Stereoselective Synthesis, Spectral Characterization, Docking and Biological Screening of Coumarin Derivatives

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Summary: The compounds being synthesized in present research are chiral in nature so for getting enantiopure compounds, stereoselective synthesis was carried out by organocatalysis. The importance of enantiopure compounds can not be overstated because the living systems are chiral in nature and response of enantiomers can be very different in living systems. The organocatalysed synthesis was accumplished using 4-hydroxycoumarin and variously substituted dibenzylideneacetones as reactants and the organocatalyst being used was 9-amino-9-deoxyepiquinine. The range of enantioselectivity achieved was 24-95%. The synthesized compounds were characterized by UV, IR, ^{1}H NMR, ^{13}C NMR, EIMS, UVCD, VCD and Chiral HPLC. The major focus of this research was to develop anticoagulant compounds and therefore the molecular docking studies were carried out with crystal structure of vitamin k epoxide reductase (3kp9) and then screened for *in-vitro* anticoagulant activity by using warfarin as positive control. Out of six synthesized compounds, four compounds **(1,2,5,6)** have shown greater binding affinity with 3kp9 than warfarin. In *in-vitro* anticoagulant studies, all compounds showed improved IC50 values than warfarin. Besides anticoagulant activity, antimocrobial activities were also carried out with six different strains of bacteria and fungi. Compound **(5)** showed 79% inhibition against *Bacillus subtillis* and 62 % inhibition against *Staphylococcus aureus*.

Keywords: Stereoselective synthesis; Coumarins; Michael Addition; Benzopyrones; Warfarin; Anticoagulant activity; Docking studies; Antifungal activity; Antibacterial activity.

Introduction

Stereoselective synthesis is a very important branch of synthetic chemistry because living systems are mostly chiral so the response of stereoisomers is different in human body in case of administering chiral drugs. In the present research, stereoselective synthesis of coumarin derivatives were done by Michael addition reaction. Asymmetric Michael addition reactions are building blocks of many drugs and natural products synthesis [1]. There are several methods for induction of stereoselectivity such as by using chiral auxillaries [2], chiral pool synthesis [3] and chiral catalysts [4]. As regards the chiral catalysis earlier metal containing chiral catalysts were mostly being used however metal containing catalysts require inert conditions and therefore handling is difficult. Moreover organocatalysts are environmental friendlier, easily available and less expensive. In the present work, stereoselctive synthesis was carried out by using organocatalysis due to all these parameters [5,6]. Different types of organocatalysts are reported such as chiral diamines [7], chiral oxazolenes [8], proleins [9], crown ethers [10], polymer supported organocatalysts [11] and cinchona alkaloids [12]. In the present work a cinchona based alkaloid, 9-amino-9-deoxyepiquinene has been selected which is reported to have excellent enantioselectivity [13, 14].

The stereoselective synthesis is very important in medicinal chemistry. Generally more than eighty persent of the drugs in use are chiral and most of them are used as recemic mixtures [15, 16]. In around 1950, a chiral drug thalidomide was used as recemic mixture to treat morning sickness in expecting mothers. This cause absence or shorten of libs in childern being born. Later studies showed that its *(R)* form is therapeutically active while *(S)* form is not only inactive but also responsible for birth defects [16]. Case of thalidomide illustrates why we pay so much attention to stereoselective synthesis.

Coumarins are naturally occuring medicinally important class of benzopyrones [17]. Different derivatives of 4-hydroxycoumarin are also called superwarfarins such as brodifacoum, bromadilone, difenavoum, warfarin, coumafuryl and coumatetralyl and used as anticoagulants and rodenticides [18]. Besides being potent anticoagaulant other derivatives also have antiviral, antitumor, antiinflammatory and antimicrobial activities [19]. Clinical studies showed that

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derivatives of 4-hydroxycoumarin are well absorbed in human gastrointestinal track [20]. Almost all superwarfarins are used in the form of recemic salts and its *(S)* isomer is more active than *(R)* isomer [21]. Superwarfarin is a vitamin K antagonist drug used to treat thrombolism by blocking vitamin K_1 epoxide reductase [22].

Computer aided drug discovery tools are widely being used and are very useful. Molecular docking gives information about the behaviour of ligand in binding site and information regarding its biological functions[23]. Scoring function of docking is equivalent to binding energy. More negative binding energy values represent strong bonding interactions such as ionic interaction, hydrogrn bonding and van der waals interactions.

Experimental

Chemicals and equipments

All chemicals were purchased from Sigma Aldrich, BDH or Fluka and used without purification.

The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$-*d* on Bruker biospin ICON-NMR or AVANCE-AV-300 spectrometer (US), using TMS as internal reference. The apparent resonance multiplicity is described as s (singlet), br (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet) and m (multiplet). Infrared measurements were recorded in 400-4000cm^{-1} on a spectrum 2000 FT-IR spectrophotometer by Perkin Elmer (USA). Melting points were determined in a capillary tube using a Gallenkamp (UK) electrothermal melting point apparatus. The enantiomeric excess was determined by chiral column, Lux 5 µm Cellulose-1, LC Column 250x4.6 mm(USA). The instrument used for this technique was HPLC Perkin Elmer (USA). Samples were analyzed with evaporation point of max. 300^{o}C; mass range: m/z 30-800 amu. VG Instruments autospec/EBEE-Geometry was used to record mass spectra. Electron impact (EI) yield mostly fragment ions. Molecular ions are not always observed. High resolutions feature with ca 6000-8000 resolution CD spectra were measured by JASCO-815 CD spectrometer (USA) in static mode.

Synthesis

General procedure for synthesis of warfarin analogues

First to prepare dibenzylideneacetone, 4ml of acetone and 10ml of substituted benzaldehyde were dissolved in methanol in a round bottom flask equipped with magnetic stirrer and then added 5ml of 10% NaOH. The reaction mixture was stirred at room temperature for 30minutes. Washed the residue with water to remove excess alkali, dried it and recrystallized by using hot ethanol [24]. Melting point and IR data of synthesized compounds were compared with reported values from chemical book data.

In second step to prepare compounds **(1-6),** 4-hydroxycoumarin (0.32mmol), substituted dibenzylideneacetone (0.2mmol), 20 mol % 9-amino-9-deoxyepiquinine were dissolved in 20 ml of dry DCM in round bottom flask and 30 mol % trifluroacetic acid (TFA) was also added as additive in the reaction mixture. The reaction mixture was stirred at room temperature for 3 to 4 days and the progress of reaction was monitored by TLC visualized under UV lamp and developed in vanillin spray [25]. Crude sample was purified by column chromatography, using different ratios of n-hexane and ethyl acetate. At the end got a single spot of product on TLC and purity was further verified by HPLC.

Circular dichroism studies

Circular dichroism (CD) spectra were measured by *JASCO-815* CD spectrophotometer in static mode. For CD measurements, synthesized derivatives were dissolved in a mixture of aqueous phosphoric acid and acetonitrile having 4:6 ratio and pH value of about 2.0. Other measuring parameters are given in Table 1.

