Enantioselective Synthesis, Computational Molecular Docking and Invitro Anticoagulant Activity of Warfarin-Based Derivatives

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Abstract

Warfarin containing coumarin ring system possesses excellent anticoagulant activity in its enantiomeric form (S). The present work designed to synthesize warfarin based derivatives enantioselectivily to explore their anticoagulant potential. The substituted chalcones were reacted with 4-OH coumarin in presence of chiral catalyst 9-amino-9-deoxyepiquinine to afford warfarin based analogues **5a-5k**. The structures of synthesized compounds **5a-5k** were confirmed by FTIR, ^{1}HNMR,^{13}CNMR and EIMS data. The enantioselectivity has been assured by determining the enantiomeric excess (ee) in chiral HPLC which exhibited 16-99%ee. The invitro anticoagulant activity of synthesized compounds **5a-5k** was evaluated by plasma recalcification time (PRT) method and it was found that most of the derivatives showed good anticoagulant activity specifically compound **5b** exhibited excellent results compared to warfarin. The compound **5b** displayed IC_{50} value 249.88μM better than standard warfarin (IC_{50} 408.70μM). The molecular docking studies has been performed against vitamin K epoxide reductase with PDBID 3kp9. The synthesized compounds binds well in active binding site of target enzyme. The derivative **5b** showed pi-stacking interactions with amino acid phenylalanine (PHE A 114).The antimicrobial activity of synthesized compounds has also been performed and results showed moderate antimicrobial activity. Based upon our results it is proposed derivative **5b** may act as lead compound to design more potent anticoagulant derivatives.

Introduction

Warfarin is a chiral compound used as an oral anticoagulant drug since 1950 to treat deep vein thrombosis, which is a process of blood clotting and movement of clots in other body parts.[1,2] The *(S)* enantiomer of warfarin possesses higher anticoagulant activity compared to its *(R)* enantiomer.[3] It decreases blood clotting by blocking an enzyme vitamin K epoxide reductase (VKOR), that activates vitamin K.[4] In spite of its advantage, there are many reported complications of warfarin such as internal bleeding,[4] tissue damage, purple toe syndrome and drug interaction.[5-7] It has been reported that tautomerism in warfarin is one of the major reasons of these unwanted interactions.[8] Tautomers of any compound mostly have different fingerprints, hydrophilicities, pKa values and electrostatic properties. Warfarin exists in roughly 40 tautomeric forms, [9,10] though two forms, keto and hemiketal, are major contributors (Fig. 1).

The computational molecular docking studies is a powerful tool currently used for rational drug designing and drug development. The insilico docking studies helps to identify the preferred orientation of the designed molecules to interact with the target protein.[11–14] The warfarin based derivatives **5a-5k** were designed on the basis of their potential to bind with target protein vitamin K epoxide reductase (VKOR). The target protein with PDBID 3kp9 was selected to perform the molecular docking of the synthesized warfarin based derivatives.

Enantioselective synthesis has versatile applications in the field of medicinal chemistry as one of the two enantiomers of any drug possesses high pharmacological activity compared to the other.[15–18] Many

small chiral organic molecule such as Cinchona alkaloids, chiral amine, Imidazolidinone and their derivatives are used as organocatalysts to attain enantioselectivity.[19–20] Cinchona alkaloids especially the quinine, have been known for their antimalarial activity also used in asymmetric organocatalysis during the last 2 decades. Beside the natural Cinchona alkaloids, a number of their derivatives, have been employed in diverse types of enantioselective syntheses by asymmetric catalysis.[21–23]

Keeping in view the aim of better alternatives of warfarin, eleven derivatives have been synthesized based upon their structural similarities with warfarin. The enantioselectivity of *(S)* enantiomer was achieved by using cinchona alkaloid based organocatalyst 9-amino-9-deoxyepiquinine. The target molecules **5a-5k** were designed in such a way to minimize the chances of tautomerism by replacing methyl group at 12th position (Fig. 1) with bulky phenyl ring.

Results And Discussion

Chemistry

Stereoselective synthesis of warfarin analogues were carried out through organocatalyzed Michael addition. The 4-hydroxycoumarin (**4a**) being Michael donor attacks at differently substituted chalcones **3a-3k** (Michael acceptors) results in the formation of title warfarin based derivatives 5**a-**5**k.** The structures of the final products 5**a-**5**k** having variables substitution pattern are presented in Fig. 2.

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 of trans iminium cation to obtain the desired *(S)* product in excess.[24] The catalyst used in this reaction was 40mol% as covalent binding of substrate requires high catalyst loading.

The FTIR spectra of final compounds showed –OH stretching absorption bands between 3200-3500cm^{-1} and intense stretching absorption for lactone C = O appears at 1700-1745cm^{-1} which confirms the structures. The presence of another stretching absorption at 1610-1640cm^{-1} is characteristic for C = O which confirms the presence of keto form in excess. The keto form is present in excess as compared to hemiketal tautomer also confirmed from ^{1}HNMR spectral data. The singlet for the hydroxyl group appeared above 8ppm which showed the presence of keto form. The aromatic protons and other signals appears in their acceptable regions which assure the formation of title compounds 5**a-**5**k**. The characteristic peaks for carbonyl carbons (153-165ppm) in the ^{13}CNMR spectra also confirms the presence of two carbonyl carbons in the structures. EIMS either gives molecular ion peak or typical fragments of coumarin ring generated in warfarin EIMS spectra at m/z 121 [$C_{11}H_7O_3^+$], 187 [$C_{11}H_7O_3^+$] and 93 [$C_6H_5O^+$] are present with different percentages.[25]

The absolute configuration of the synthesized compounds was determined by ultraviolet circular dichroism (UVCD) spectrum in the range 180-400nm while chiral HPLC was employed to find out the

enantiomeric excess. The intense band in the range of 190-223nm which is also present in the UV spectrum of the respective compound is due to allowed π-π* transition (Fig. 3a). The UVCD spectrum assured the (*S*)-configuration of the most potent derivative **5b** as the same negative cotton effect was observed in the UVCD spectrum of the standard (*S*)-warfarin.

All the synthesized analogues showed a negative cotton effect due to π-π* transition in the range of 190-223nm (Table 1). The assigned configuration of all the synthesized compounds is based on this comparison.

