 1626 (C=C str. coumarin), 855 (Ar-CH def); m/z: 299 (100.0%)**.**

4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one (4c)
IR (KBr)cm^{-1} 3230 (OH str.), 320 (Ar-H), 1725 (C=O str. of lactone), 1463(-N=N-), 1616 (C=C str. coumarin), 825 (Ar-CH def); m/z: 344 (100.0%)**.**

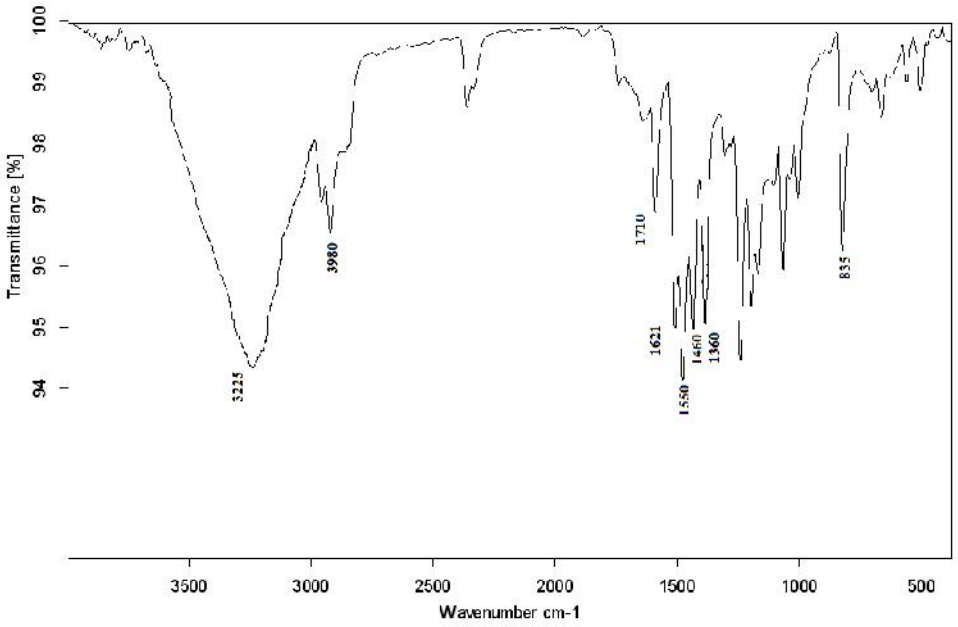


Figure (1): IR spectra of azo compound 4a

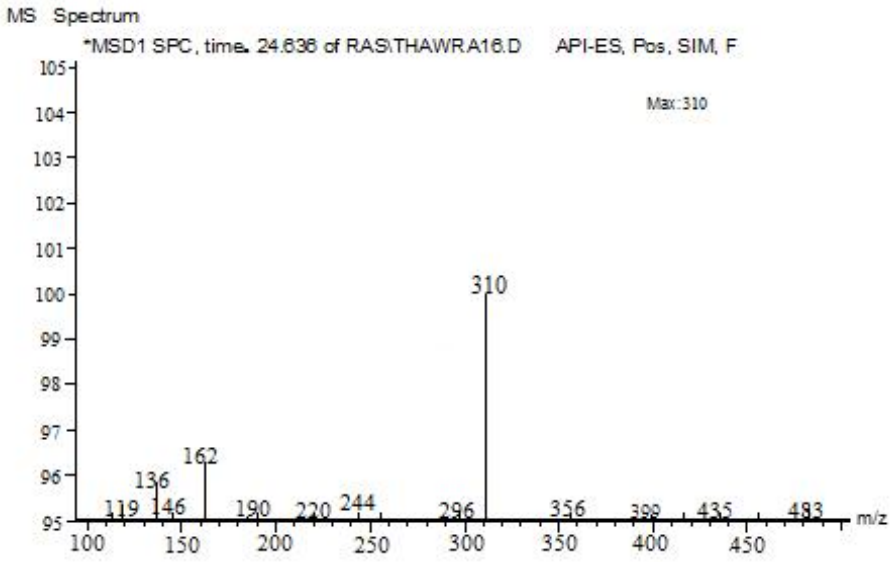


Figure (2): HPLC/MS ofazo compound 4a

5.2. Prothrombin Time PT Test:

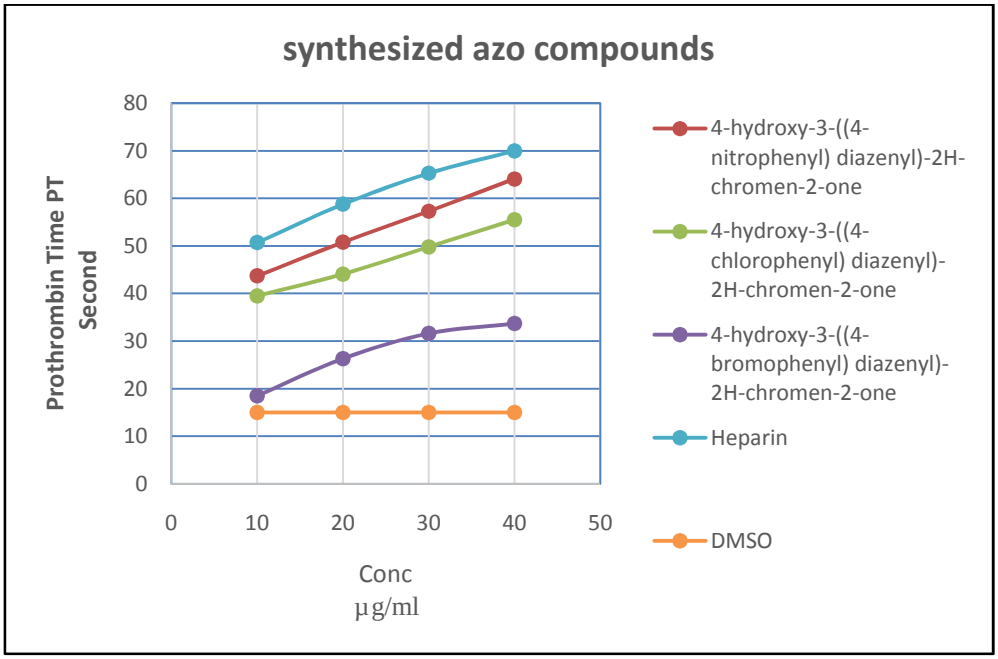
The synthesized azo compounds were tested for blood coagulation effects in normal human plasma and found to be significantly prolonged the prothrombin time (PT) of normal human plasma.

Table 3: Effect of synthesized azo compounds on Prothrombin Time (PT) of Normal Human Plasma

synthesized azo compounds	Conc (µg/ml)	Prothrombin time (PT) (Second)
4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one	10	43.7±0.05
	20	50.8±0.02
	30	57.3±0.01
	40	64.1±0.07
4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one	10	39.5±0.05
	20	44.1±0.04
	30	49.8±0.03
	40	55.5±0.04
4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one	10	18.5±0.01
	20	26.3±0.03
	30	31.6±0.04
	40	33.7±0.07
Heparin	10	50.6±0.01
	20	58.5±0.04
	30	65.2±0.01
	40	70.0±0.05
DMSO 2%	10	15±0.01
	20	15±0.04
	30	15±0.03
	40	15±0.05

All of the data are expressed as mean ±SD (n = 3).

The synthesized azo compounds were studied for the in vitro anticoagulant activity using the prothrombin time assay. Observed from Table 3, the4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one showed the highest blood clotting time compared to other compounds, 64.1 sec, 57.3sec, 50.8sec, 43.7secat40µg/ml, 30µg/ml, 20µg/ml, 10µg/mlconcentrations respectively, Followed by a 4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one, Whereas4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one exhibited lower activity,whene were comparable with polar extracts.



Figure(2):Effect of synthesized azo compounds on Prothrombin Time (PT) of Normal Human Plasma

5.3. Activated Partial ThromboplastinTime APTT Test:

The synthesized azo compounds were tested for blood coagulation effects in normal human plasma and found to be significantly prolonged the activated partial thromboplastintime (APTT) of normal human plasma.