Table-1: CD measuring parameters.

Parameter	Value
Concentration of sample	200µg/ml
Wavelength	170-400nm
Data interval	0.1nm
Response time	2sec
Spectral band width	1nm
Number of accumulations	3 time
Optical path length	1mm

Chiral HPLC

Enantiomeric excess was determined by chiral stationary phase HPLC using Lux 5 µm cellulose-1, LC column 250x4.6 mm. Mobile phase is n-hexane, isopropyl alcohol in ratio of 60:40 and 0.1% formic acid as eluent with flow rate of 1ml/min and data acquisition time was 10min.

4-hydroxy-3-[(4E)-3-oxo-1,5-diphenylpent-4-en-1-yl]-2H-chromen-2-one. **(1)**

The product obtained was white solid, Yield: 60%, mp:157-19 °C, ee: 95% *[S]*, UV λmax(nm): 214, IR *ν*(cm^{-1}):1780 (C=O), 1710 (C=O of coumarin ring), 1610 (Ar C-H), 1310 (C-OH) , ^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ(ppm): 7.63 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.56 (m, 1H), 7.20 (s, 6H), 7.17 (d, *J* = 1.9 Hz, 3H), 7.15 (s, 2H), 7.12 (d, *J* = 3.2 Hz, 1H), 6.94 (d, *J* = 8.3 Hz, 1H), 6.83 (d, *J* = 8.3 Hz, 1H), 4.28 (d, *J* = 4.8 Hz, 1H), 4.22 (d, *J* = 4.8 Hz, 2H), 3.66 (d, *J* = 4.7 Hz, 1H), 3.54 (m, 1H), 0.12 (s, 1H),^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ(ppm): 208.20, 194.02, 137.09, 136.91, 136.88, 136.41, 128.76, 128.67, 128.14, 127.97, 126.50, 125.96, 124.72, 116.90, 116.80, 51.2, 43.29, EIMS m/z: 396.1 [$C_{26}H_{20}O_4^+$] (33%), 292 [$C_{19}H_{16}O_3^+$] (17%), 265.1[$C_{17}H_{11}O_3^+$] (45%), 264 [$C_{17}H_{11}O_3^+$] (92%), 249 [$C_{17}H_{13}O_2^+$] (27%), 144 [$C_{10}H_8O^+$] (18%), 131 [$C_9H_7O^+$] (10%), 121 [$C_7H_5O_2^+$] (35%), 104 [$C_7H_4O^+$] (57%), 92 [$C_7H_8^+$] (19%), 77 [$C_6H_5^+$] (16%), 28 [$C_2H_4^+$] (9%).

3-[(4E)-1,5-bis(4-fluorophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(2)**

The product obtained was brown solid, Yield: 62%, mp:191-197 °C, ee: 24% *[S]*, UV λmax(nm): 214, IR *ν*(cm^{-1}):1770 (C=O), 1720 (C=O of coumarin ring), 1610 (Ar C-H), 1250 (C-OH),^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ(ppm): 7.65 (dd, *J* = 6.0, 2.7 Hz, 1H), 7.58 (dd, *J* = 7.6, 3.4 Hz, 1H), 7.55 (m, 1H), 7.45 (dd, *J* = 5.8, 3.3 Hz, 2H), 7.30 (m, 1H), 7.07 (d, *J* = 5.2 Hz, 1H), 7.03 (d, *J* = 1.9 Hz, 1H), 7.00 (t, *J* = 2.6 Hz, 1H), 6.92 (d, *J* = 15.9 Hz, 2H), 6.76 (d, *J* = 1.9 Hz, 1H), 6.72 (d, *J* = 1.9 Hz, 1H), 6.68 (d, *J* = 2.1 Hz, 1H), 4.13 (d, *J* = 1.8 Hz, 1H), 3.96 (d, *J* = 4.4 Hz, 1H), 3.62 (d, *J* = 5.5 Hz, 1H), 2.58 (m, 1H), 1.62 (s, 1H),^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ (ppm): 203.02, 165.72, 140.09, 135.36, 130.46, 128.84, 128.31, 128.20, 128, 127.74, 127.63, 126.78, 124.45, 123, 114.93, 114.29, 114.00, 113.87, 113.58, 66.16, 47.94, 28.37, EIMS m/z: 432.1[$C_{26}H_{18}O_4F_2^+$] (4%), 391.3 [$C_{24}H_{17}O_3F_2^+$] (3%), 282.0 [$C_{17}H_{17}O_3F^+$] (14%), 167.0 [$C_9H_8O_2F^+$] (30%), 149 [$C_9H_6OF^+$] (100%), 121 [$C_7H_5O_2^+$] (19%), 71 [$C_4H_4F^+$] (17%), 57 [$C_3H_5O^+$] (28%), 43 [$C_3H_7^+$] (20), 29 [$C_2H_5^+$] (12%).

3-[(4E)-1,5-bis(4-chlorophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(3)**

The product obtained was white solid, Yield: 55%, mp: 235-240 °C, ee: 24% *[S]*; UV: λmax(nm) 204, IR: *ν*(cm^{-1}): 1730 (C=O), 1710 (C=O of coumarin ring), 1610 (Ar C-H), 1420 (C-OH),^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ(ppm): 7.63 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.56 (m, 1H), 7.20 (s, 6H), 7.17 (d, *J* = 1.9 Hz, 3H), 7.15 (s, 2H), 7.12 (d, *J* = 3.2 Hz, 1H), 6.94 (d, *J* = 8.3 Hz, 1H), 6.83 (d, *J* = 8.3 Hz, 1H), 4.28 (d, *J* = 4.8 Hz, 1H), 4.22 (d, *J* = 4.8 Hz, 2H), 3.66 (d, *J* = 4.7 Hz, 1H), 3.54 (m, 1H), 0.11 (s, 1H), ^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ (ppm): 200.27, 141.39, 139.47, 139.16, 135.58, 133, 132, 131.75, 129.32, 129.21, 129.18, 129.14, 129.12, 129, 128.9, 128.89, 128.81, 128.66, 128.60, 128.49, 127.92, 127.30, 127.14, 126.28, 123.67, 122.22, 166.22, 53.23, 45.05, 43.98, EIMS m/z: 448.0 [$C_{26}H_{15}O_3Cl_2^+$] (46%), 446.0 [$C_{26}H_{13}O_3Cl_2^+$] (66%), 335.0 [$C_{17}H_{12}O_3Cl_2^+$] (72%), 304.0 [$C_{17}H_{13}OCl_2^+$] (46%), 302.0 [$C_{17}H_{11}OCl_2^+$] (74%), 267.0 [$C_{17}H_{12}OCl^+$] (62%), 239 [$C_{16}H_{15}O_2^+$] (24%), 204 [$C_{15}H_8O^+$] (56%), 149 [$C_9H_9O_2^+$] (22%), 167 [$C_{10}H_{15}O_2^+$] (28%), 165 [$C_{12}H_5O^+$] (74%), 137.0 [$C_8H_9O_2^+$] (63%), 102 [$C_8H_6^+$] (86%), 101.0 [$C_8H_5^+$] (100%), 77 [$C_6H_5^+$] (13%),75 [$C_6H_3^+$] (52%), 63 [$C_5H_3^+$] (12%), 51 [$C_4H_3^+$] (25%), 27 [$C_2H_3^+$] (16%).