Table 1
CD data of synthesized warfarin analogues

Compound	%ee	CD: λ_{max} [nm] (mdeg)
5a	91*(S)*	215 (-54), 224 (+ 20)
5b	16 *(S)*	206 (-20), 204 (-20), 209 (+ 20)
5c	70*(S)*	193 (-58), 196(-18), 204(-5)
5d	60*(S)*	190 (-7), 203(-5)
5e	51*(S)*	194 (-20), 204(-28)
5f	29*(S)*	195 (-7), 202 (-7), 209 (-4)
5g	54*(S)*	203 (+ 20), 206 (-12), 222 (-30)
5h	41*(S)*	206 (-25), 216 (-5)
5i	24*(S)*	202 (-30), 204 (+ 15), 203 (-12), 213 (-26)
5j	98*(S)*	201 (-5), 204 (+ 40), 208 (-30)
5k	96*(S)*	204 (+ 10), 206 (-30), 210 (+ 10)

The enantiomeric excess (ee) determined by chiral HPLC showed that enantiomer present in excess is the (*S*) having 16%ee (Fig. 3b). The large peaks in HPLC chromatogram are due to *(R), (S)* of keto form while the very small peaks showed *(R), (S)* of hemiketal form respectively.

Biological Evaluation

Invitro Anticoagulant Activity

Venous blood was collected from healthy volunteer and extracted plasma from it and tested for coagulation time at 37^{o}C in comparison to warfarin (Fig. 4). The warfarin based derivatives 5**a**-5**k** were designed to restrict the formation of tautomeric forms as in compounds 5**a**-5**k** methyl group is substituted with aryl group in order to decrease rotation across dihedral bond and to avoid formation of hemiketal form.

According to *invitro* anticoagulant studies most of the synthesized compounds showed better activity as compared to standard warfarin. The compound **5b**, having unsubstituted phenyl rings on coumarin nucleus showed excellent activity with IC_{50} 249.88μM (Fig. 5) values in comparison to all synthesized compounds and standard warfarin (IC_{50} = 408.70μM). The compounds **5c, 5f** and **5g** have similar anticoagulant activity (IC_{50} = 299.93μM) are more active than standard warfarin but are less potent than compound **5b**. All of the synthesized derivatives possess substituted phenyl rings on coumarin nucleus but the most potent compound has unsubstituted phenyl rings. The presence of unsubstituted phenyl rings in compound **5b** play vital role in its anticoagulant activity. However, in case of the analogue in which chloro group is substituted on both aromatic rings (compound **5i**), the coagulation time was greater than warfarin but is less active based upon its IC_{50} value (419.22μM).

Antimicrobial Activity

It is already reported that many natural and synthetic coumarins possesses antimicrobial activities.[26, 27] The warfarin based derivatives 5**a**-5**k** have also been evaluated for their potential against bacterial and fungal strains. The results showed that the derivatives displayed moderate to good antimicrobial activity against the selected bacterial and fungal strains. Compound **5d** showed 68% inhibition against *staphylococcus aureus* and compound **5g** showed 68% inhibition against *Bacillus subtillis*. The compound **5f** showed 70% inhibition against *candida albicans* which possesses amino substituted phenyl ring. The rest of the derivatives showed moderate to no activity against different tested strains (Table 2).

Table 2
Percentage (%) inhibition of synthesized warfarin analogues for antibacterial screening and antifungal activity.

	Antibacterial activity						Antifungal activity					
	E.c	**B.s**	**S.a**	**P.a**	**S.t**		**T.r**	**C.a**	**A.n**	**M.c**	**F.l**	**C.g**
Warfarin	-	20.9	-	-	-		-	-	-	-	-	2.5
5a	-	22	-	-	-		-	-	-	2.5	-	-
5b	-	-	-	-	-		-	-	-	-	-	-
5c	-	-	-	-	-		-	-	-	2.5	-	-
5d	-	24.5	68	-	-		-	-	-	-	-	-
5e	-	-	-	-	-		-	-	-	-	-	-
5f	-	-	30	-	-		-	70	-	10	-	-
5g	-	68	-	-	-		-	-	-	15	-	-
5h	-	-	34	-	-		-	-	-	-	-	-
5i	-	-	-	-	-		-	-	-	-	-	-
5j	-	-	-	-	-		-	-	-	-	-	-
5k	-	35	-	-	-		-	-	-	-	25	
Ofloxacin	92	94	87	95	90	Miconazole	113	97.8	-	98.1	73.5	49.5
(-) no activity												
E.c *(Echerichia coli)*, **B.s** *(Bacillus subtillis)*, **S.a** *(Staphylococcus aureus)*, **P.a** (*Pseudomonas aeruginosa)*, **S.t** *(Salmonella typhus);* **T.r** *(Trichphyton rubrum)*, **C.a** *(Candida albicans)*, **A.n** *(Aspergillus Niger)*, **M.c** *(Microsporum canis)*, **F.l** *(Fusarium lini)*, **C.g** *(Canadida Glabrata)*												

Docking Studies of Compounds 5**a**-5**k**

The computational molecular docking studies of the synthesized warfarin based derivatives 5a-5k was performed against vitamin K epoxide reductase with PDBID 3kp9. The structure provided by Li et. al. (PDB ID 3kp9) and multiple sequence alignment comparison predicted that the core of all VKOR's consist of four TMs including those in mammals.[28] The homologues of vitamin K epoxide reductase (VKOR) have been identified in several different organisms, including various plants, archaea, bacteria, etc.[29–31] The structural homology has been observed in different species i.e reduced (thiol) and oxidized (disulphide-bonded) states as well as pair of cysteine and a conserved serine/threonine. The docking results of synthesized derivatives 5a-5k were compared with the standard warfarin which is well known vitamin K antagonist. Most of synthesized compounds have better binding affinity with the target protein compared to the standard warfarin (Fig. 6).

The most potent derivative **5b** possess unsubstituted phenyl ring showed good binding affinity with binding energy value − 9.5 kcal/mol. In compound **5b** instead of methyl group the bulky phenyl rings are present which form pi-stacking interaction with the target protein (Fig. 7). The binding affinity of the compound **5b** is found to be better than the standard warfarin − 8.8 kcal/mol.