Table (4): Effect of synthesized azo compounds on Prothrombin Time (PT) of Normal Human Plasma

synthesized azo compounds	Conc (µg/ml)	Activated Partial Thromboplastin Time APTT (Second)
4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one	10	95.4±0.04
	20	100.4±0.04
	30	106.6±0.05
	40	115.5±0.02
4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one	10	90.1±0.02
	20	94.5±0.05
	30	97.8±0.03
	40	101.9±0.01
4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one	10	75.2±0.04
	20	79.7±0.03
	30	82.4±0.05
	40	84.9±0.06
Heparin	10	101.5±0.01
	20	108.8±0.03
	30	112.4±0.02
	40	120.9±0.01
DMSO 2%	10	45±0.02
	20	45±0.05
	30	45±0.03
	40	45±0.02

All of the data are expressed as mean ±SD (n = 3).

The synthesized azo compounds were studied for the in vitroanticoagulant activity using the activated partial thromboplastintime (APTT) assay. Observed from Table 4, the4-hydroxy-3-((4-nitrophenyl) diazenyl)-2H-chromen-2-one showed the highest blood clotting time compared to other compounds,95.4 sec, 100.4sec, 106.6sec, 115.5secat40µg/ml, 30µg/ml, 20µg/ml, 10µg/ml concentrations respectively,Followed by a 4-hydroxy-3-((4-chlorophenyl) diazenyl)-2H-chromen-2-one, Whereas 4-hydroxy-3-((4-bromophenyl) diazenyl)-2H-chromen-2-one exhibited lower activity,whene were comparable with polar extracts.

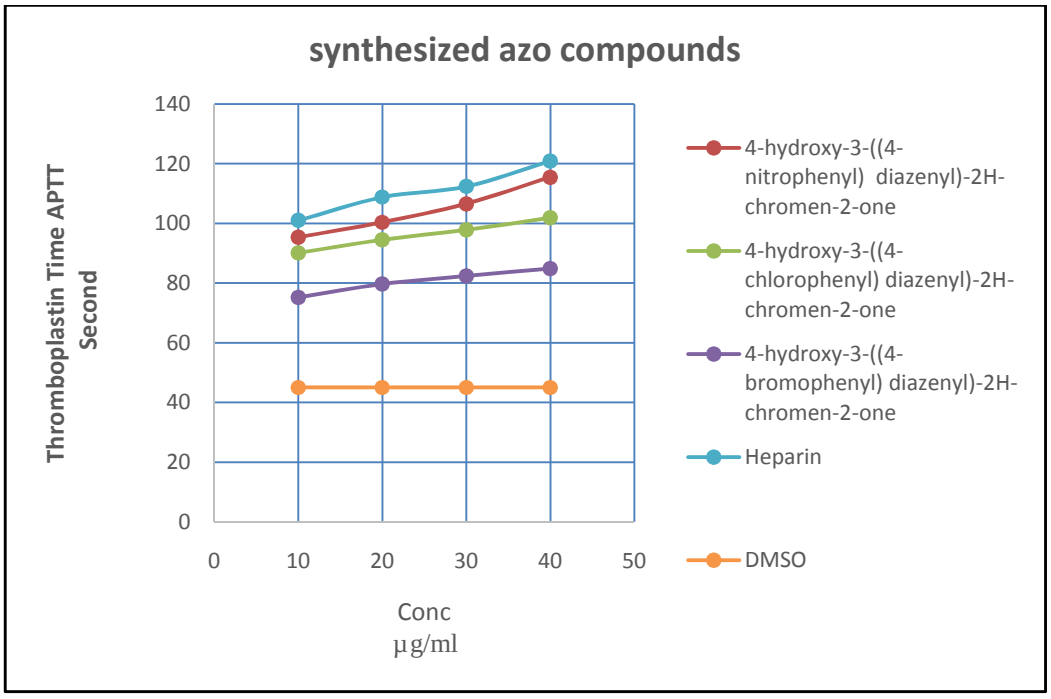


Figure (3):Effect of synthesized azo compounds on Activated Partial Thromboplastin Time APTT of Normal Human Plasma

Conclusions

The present research work involves the synthesis of novel 3-arylazo 4-hydroxy 4-methyl coumarin candidates and toexplore their anticoagulants activity. All compounds have remarkable activity compared to Heparin.Hence, it is concluded that there is a scope of further study the synthesis azo compound In vivo coagulation studies.

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