3-((4E)-1,5-bis(4-methoxyphenyl)-3-oxopent-4-enyl)-4-hydroxy-2H-chromen-2-one. **(4)**

The product obtained was yellow solid, Yield: 80%, mp:150-156 °C, ee: 89% *[S]*, UV λmax (nm): 218, IR *ν*(cm^{-1}): 1770 (C=O), 1720 (C=O of coumarin ring), 1600 (Ar C-H), 1380 (C-OH),^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ(ppm): 7.99 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.63 (m, 2H), 7.44 (m, 2H), 7.38 (m, 1H), 7.14 (m, 2H), 7.02 (s, 1H), 6.82 (m, 1H), 6.67 (d, *J* = 8.6 Hz, 1H), 6.55 (m, 1H), 6.45 (d, *J* = 4.2 Hz, 2H), 5.82 (s, 1H), 4.95 (m, 1H), 4.08 (d, *J* = 8.8 Hz, 1H), 3.83 (s, 3H), 3.79 (d, *J* = 3.2 Hz, 1H), 3.72 (d, *J* = 2.3 Hz, 1H), 2.14 (s, 3H), ^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ (ppm): 202.6, 161.53, 158.60, 157.19, 152.63, 142.39, 134.22, 133.99, 131.76, 131.69, 130.45, 130.10, 130.01, 129.94, 129.86, 129.20, 129.10, 128.54, 127.13, 116.52, 68.85, 40.80, EIMS m/z: 457.2 [$C_{28}H_{25}O_6^+$] (8%), 439 [$C_{28}H_{23}O_5^+$] (26%), 411 [$C_{27}H_{23}O_4^+$] (15%), 383 [$C_{24}H_{15}O_5^+$] (20%), 367 [$C_{25}H_{19}O_3^+$] (6%), 311 [$C_{22}H_{15}O_2^+$] (13%), 257 [$C_{18}H_9O_2^+$] (28%), 211[$C_{14}H_{11}O_2^+$] (18%), 183 [$C_{13}H_{11}O^+$] (100%), 127.1 [$C_7H_{11}O_2^+$] (25%), 83.9 [$C_5H_8O^+$] (30%), 71 [$C_4H_7O^+$] (35%), 57 [$C_3H_5O^+$] (65%),43 [$C_2H_3O^+$] (58%), 41 [$C_3H_5^+$] (27%), 29 [CHO^+] (13%).

3-[(4E)-1,5-bis(4-bromophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(5)**

The product obtained was brown solid, Yield: 75%, mp:140-142 °C, ee: 25% *[S]*, UV λmax(nm): 221, IR *ν*(cm^{-1}): 1760 (C=O), 1725 (C=O

of coumarin ring), 1620 (Ar C-H), 1410 (C-OH),^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ(ppm): 9.85 (s, 1H), 7.63 (m, 1H), 7.56 (m, 2H), 7.49 (m, 1H), 7.38 (m, 2H), 7.26 (m, 1H), 7.13 (m, 2H), 7.03 (m, 2H), 6.94 (m, 1H), 6.88 (d, *J* = 2.0 Hz, 2H), 4.13 (m, 1H), 4.00 (d, *J* = 4.1 Hz, 1H), 3.81 (dd, *J* = 13.6, 3.5 Hz, 1H), 3.64 (dd, *J* = 14.8, 5.1 Hz, 1H), ^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ (ppm): 205.03, 190.01, 164.00, 142.15, 135.79, 132.26, 132.26, 131.88, 129.82, 129.75, 129.66, 125.80, 122.35, 122.02, 77.22, 50.17, 43.30, EIMS m/z: 553.9 [$C_{26}H_{18}O_4Br_2^+$] (12%), 393.8 [$C_{17}H_{13}OBr_2^+$] (20%), 391.9 [$C_{17}H_{12}OBr_2^+$] (41%), 341 [$C_{17}H_{10}O_3Br^+$] (56%), 311 [$C_{16}H_8O_2Br^+$] (31%), 263 [$C_{11}H_{19}O_2Br^+$] (15%), 249 [$C_{12}H_9OBr^+$] (18%), 224 [$C_{10}H_8OBr^+$] (5%), 210.9 [$C_9H_6OBr^+$] (24%), 208.9 [$C_{10}H_9Br^+$] (30%), 204.1[$C_{13}H_{16}O_2^+$] (51%), 181.9 [$C_8H_6Br^+$] (60%), 149.0 [$C_9H_9O_2^+$] (34%), 121.0 [$C_7H_5O^+$] (44%), 102.0 [$C_6H_8^+$] (100%),101.0 [$C_5H_9O_2^+$] (35%), 92 [$C_6H_4O^+$] (22%), 75 [$C_6H_3^+$] (21%), 50 [$C_4H_2^+$] (16%), 26.9 [$C_2H_3^+$] (16%).

3-((4E)-1,5-bis(3,4,5-trimethoxyphenyl)-3-oxopent-4-enyl)-4-hydroxy-2H-chromen-2-one. **(6)**