Conclusion

The eleven warfarin analogues were successfully synthesized having different substitution pattern to evaluate their role in anticoagulant potential. The enantioselectivity ranged from 16–99% confirmed by chiral HPLC and UVCD results assured that derivatives have *(S)* enantiomer as major product. Moreover, docking studies clearly showed the better binding affinity of the keto form than hemiketal form of warfarin. The invitro anticoagulant activity results evaluated by plasma recalcification time (PRT) method showed that most of the derivatives have good anticoagulant activity especially derivative **5b** displayed excellent results. The most potent derivative **5b** having IC_{50} value 249.88μM better than standard warfarin (IC_{50} 408.70μM). The molecular docking results performed against vitamin K_1 epoxide reductase with PDB ID 3kp9, showed most of the synthesized derivatives have good binding affinity as compared to standard warfarin. The most potent compound **5b** showed pi-stacking interactions with amino acid phenylalanine (PHE A: 114). The antimicrobial activity of the synthesized compounds has also been performed and results showed moderate antimicrobial activity. Based upon our results it has been concluded that derivative **5b** may act as lead compound to design more potent anticoagulant derivatives.

Materials And Methods

All reagents and chemicals were purchased from sigma Aldrich, Fluka, BDH and used as received. The ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 or $CDCl_3$-*d* on Bruker biospin ICON-NMR spectrometer and AVANCE AV-300 spectrometer (US) using TMS as internal reference. The apparent resonance multiplicity is described as s (singlet), bs (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 FTIR spectrophotometer by Perkin Elmer (USA). Melting point was determined in a capillary tube using a Gallenkamp (UK) electrothermal melting point apparatus. The enantiomeric excess was determined by chiral column, Lux 5μm Cellulose-1, LC Column 250x4.6 mm (USA). The instrument used for this technique was HPLC PerkinElmer (USA). Analysis of sample with an 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 (EIMS) yield mostly fragment ions. Molecular ions are not always observed. High resolution features with ca 6000–8000 resolution. CD spectra were measured by JASCO-815 CD spectrometer (USA) in static mode. For CD measurements, the warfarin analogues were dissolved in a mixture of aqueous phosphoric acid and acetonitrile having ratio 4:6 and pH 2.

General procedure for synthesis of analogues of 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one

Scheme 1 presented complete synthetic strategies. In first step α,β unsaturated ketones (**3a-3k)** (chalcones) were synthesized[32,33] by taking 1:1 mole ratio of substituted aldehyde (**1a-1e)** and substituted ketone (**2a-2f)** in a two neck round bottom flask equipped with magnetic stirrer and dissolve it in minimum amount of ethanol/methanol at room temperature and then add 10% NaOH drop wise in above solution and leave for 2hrs at room temperature and at the end of reaction neutralized by dilute HCl and extracted by ethanol and dried in a rotary evaporator. Solid product obtained was purified by column chromatography by using 7:3 ratio of n-Hexane and ethyl acetate respectively and recrystallization in ethanol and their structures were confirmed by the comparison of their melting points and IR data with the reported values.

Then to prepare warfarin analogues **5a-5k**; 4-hydroxycoumarin (0.32mmol), α,β unsaturated ketones (0.2mmol), and 20mol % 9-amino-9-deoxy epiquinine were dissolved in 20ml of dry DCM in a two neck round bottom flask equipped with magnetic stirrer, followed by addition of 40 mol % trifluoracetic acid (TFA) as additive.[24] The reaction mixture was stirred at room temperature for 3 to 4 days at room temperature and the progress of reactions was monitored by TLC visualized under UV lamp and developed in vanillin spray. The crude products were purified by column chromatography using different ratios of n-hexane and ethyl acetate. A single spot of product was obtained on TLC after column chromatography and purity was further verified by HPLC.

Chiral HPLC

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

Enantiomeric excess (% ee) = $\frac{[S]-[R]}{[S]+[R]}X100$

Circular Dichroism Studies

CD spectra were measured by *JASCO-815* CD spectrophotometer in static mode. For CD measurements, 200µg/ml of samples were prepared by dissolving synthesized compounds in a mixture of aqueous phosphoric acid and acetonitrile (4:6) having pH 2.0.

As regards the measuring parameters, wavelength range was 170-400nm, data interval time 1sec, response time 2sec, spectral band width 1nm, number of accumulations 3, and optical path length 10mm were used as provided in *JASCO* CD spectrometer's instrumental manual for CD measurement of warfarin.

All the synthesized products showed maximum UV absorbance band between 190 to 220nm. In CD, the cotton effect of the band at the value of the maximum absorption of UV was compared with the reported cotton effect of warfarin provided in *JASCO* CD spectrometer's instrumental manual. A negative cotton

effect in CD in particular absorption indicates the *(S)* enantiomer in excess. The CD spectra are given in Fig. 8.

Tautomerism

The percentage of keto-hemiketal tautomers was determined by following the already reported method.[34] Briefly, the ^{1}HNMR peaks integration from simple one dimensional spectra are used to determine the ratio of tautomers. The percentages of the keto-hemiketal tautomers is presented in Table 3.

Table 3
The keto-hemiketal percentage of synthesized warfarin analogues

Compound	Solvent	Keto%	Hemiketal%
5a	$CDCl_3$	64	36
5b	$CDCl_3$	69	31
5c	$CDCl_3$	52	48
5d	$CDCl_3$	85	15
5e	$CDCl_3$	60	40
5f	DMSO	62	38
5g	$CDCl_3$	43	53
5h	$CDCl_3$	49	51
5i	$CDCl_3$	60	40
5j	$CDCl_3$	54	46
5k	$CDCl_3$	51	49

4-hydroxy-3-[1-(4-hydroxy-3-methoxyphenyl)-3-oxobutyl]-2H-chromen-2-one (5a)

Solid; Yield 90%; mp 139-141^{o}C; ee 91% *[S]*; IR (acetone) ν_{max} 1720 (C = O), 1625 (C = O), 1510 (C-H), 1380 (O-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.69 (1H, bs, OH), 7.80 (1H, m, ArH), 7.43 (1H, m, ArH), 7.20 (2H, m, ArH), 6.88 (3H, m, ArH), 5.46 (1H, s, OH), 4.08 (2H, m, CH_2 Keto), 3.77 (3H, s, OCH_3), 2.35 (1H, m, CH of steric center), 1.66 (2H, s, CH_2); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 162.1 (C = O), 161.2 (C = O), 159.6 (C-OH), 158.6 (ArC), 152.9 (ArC), 145.0 (ArC), 135.0 (ArC), 132.9 (ArC), 132.0 (ArC), 123.9 (ArC-OH), 123.6 (ArC), 122.74 (ArC), 117.0 (ArC), 116.6 (ArC), 115.0 (ArC), 114.0 (ArC), 111.1 (ArC), 99.0 (CH), 60.4 (CH_2 Keto), 56.0 (CH), 42.7 (CH_2), 39.8 (CH_3), 33.6, 29.6 (CH); EI MS *m/z* [M^{+1}] 354.1 [$C_{20}H_{18}O_6^{+}$] (40%).