The product obtained was brown solid, Yield: 72%, mp:171°C, ee: 90% *[S]*, UV λmax(nm): 218, IR *ν*(cm^{-1}): 1740 (C=O), 1730 (C=O of coumarin ring), 1650 (Ar C-H), 1340 (C-OH),^{1}H NMR (200 MHz, $CDCl_3$-*d*) δ (ppm): 11.46 (s, 1H), 7.94 (m, 2H), 7.81 (d, *J* = 8.1 Hz, 1H), 7.64 (m, 2H), 7.50 (m, 1H), 7.37 (d, *J* = 0.9 Hz, 1H), 7.31 (d, *J* = 7.8 Hz, 1H), 7.28 (d, *J* = 4.2 Hz, 1H), 6.35 (d, *J* = 1.2 Hz, 1H), 4.26 (d, *J* = 4.3 Hz, 1H), 4.04 (d, *J* = 5.9 Hz, 1H), 3.88 (s, 1H), 3.70 (m, 1H), 2.28 (s, 3H), 2.24 (s, 6H), 2.20 (s, 3H), 0.81 (s, 6H), ^{13}C NMR (50 MHz, $CDCl_3$-*d*) δ (ppm): 173.35, 172.93, 132.79, 124.18, 123.83, 116.80, 116.72, 116.70, 116.27, 105.46, 104.52, 104.34, 104.25, 77.43, 56.15, 29.47, 29.34, 29.27, 29.12, 29.08, 24.90, 24.86, EIMS m/z: 574.2 [$C_{32}H_{32}O_{10}^+$] (20%), 495.4 [$C_{26}H_{23}O_{10}^+$] (13%), 467.3 [$C_{25}H_{23}O_9^+$] (30%), 439.3 [$C_{28}H_{23}O_5^+$] (41%), 411.3 [$C_{23}H_{24}O_7^+$] (14%), 383.3 [$C_{22}H_{23}O_8^+$] (10%), 367.3 [$C_{22}H_{23}O_7^+$] (6%), 339.2 [$C_{21}H_{22}O_6^+$] (11%), 311.2 [$C_{19}H_{18}O_6^+$] (13%), 285.2 [$C_{24}H_{16}O_6^+$] (10%), 257.2 [$C_{18}H_9O_2^+$] (33%), 239.2 [$C_{18}H_8O^+$] (8%), 211.2 [$C_{14}H_{17}O_2^+$] (28%), 182.1 [$C_{13}H_{11}O^+$] (100%), 171 [$C_{12}H_{11}O^+$] (11%), 121 [$C_7H_5O^+$] (22%), 98 [$C_5H_6O_2^+$] (28%), 85 [$C_4H_5O_2^+$] (24%), 71[$C_4H_7O^+$] (36%), 57.1 [$C_3H_5O^+$] (76%), 43 [$C_2H_3O^+$] (64%), 41 [$C_3H_5^+$] (33%), 26.9 [C_2H_5] (14%).

Bioassay

In-vitro anticoagulant studies by plasma recalcification time (PRT) method

Anticoagulant potential of test compounds was determined by PRT method [26]. The blood samples were obtained from healthy volunteers in tubes containing 3.8% sodium citrate (9:1) in order to prevent the clotting process. Centrifugation (15 min, 3000 rpm) was carried out to obtain platelet poor plasma. The plasma 0.2 ml and 0.1 ml of different concentrations of test compounds (100, 300 and 1000 μM) and 0.3 ml of $CaCl_2$ (25 mM) were added together in a clean fusion tube and incubated at 37°C in a water bath. Warfarin was used as positive control. The clotting time was recorded with a stopwatch by tilting the test tubes every 5 second, results are presented in Fig 8. Calculted IC50 values of synthesized derivatives **(1-6)** and warfarin are summarized in Fig 9.

Antibacterial activities of coumarin derivatives by microplate almar blue assaymethod

Antibacterial activities were measured by microplate almar blue assay method by using DCM as solvent. Synthesized derivatives **(1-6)** of 4-hydroxycoumarin including warfarin were screened for their antibacterial activities against *Bacillus subtillis, Staphylococcus aureus* (gram-positive) and *Pseudomonas aeruginosa* , *Salmonella typhi, Escherichia coli* (gram-negative) strains of bacteria. The ofloxacin was used as a standard drug [27]. The applied concentration of compounds was 200μg/ml. The zone of inhibition was measured in mm (millimeters) and then %inhibition was calculated. Their results are summarized in Table-4. Among six synthesized derivatives only compound **(5)** showed good activity against both strains of gram positive bacteria.

Antifungal activities by agar tube dilution method

Antifungal activities of all synthesized derivatives **(1-6)** of 4-hydroxycoumarin were evaluated with the help of agar tube dilution method. Concentrations of samples were 400μg/ml in DMSO, the incubation temperature was 27°C and incubation period was seven days [28]. The tested fungal strains were *Trichphyton rubrum, Candida albicans, Aspergillus niger, Microsporum canis, Fusarium lini* and *Canadida glabrata*. The amphotericin B was used as standard drug. The antifungal activities of the compounds were measured in % inhibition. Synthesized compounds did not show antifungal activity.

Results and Discussion

Chemistry

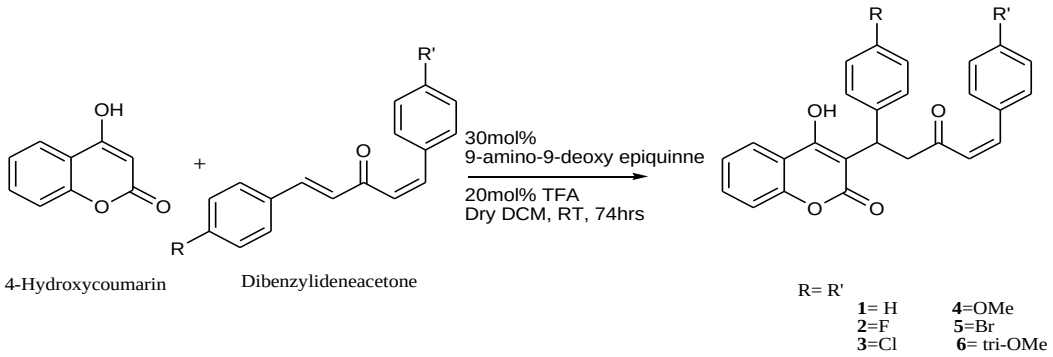
Syntheses involved two steps and in the first step dibenzylideneacetones were prepared by claisen-

schmidt condensation of acetone with variously substituted benzaldehydes in presence of NaOH. Synthesized dibenzylideneactones were recrystallized in ethanol. In the second step, 4-hydroxycoumarin was used as nucleophile in Michael addition reaction to the dibenzylideneacetone in presence of chiral catalyst 9-amino-9-deoxyepiquinine (Scheme 1). Such reactions are very effective and efficient for C-C bond formation and for creating chirality in the products.

In this case both 4-hydroxycoumarin (Michael donor) and dibenzylideneacetone (Michael acceptor) are achiral, whereas the product is chiral and chirality was induced by using 9-amino-9-deoxyepiquinine as chiral catalyst. Dried solvents were used in the reactions and 20 mol % trifluroacetic acid (TFA) was used as additive to enhance catalytic activity.

Mechanism involves nucleophilic attack of primary amine of the catalyst to carbonyl group of α,β-unsaturated ketones leading to formation of an intermediate which dehydrate to form trans-iminium cation. Then nucleophilic attack of 4-hydroxycoumarin takes place from the *si* face [25] of trans-iminium cation to obtain the desired S product in excess. At the end, the catalyst regenerates by hydrolysis. This mechanism is typical for covalent organocatalysis. Covalent binding of substrate normally requires high catalyst loading, typically ranging from 20-30 mmol%.

Enantiomeric excess of the products were measured by chiral HPLC. To find out configuration of major enantiomer in synthesized products, CD studies were carried out. The data in this regard is shown in Table 2. The CD values of synthesized compounds were compared with reported values of warfarin, provided in *JASCO* CD spectrometer's instrumental manual. Reported CD spectrum of *(S)* warfarin showed negative cotton effect at 220nm. All the synthesized compounds also showed negative cotton effect at 200 to 220nm indicating *(S)* enantiomer in excess. CD and VU absorption spectrum of compound (1) is given in Fig 1.