4-hydroxy-3-(3-oxo-1,3-diphenylpropyl)-2H-chromen-2-one (5b)

Solid; Yield 80%; mp150^{o}C; ee 16% *[S]*; IR (acetone) ν_{max} 1741 (C = O), 1677 (C = O), 1570 (C-H), 1380 (O-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.78 (1H, s, OH), 8.03 (2H, m, ArH), 8.00 (1H, s, ArH), 7.94 (1H, d, J = 1.7 Hz, ArH), 7.90 (1H, d, J = 1.7 Hz, ArH), 7.54 (1H, m, ArH), 7.46 (1H, d, J = 1.4 Hz, ArH), 7.42 (1H, q, J = 1.7 Hz, ArH), 7.38 (1H, m, ArH), 7.32 (1H, s, ArH), 7.27 (1H, m, ArH), 7.24 (2H, d, J = 1.0 Hz, ArH), 7.14 (1H, m, Ar H), 4.86 (1H, d, J = 2.4 Hz, CH_2), 4.05 (1H, m, CH of steric center), 3.77 (1H, d, J = 2.4 Hz, CH_2), 3.68 (1H, d, J = 2.4 Hz, CH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 161.1 (C = O), 152.9 (C = O), 139.9 (C-OH), 134.42 (C-OH), 131.7 (ArC), 128.9 (ArC), 128.7 (ArC), 128.2 (ArC), 126.7 (ArC), 124.0 (ArC), 116.2 (ArC), 77.2 (CH), 45.1 (CH), 35.2 (CH), 29.6 (CH_2); EI MS m/z [M^{+1}] 370.2 [$C_{24}H_{18}O_4^{+}$] (15%).

4-hydroxy-3-(1-(4-hydroxy-3-methoxyphenyl)-3-oxo-3-phenylpropyl)-2H-chromen-2-one (5c)

Solid; Yield 68%; m.p. 154^{o}C; ee 70% *[S]*; IR (acetone) ν_{max} 1743 (C = O), 1607 (C = O), 1513 (C-H), 1360 (O-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.76 (1H, s, OH), 7.66 (1H, s, ArH), 7.63 (1H, s, ArH), 7.64 (1H, s, ArH),7.62 (1H, s, ArH), 7.61 (2H, s, ArH), 7.48 (2H, s, ArH), 7.46 (2H, s, ArH), 7.45 (1H, s, ArH), 7.35 (1H, s, ArH), 5.35 (1H, s, Ar-OH), 4.47 (1H, d, CH_2, J = 4.0 Hz), 3.83 (1H, d, CH, J = 6.0 Hz), 3.80 (3H, s, OCH_3), 3.76 (1H, m, CH), 3.55 (1H, d, J = 6.0 Hz, CH); ^{13}C NMR (DMSO, 50 MHz) δ 206.5 (C = O), 165.7 (C = O), 161.9 (C-OH), 153.5 (ArC), 132.7 (ArC), 123.9 (ArC), 123.2 (ArC), 116.3 (ArC), 115.8 (ArC), 90.8 (CH), 56.0 (CH), 55.5 (CH_2), 30.6 (OCH_3), 19.6 (CH); EI MS m/z [M^{+1}] 416.1 [$C_{25}H_{20}O_6^{+}$] (5%).

4-hydroxy-3-(3-(naphthalen-3-yl)-3-oxo-1-phenylpropyl)-2H-chromen-2-one (5d)

Solid; Yield 60%; mp 210 ºC; ee 60% *[S]*; IR (acetone) ν_{max} 1715 (C = O), 1670 (C = O), 1508 (C-H), 1384 (O-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.88 (1H, s, OH), 8.18 (1H, d, ArH, J = 1.8 Hz), 8.05 (1H, m, ArH), 7.86 (1H, d, ArH J = 6.0 Hz), 7.79 (2H, d, J = 5.3 Hz, ArH), 7.69 (1H, dd, J_1 = 8.7 Hz, $J_2$1.8 Hz, ArH), 7.55 (1H, m, ArH), 7.37 (2H, q, ArH, J = 1.6 Hz), 7.31 (1H, m, ArH), 7.27 (2H, q, J = 1.5 Hz, ArH), 7.23 (1H, m, ArH), 5.92 (1H, d, J = 5.0 Hz, CH_2), 4.70 (1H, d, J = 4.9 Hz, CH_2), 2.10 (1H, s, CH), 1.17 (1H, m, CH, steric center); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 167.1 (C = O), 133.4 (C-OH), 132.9 (ArC), 129.7 (ArC), 128.6 (ArC), 127.1 (ArC), 126.6 (ArC), 123.9 (ArC), 122.6 (ArC), 77.1 (CH), 36.7 (CH_2), 32.4 (CH_2). 27.28 (CH); EIMS peak m/z 403.1 [$C_{27}H_{15}O_4^{+}$] (20%), 402.1 [$C_{27}H_{14}O_4^{+}$] (65%), 403.1 [$C_{27}H_{15}O_4^{+}$] (20%), 326.1 [$C_{21}H_{10}O_4^{+}$] (28%), 325.1 [$C_{21}H_9O_4^{+}$] (100%), 282.1 [$C_{17}H_{14}O_4^{+}$] (16%), 265 [$C_{17}H_{13}O_3^{+}$] (3%), 252.1 [$C_{16}H_{12}O_3^{+}$] (12%), 176.1 [$C_{11}H_{12}O_2^{+}$] (6%), 127 [$C_6H_7O_3^{+}$] (14%), 121 [$C_7H_5O_2^{+}$] (12%), 93 [$C_6H_5O^{+}$] (3%), 77.1 [$C_6H_5^{+}$] (6%), 51.1 [$C_4H_3^{+}$] (4%), 26.9 [$C_2H_3^{+}$] (6%).

4-hydroxy-3-(1-(4-hydroxy-3-methoxyphenyl)-3-(2-hydroxyphenyl)-3-oxopropyl)-2H-chromen-2-one (5e)

Solid; Yield 75%; mp 192ºC; ee 51% *[S]*; IR (acetone) ν_{max} 1700 (C = O), 1620 (C = O), 1480 (C-H), 1390 (O-H) cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 7.94 (3H, d, J = 8.4 Hz, ArH), 7.64 (1H, m, ArH), 7.54 (1H, dd, J = 7.1 1.5 Hz, ArH), 7.47 (1H, m, ArH), 7.35 (1H, d, J = 5.3 Hz, ArH), 7.32 (2H, s, ArH), 6.80 (1H, d, J = 8.2 Hz, ArH), 6.65 (1H, m, ArH), 6.0 (1H, s, OH), 5.25 (1H, m, CH), 4.26 (2H, d, J = 4.3 Hz, CH_2), 4.21 (1H, s, OH), 4.15 (1H, dd, J_1 = 1.7 Hz, J_2 = 5.9 Hz, CH), 4.09 (1H, s, Ar-OH), 3.79(1H, d, CH), 3.68 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 200.4 (C = O), 166.0 (C = O), 162.2 (C-OH), 161.7 (C-OH), 148.3 (ArC), 146.7 (ArC), 146.6 (ArC), 135.8 (ArC), 135.3 (ArC), 132.5 (ArC), 130.2 (ArC),124.5 (ArC), 124.4 (ArC), 121.6 (ArC), 120.8 (ArC), 120.5 (ArC),116.3 (ArC), 116.1 (ArC), 112.2 (ArC), 102.5 (CH), 56.2 (OCH_3), 47.1 (CH), 38.1(CH_2); EI MS m/z [M^{+1}] 432.1 [$C_{25}H_{20}O_7^+$] (3%).

3-[3-(4-aminophenyl)-3-oxo-1-phenylpropyl]-4-hydroxy-2H-chromen-2-one (5f)

Solid; Yield 80%; mp 137 ºC; ee 29% *[S]*; IR (acetone) ν_{max} 3400 (N-H), 1700 (C = O), 1610 (C = O),1585 (N-H), 1495 (C-H), 1300 (O-H) cm^{-1}; ^1H NMR (DMSO, 200 MHz) δ 7.86 (2H, d, J = 1.7 Hz, ArH), 7.82 (2H, d, J = 1.9 Hz, ArH), 7.66 (2H, ddd, J_1 = 8.6 Hz, J_2 = 7.1 Hz, J_3 = 1.7 Hz, ArH), 7.39 (4H, m, ArH), 7.27 (2H, m, ArH), 7.11 (1H, m, ArH), 5.61 (2H, s, NH_2), 2.24 (1H, m, CH), 2.22 (1H, m, CH, steric center), 2.0 (1H, m, OH), 1.25 (1H, d, CH2, J = 7.2 Hz), 1.09 (1H, m, CH_2); ^{13}C NMR (DMSO, 50 MHz,) δ 167.5 (C = O), 165.6 (C = O), 161.9 (C-OH), 153.5 (ArC-NH_2), 152.4 (C-OH) 132.7 (ArC-OCH_3), 123.9 (ArC), 123.2 (ArC),119.8 (ArC), 116.3 (ArC), 115.7 (ArC-OH), 103.96 (ArC), 90.9 (C-H), 20.7 (CH_2), 14.4 (CH); EI MS peak m/z 367.1 [$C_{24}H_{17}O_3N^+$] (9%), 290.1 [$C_{19}H_{16}O_2N^+$] (16%), 223.1 [$C_{14}H_{511}O_2N^+$] (14%),162 [$C_9H_6O_3^+$] (43%) 121 [$C_7H_5O_2^+$] (12%), 120.0 [$C_7H_4O_2^+$] (100%), 93 [$C_6H_7N^+$] (12%), 92 [$C_6H_6N^+$] (71%), 77.1 [$C_6H_5^+$] (15%), 51.0 [$C_4H_3^+$] (14%), 26.9 [$C_2H_3^+$] (6%).

3-[3-(4-chlorophenyl)-3-oxo-1-phenylpropyl]-4-hydroxy-2H-chromen-2-one (5g)

Solid; Yield 76%; mp166 ºC; ee 54% *[S]*; IR (acetone) ν_{max} 1720 (C = O), 1625 (C = O), 1500 (C-H), 1300 (O-H) cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz,) δ 9.52 (1H, s, OH), 7.97 (1H, t, J = 1.9 Hz, ArH), 7.91 (2H, m, ArH), 7.66 (1H, t, ArH, J = 3.4 Hz), 7.62 (1H, d, ArH, J = 3.4 Hz), 7.49 (1H, d, ArH, J = 4.0 Hz), 7.45 (1H, d, ArH, J = 2.4 Hz), 7.42 (2H, t, J = 1.9 Hz, ArH), 7.38 (1H, d, J = 1.6 Hz, ArH), 7.26 (2H, m, ArH), 7.14 (1H, m, ArH), 4.86 (1H, dd, J_1 = 9.9 Hz, J_2 = 2.5 Hz, CH_2), 4.37 (1H, m, CH, steric center), 3.68 (1H, dd, J_1 = 19.1 Hz, J_2 = 2.6 Hz, CH_2), 2.28 (1H, s, OH), 1.60 (1H, d, J = 6 Hz, CH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 201.2 (C = O), 167.7 (C = O), 152.8 (C-OH), 135.2 (ArC), 132.5 (ArC), 132.2 (ArC), 131.8 (ArC), 130.8 (ArC), 130.0 (ArC), 129.2 (ArC), 128.8 (ArC), 128.2 (ArC), 128.0 (ArC), 116.2 (C-H), 68.2 (CH_2), 38.8 (CH), 22.9 (CH); EI MS m/z [M^{+1}] 404.2 [$C_{24}H_{17}O_4Cl^+$] (5%).