Scheme-1: General Scheme of synthesis.

Table-2: Enantiomeric excess (ee) and major CD absorption.

Compound	%ee	CD: λ_{max} [nm](mdeg)
1	**95*(S)***	**216 (-9)**
2	**24*(S)***	**212 (-30)**
3	**24*(S)***	**214 (-22)**
4	**89*(S)***	**218 (-40)**
5	**25*(S)***	**214 (-12)**
6	**90*(S)***	**216 (-30)**

Table-3: Binding affinity values of synthesized compounds with 3kp9.

Ligand	Binding Affinity kcal/mol
Warfarin	**-8.7**
Compound (1)	**-9.8**
Compound (2)	**-10.4**
Compound (3)	**-8.7**
Compound (4)	**-8.4**
Compound (5)	**-9.7**
Compound (6)	**-7.0**

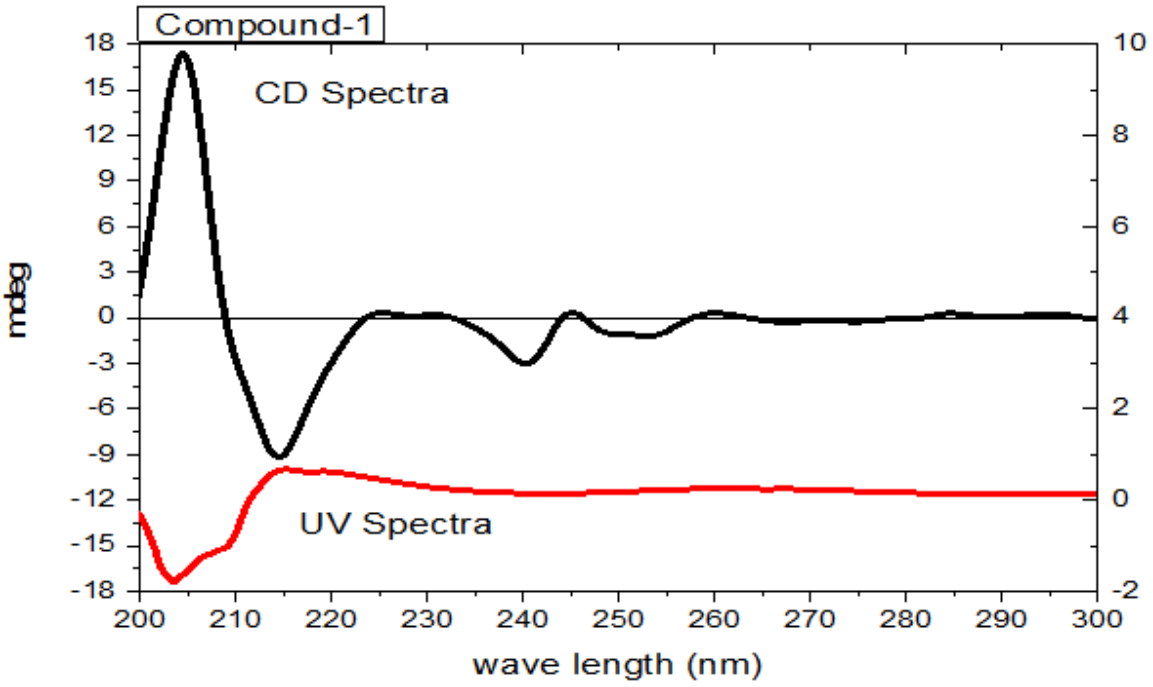


Fig. 1: CD and UV absorption spectra of compound **(1).**

Molecular docking analysis

Docking studies were carried out by using Discovery studio 2016, Chemsketch, AutoDock tools-1.5.6 and PyRx. First acquired crystal structure of 3kp9 in PDB (protein data bank) format from RCSB Protein data bank then removed already attached ligand. Drew synthesized ligands' structures in chemsketch and assigned smile notation and then in open babel added hydrogens and 3D coordinates to convert structure in PDB format. Then auto dock tools were used to add polar hydrogen, kollman charges, compute gaster charges and set grid box in protein structure and saved it as PDBQT format and then opened ligand in it and chose torsion for autodock upto 12 torsional degree of freedom (DOF) and then saved it too in PDBQT format. Opened both ligand and macromolecule in PyRx for docking and run AutoDock Vina [29].

The binding affinity values of synthesized derivatives **(1-6)** with 3kp9 are presented in Table 3.

Docking studies of 4-hydroxy-3-[(4E)-3-oxo-1,5-diphenylpent-4-en-1-yl]-2H-chromen-2-one. **(1)**

Binding affinity of compound **(1)** is greater than that of warfarin. It showed conventional van der waals forces of attraction with few amino acids of 3kp9 Fig 2. Also showed ***π***-sulfur, sulfur-***x***, ***π-π*** stacked and ***π***-alkyl bonding with, phenylalanine, cysteine, methionine, valine, threonine, and leucine

Docking studies of 3-[(4E)-1,5-bis(4-fluorophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(2)**

Compound **(2)** has highest binding affinity values i-e -10.4kcal/mol. Due to methyl substitution it also showed hydrogen bonding with leucine of 3kp9 (Fig 3).

Docking studies of 3-[(4E)-1,5-bis(4-chlorophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(3)**

Binding affinity of compound **(3)** is same as warfarin but connectivity with pocket amino acids of 3kp9 is different than warfarin. Warfarin showed carbon hydrogen bonding with glycine while compound **(3)** don not show any binding with glycine (Fig 4). Warfarin also showed conventional hydrogen bonding with threonine but Compound **(3)** gives week van der waal forces of attraction with threonine.

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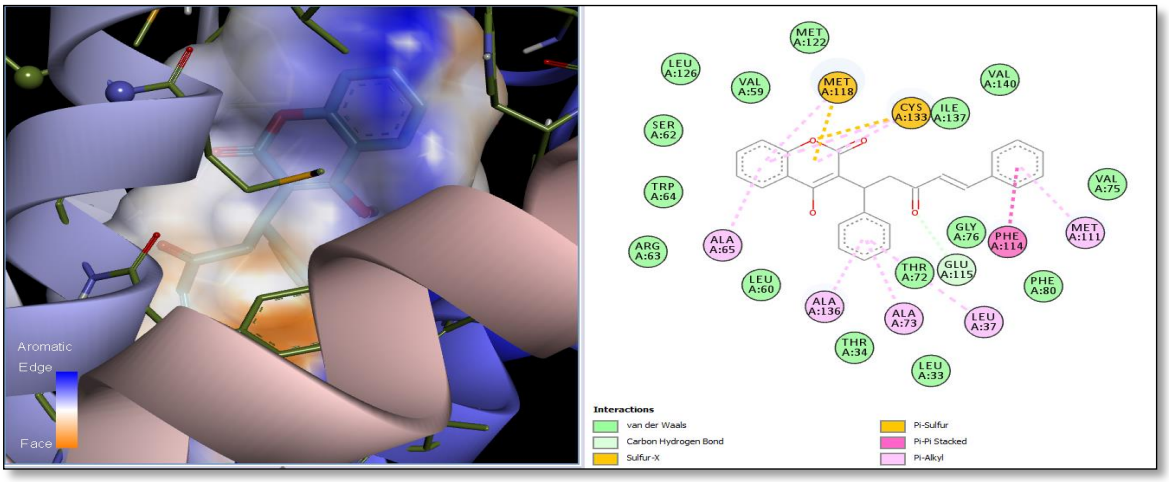