3-(3-(4-chlorophenyl)-1-(3,4,5-trimethoxyphenyl)-3-oxopropyl)-4-hydroxy-2H-chromen-2-one (5h)

Solid; Yield 65%; mp 110 ºC; ee 41% *[S]*; IR (acetone) ν_{max} 1725 (C = O), 1600 (C = O), 1510 (C-H), 1330 (O-H) cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 7.95 (1H, m, ArH), 7.65 (1H, t, J = 3.0 Hz, ArH), 7.61 (1H, d, J = 2.5 Hz, ArH), 7.46 (1H, m, ArH), 7.37 (1H, dd, J_1 = 8.6 Hz, J_2 = 3.8 Hz, ArH), 7.29 (1H, s, ArH), 7.13 (1H, m, ArH), 7.03 (1H, m, ArH), 6.29 (1H, s, ArH), 6.12 (1H, s, ArH), 4.16 (1H, d, J = 1.6 Hz, CH_2), 4.14 (1H, d, J = 1.8 Hz, CH_2), 3.85 (3H, s, OCH_3), 3.69 (1H, m, OH), 3.63 (1H, m, OCH_3), 3.58 (1H, s, CH, steric center), 3.45 (1H, d, J = 2.6 Hz, CH), 3.44 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 206.9 (C = O), 167.7 (C = O), 153.0 (C-OH), 152.9 (ArC), 134.3 (ArC-Cl), 132.5 (ArC), 131.5 (ArC), 130.9 (ArC), 129.4 (ArC), 128.8 (ArC), 128.7 (ArC), 128.50 (ArC), 126.55 (ArC), 107.4 (ArC), 106.2 (CH), 105.2 (ArC), 68.2 (CH_2), 56.2 (CH), 56.16 (OCH_3), 55.82 (OCH_3), 38.7 (OCH_3), 30.4 (OCH_3), 22.9 (OCH_3); EIMS peak m/z 383.5 [$C_{21}H_{16}O_5Cl^+$] (2%), 279.2 [$C_{18}H1_5O_3^+$] (6%), 212.1 [$C_{14}H_{12}O_2^+$] (18%), 167.1 [$C_{12}H_7O^+$] (28%), 149.0 [$C_9H_9O_2^+$] (100%), 121.1 [$C_7H_5O^+$] (10%), 71[$C_4H_7O^+$] (21%), 57 [$C_3H_5O^+$] (35%), 43[$C_2H_3O^+$] (24%).

3-(1,3-bis(4-chlorophenyl)-3-oxopropyl)-4-hydroxy-2H-chromen-2-one (4i)

Solid; mp 122 ºC; Yield 62%; ee 24% *[S]*; IR (acetone) ν_{max} 1710 (C = O), 1625 (C = O), 1500 (C-H), 1360 (O-H) cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 9.64 (1H, s, OH), 7.96 (1H, d, J = 1.9 Hz, ArH), 7.93 (2H, d, J = 2.0 Hz, ArH), 7.89 (1H, d, J = 1.7 Hz, ArH), 7.42 (2H, d, J = 2.1 Hz, ArH), 7.29 (1H, d, J = 2.2 Hz, ArH), 7.26 (2H, d, J = 2.6 Hz, ArH), 7.22 (1H, s, ArH), 7.12 (1H, m, ArH), 5.19 (1H, dd, J_1 = 10.2 Hz, J_2 = 1.6 Hz, CH_2), 4.80 (1H, dd, J_1 = 10.2 Hz, J_2 = 2.3 Hz, CH_2), 4.27 (1H, m, CH, steric center), 3.67 (1H, d, J = 2.3 Hz, CH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 201.1 (C = O), 173.28 (C = O), 165.2 (C-OH), 157.8 (ArC), 147.3 (ArC-Cl), 133.1 (ArC-Cl), 131.9 (ArC), 130.0 (ArC), 129.5 (ArC), 129.2 (ArC), 128.2 (ArC), 123.9 (ArC), 117.5 (ArC), 116.2 (ArC), 62.11(CH_2), 34.0 (CH), 29.5 (CH), 14.10 (CH); EIMS peak m/z 429.1 [$C_{23}H_{18}O_4Cl_2^+$] (3%), 383.4 [$C_{22}H_{20}O_4Cl^+$] (3%), 265 [$C_{17}H_{13}O_3^+$] (2%), 183 [$C_{13}H_{11}O^+$] (10%), 167 [$C_9H_{11}O_3^+$] (30%), 149.0 [$C_9H_9O_2^+$] (100%),121 [$C_{11}H_7O_3^+$] (8%), 93 [$C_6H_5O^+$] (12%), 71 [$C_4H_7O^+$] (33%), 57 [$C_3H_5O^+$] (60%).

3-(3-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-oxopropyl)-4-hydroxy-2H-chromen-2-one (5j)

Solid; As product was sticky so melting point cannot be determined. Yield 60%; ee 99.98% *[S]*; IR (acetone) ν_{max} 1745, 1600, 1480, 1300 cm^{-1}; ^1H NMR ($CDCl_3$, 200 MHz) δ 7.71 (1H, s, ArH), 7.65 (2H, d, J = 3.3 Hz, ArH), 7.64 (1H, s, ArH), 7.62 (2H, d, J = 3.3 Hz, ArH), 7.55 (1H, d, J = 19.5 Hz, ArH), 7.48 (2H, s, ArH), 7.46 (2H, s, ArH), 7.45 (1H, s, ArH), 7.43 (1H, s, ArH), 5.26 (1H, d, J = 5.3 Hz, CH_2), 5.22 (1H, d, J = 1.5 Hz, CH_2), 5.19 (1H, d, J = 1.6 Hz, CH), 5.16 (1H, m, CH, steric center), 4.16 (3H, s, OCH_3), 4.03 (1H, bs, OH). ^{13}C NMR ($CDCl_3$, 50 MHz): δ_C 203.5 (C = O), 173.3 (C = O),167.73 (C-OH), 162.3 (C = O), 137.0 (ArC), 132.4 (ArC), 130.8 (ArC-Cl), 128.8 (ArC-OCH_3), 121.8 (ArC), 118.0 (ArC), 113.8 (ArC), 102.8 (ArC), 68.1 (CH_2), 38.7 (CH), 29.3 (OCH_3);

EI MS peak m/z 439.4 [$C_{24}H_{23}O_5Cl^+$] (22%), 383.5 [$C_{21}H_{16}O_5Cl^+$] (23%), 257 [$C_{18}H_9O_2^+$] (28%), 121 [$C_{11}H_7O_3^+$] (2%), 187 [$C_{11}H_7O_3^+$] (2%), 183 [$C_{13}H_{11}O^+$] (100%), 93 [$C_6H_5O^+$] (12%), 71[$C_4H_7O^+$] (33%), 57 [$C_3H_5O^+$] (80%), 43 [$C_3H_3O^+$] (54%).

3-[3-(4-chlorophenyl)-1-(4-fluorophenyl)-3-oxopropyl]-4-hydroxy-2H-chromen-2-one (5k)

Solid; mp 134^{o}C; Yield 72%; ee 99% *[S]*; IR (acetone) ν_{max} 1720 (C = O), 1610 (C = O), 1490 (C-H), 1300 (O-H); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.62 (1H, s, OH), 7.96 (1H, d, J = 1.9 Hz, ArH), 7.93 (2H, d, J = 1.9 Hz, ArH), 7.63 (1H, m, ArH), 7.46 (1H, m, ArH), 7.43 (2H, d, J = 1.9 Hz, ArH), 7.39 (1H, d, J = 2.1 Hz, ArH), 7.29 (1H, m, ArH), 7.15 (1H, dd, J_1 = 8.1 Hz, J_2 = 1.3 Hz, Ar H), 6.94 (2H, d, ArH, J = 8.6 Hz), 4.82 (1H, d, J = 10.0 Hz, CH_2), 4.37 (1H, dd, J_1 = 19.1 Hz, J_2 = 10.2 Hz, CH_2), 4.16 (1H, d, J = 1.6 Hz, CH), 3.68 (1H, s, CH of stereogenic center); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 206.9 (C = O), 167.7 (C = O), 160.9 (C-OH), 152.8 (ArC), 131.8 (ArC-Cl), 130.8 (ArC-F), 130.0 (ArC), 129.8 (ArC), 129.6 (ArC), 129.2 (ArC), 128.8 (ArC), 123.9 (ArC), 116.26 (ArC), 115.18 (ArC), 114.76 (ArC), 68.1 (CH_2), 38.7 (CH), 30.9 (CH); EIMS m/z [$M^{+\,1}$] 422.2 [$C_{24}H_{16}O_4FCl^+$] (10%).

Bilogical experiments

Plasma recalcification time (PRT) method.

Anticoagulant potential of test compounds **5a-5k** was determined by *PRT* method.[35] 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. at rate 3000 rpm) was carried out to obtain platelet poor plasma. 0.2 ml plasma, 0.1 ml of different concentration 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^{o}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 sec.

Microplate alamar blue assay (maba) method.

Different derivatives **5a-5k** of 4-hydroxycoumarin including warfarin were screened for their antibacterial activities against *Escherichia coli* and *Pseudomonas aeruginosa* (gram-negative), *Bacillus subtills, Staphyloccus aureus*, and *Salmonella typhi* (gram-positive) by microplate alamar blue assay (maba)[36] using DCM as solvent. The ofloxacin was used as a standard drug. The applied concentration of compounds was 200µg/ml. The zone of inhibition was measured in mm (millimeters) and then %inhibition was calculated.

Agar tube dilution method.

The antifungal activities of all synthesized derivatives **5a-5k** of 4-hydroxycoumarin were evaluated with the help of agar tube dilution method.[37] Concentration of samples was 400µg/ml of DMSO Incubated at 37^{o}C and incubation period was 7days. 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.

In silico molecular docking studies

Molecular docking studies were carried out by using Discovery studio 2016, Chemsketch, AutoDock tools-1.5.6 and PyRx. First acquired crystal structure of VKOR1 (PDB ID:3kp9) in PDB format from RCSB Protein data bank,[28] then already attached ligand was removed. Ligands **5a-5k** were drawn in chemsketch and assigned smile notation and then open babel was used to add hydrogens and 3D coordinates to convert structures in PDB format. AutoDock tools were used to add polar hydrogens, kollman charges, compute gasteiger charges and set grid box in protein structure and saved it as PDBQT format. Then opened ligand in it and chose torsion for AutoDock using upto 12 torsional degree of freedom (DOF) and then saved it too in PDBQT format. Finally, docking of ligands with VKOR1 was carried out using AutoDock Vina.[38]

Declarations

Conflict of Interest

The authors declared that there is no any conflict of interest.

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Scheme 1

Scheme 1 is available in Supplemental Files section.