Fig. 2: 2D interaction of 3kp9 with compound **(1).**

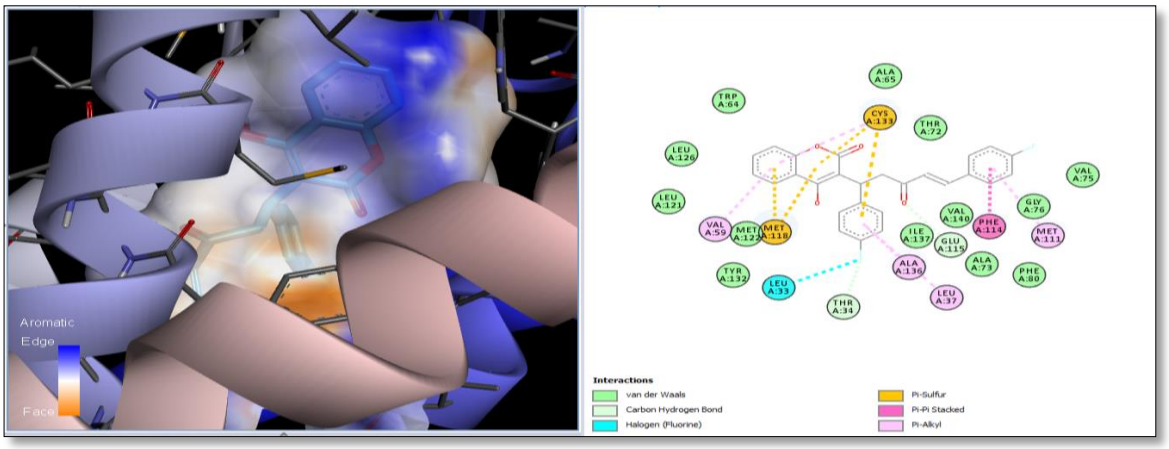

Fig. 3: 2D interaction of 3kp9 with compound **(2).**

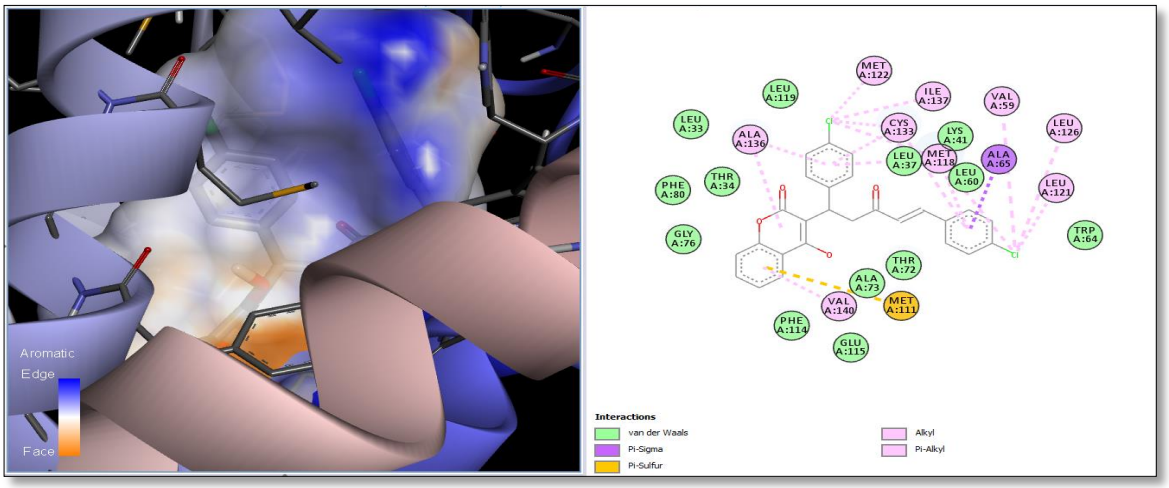

Fig. 4: 2D interaction of 3kp9 with compound **(3).**

*Docking studies of 3-((E)-1,5-bis(4-methoxyphenyl)-3-oxopent-4-enyl)-4-hydroxy-2H-chromen-2-one***. (4)**

Compound **(4)** do not shows conventional hydrogen bonding but like warfarin showed van der waal , Carbon hydrogen bond, π -sigma, π-alkyl and π-sulfur bonding with different amino acids of 3kp9 (Fig 5).

Docking studies of 3-[(4E)-1,5-bis(4-bromophenyl)-3-oxopent-4-en-1-yl]-4-hydroxy-2H-chromen-2-one. **(5**)

Compound **(5)** showed amide π-stacked and π-π stacked bonding with tryptophan and phenylalanine which is not present in other compounds (Fig 6). Its binding affinity with 3kp9 is -9.7kcal/mol which is much higher than warfarin.

Docking studies of 3-((E)-1,5-bis(3,4,5-trimethoxyphenyl)-3-oxopent-4-enyl)-4-hydroxy-2H-chromen-2-one. **(6)**

Compound **(6)** shows low binding affinity with 3kp9 than warfarin and in comparison to its *in-vitro* anticoagulant activity also shows less coagulation time than warfarin. But due to presence of methoxy groups rather than halo groups its toxicity might be lower than other analogues (Fig 7).

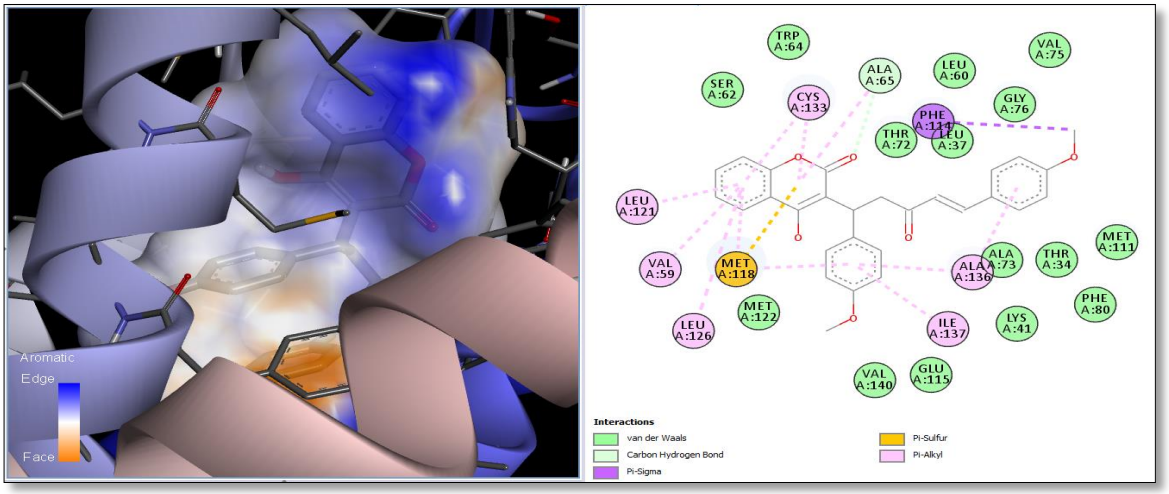

Fig. 5: 2D interaction of 3kp9 with compound **(4)**

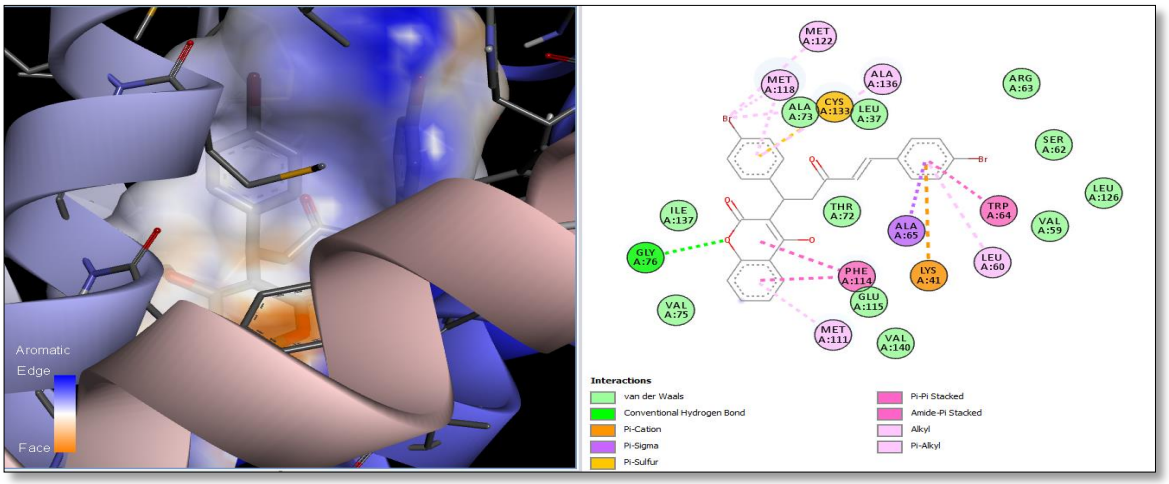

Fig. 6: 2D interaction of 3kp9 with compound **(5).**

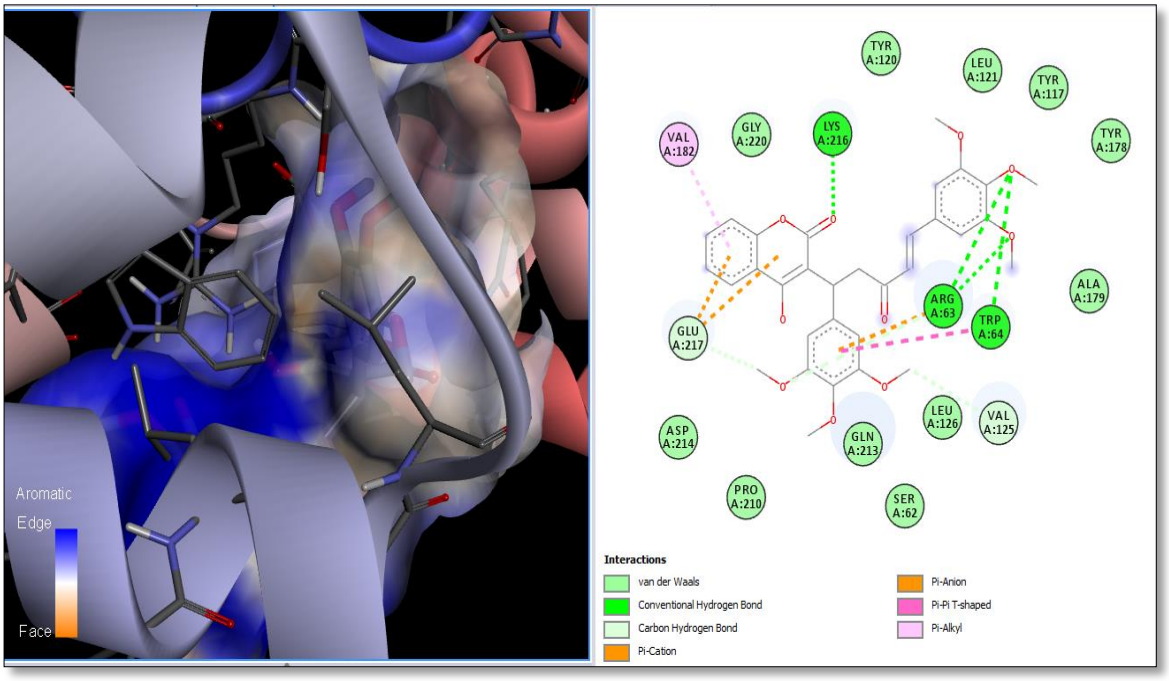

Fig. 7: 2D interaction of 3kp9 with compound **(6).**

Anticoagulant Activity

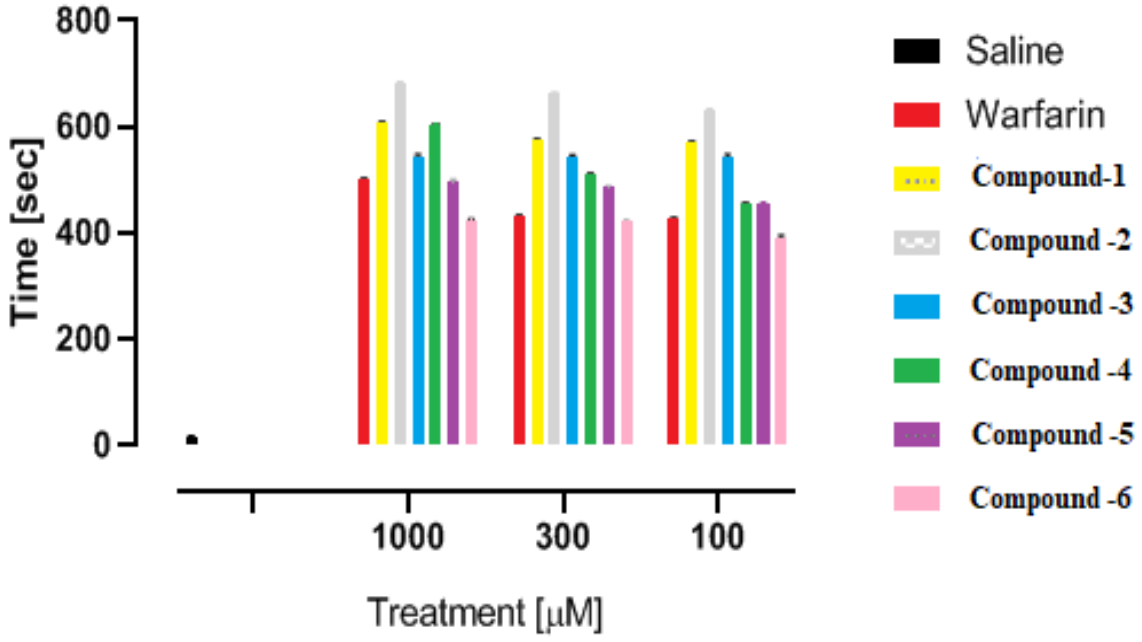

Fig. 8: Bar chart showing increase in plasma recalcification time (PRT) caused by different concentrations of Compound **1, 2, 3, 4, 5, 6** and warfarin. Data expressed as mean ± SEM, n=5, $^{***}P< 0.001$ vs. saline group, one way ANOVA with post-hoc Tukey's test.

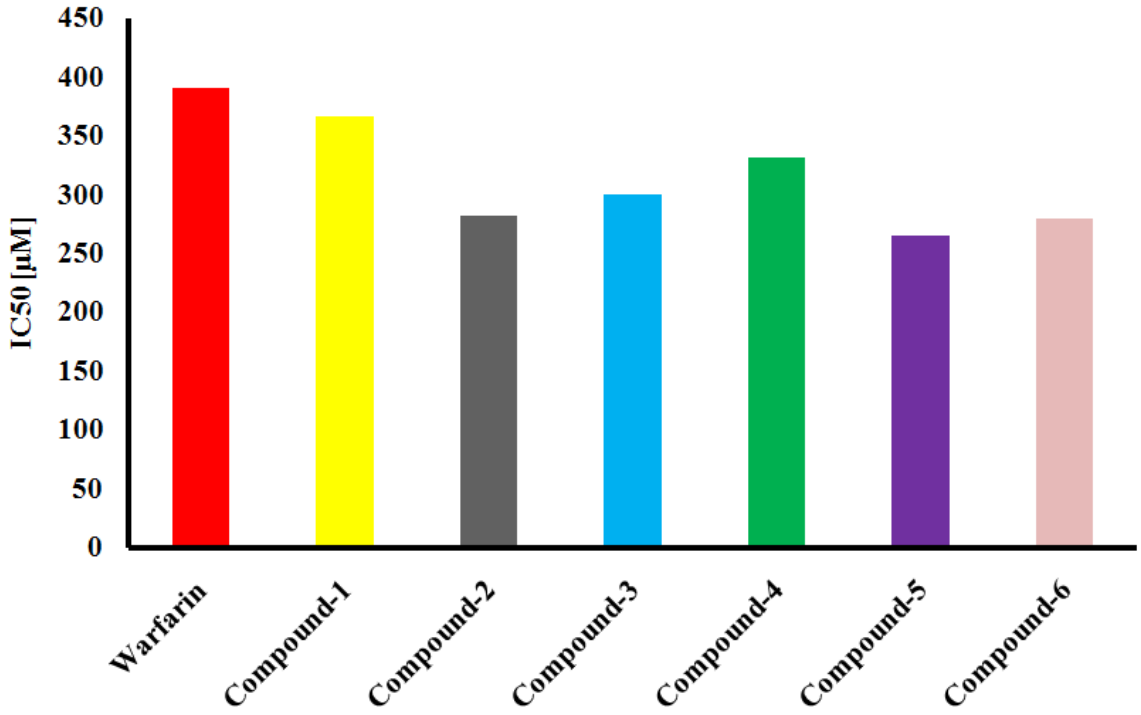

Fig. 9: Bar chart showing IC50 values of all synthesized compounds **(1-6)** in comparison with warfarin.

Table-4: Antibacterial activities of syntheiszed compounds.

Compound	% Inhibition *Bacillus subtillis*	% Inhibition *Staphylococcus aureus*	% Inhibition *Pseudomonas aeruginosa*	% Inhibition *Salmonella typhi*	% Inhibition *Escherichia coli*
Warfarin	**20.9**	-	-	-	**1.2**
(1)	**14.57**	-	**10.12**	-	**3.66**
(2)	-	-	-	-	-
(3)	-	-	-	-	-
(4)	**14.67**	**3.93**	**3.60**	-	-
(5)	**79**	**62**	-	-	-
(6)	-	-	-	-	-
Ofloxacin	**94**	**87**	**95**	**90**	**92**

"-" means that compoud showed no activity

IC50 values of warfarin are 390.70µM and IC50 of synthesized compounds are ranging from 366.70 to 264.51µM as given in Fig 9. All synthesized compounds showed better values as compared to standard drug warfarin. Compound **(5)** which has 4-bromophenyl substituent show IC50 value 264.51µM which is much better than warfarin. Compound **(2)** have 4-fluorophenyl substituent gives highest binding affinity values among all synthesized analogues i.e. -10.4kcal and IC50 values 282.53 µM. Which also show's a correlation in results of *in-silico* and *in-vitro* activity.

Antibacterial Activity

Results of antibacterial activity are sumerized in Table 4. Compound **(5)** showed good % inhibation against gram positive bacteria, while other compounds showed very low or no activity. Compound **(5)** gives 79% inhibation against *Bacillus subtillis* and 62% inhibition against *Staphyloccus aureus* strains of bacteria. This might be due to 4-bromophenyl substituent. It was observed that warfarin also did not show considerable % inhibition against these five strains of gram positive and gram negative bacteria.

Conclusions

Main objective of this research was stereoselective synthesis of chiral coumarin derivatives having mainly *(S)* configuration, keeping in view their medicinal potential and greater activity. The targeted synthesis was successfully done by reacting 4-hydroxycoumarin and substituted dibenzylideneacetones using chiral catalyst (9-amino-9-deoxyepiquinine). Synthesized products showed moderate to excellent enantiomeric excess ranging

from 24-95% as determined by chiral HPLC. Circular dichorism studies showed that S enantiomers were produced in excess amount, as literature studies of warfarin showed that S enantiomer is more active than *(R)* so getting *(S)* enantimer in excess was targeted.

Out of six, four compounds showed improved anticoagulant *in-silico* activitiy and all synthesized compounds showed improved *in-vitro* anticoagulant activity(IC50=366.70 to 264.51µM) as compared to standard drug warfarin(1C50=390.70µM). Besides anticoagulant activity, compound **(5)** showed 79% inhibition againt *Bacillus subtillis* and 62 % inhibition against *Staphylococcus aureus.* The synthesized compounds appear to be good candidates for further investigations to develop as anticoagulant drugs.

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