Figures

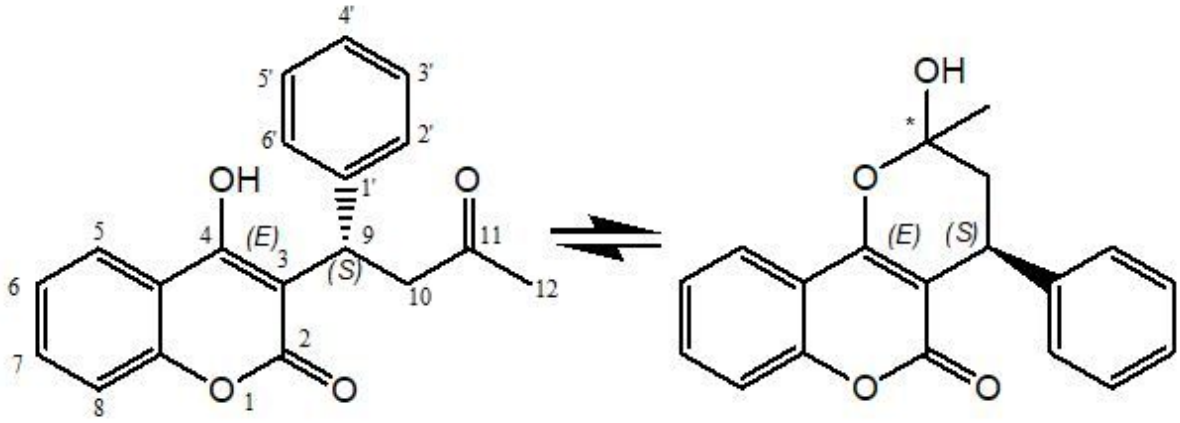

Figure 1

Major tautomeric forms, keto and hemiketal, of (S) warfarin

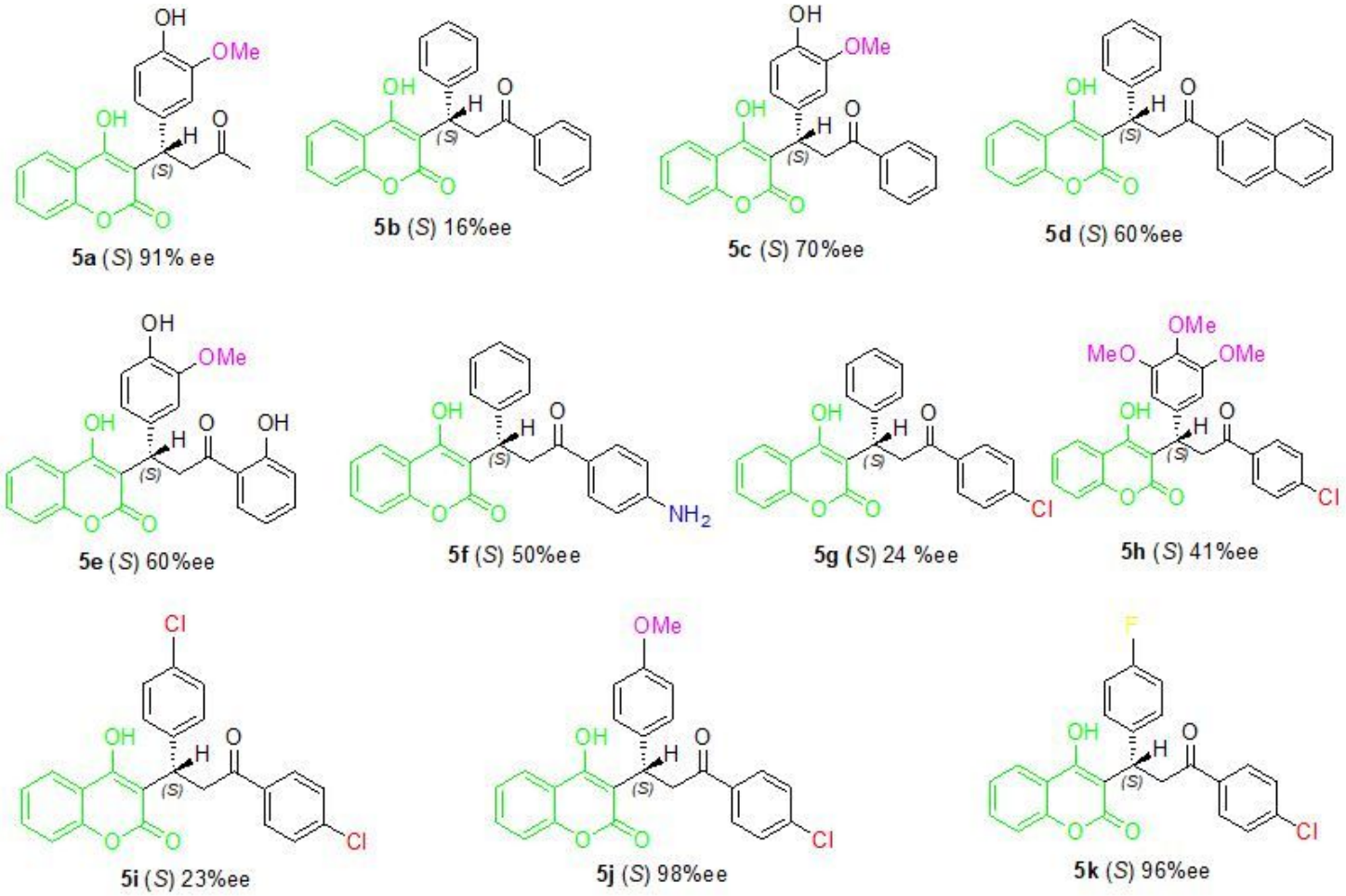

Figure 2

The chemical structures with their absolute configuration (*S*) of the synthesized warfarin based derivatives ***5a-5k***.

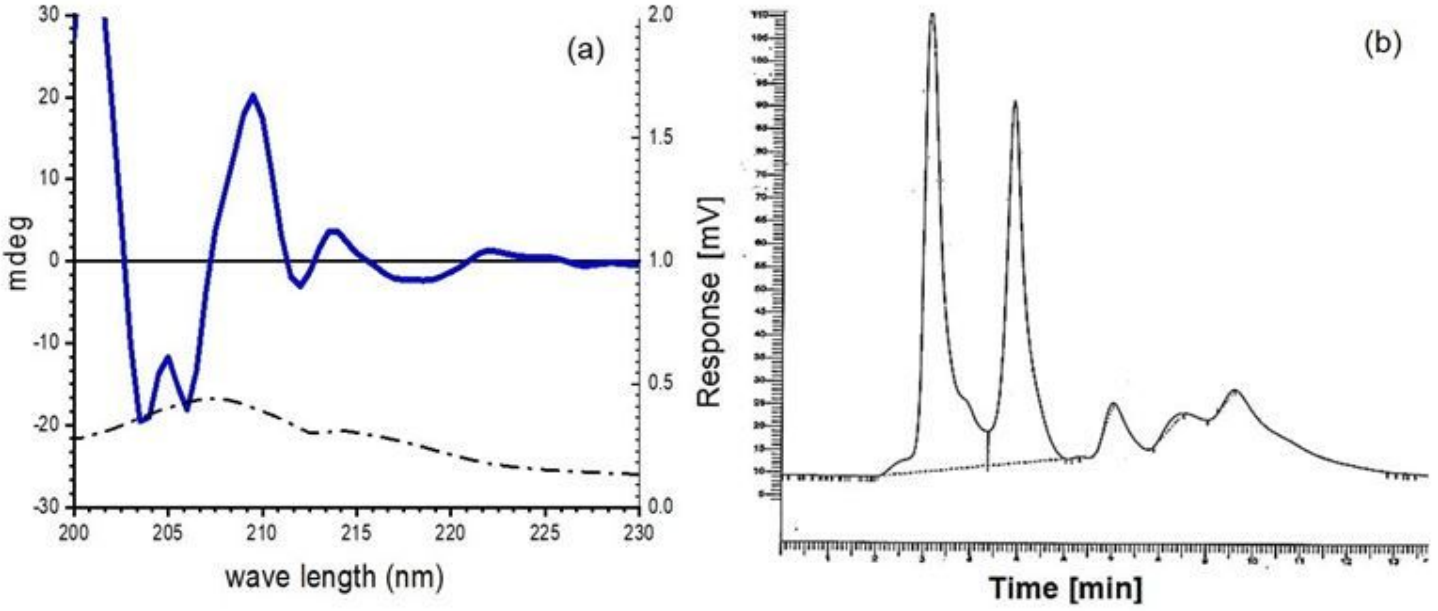

Figure 3

a) Experimental UVCD spectrum of compound **5b** in a mixture of aqueous phosphoric acid and acetonitrile (4:6) having pH 2.0 showing negative cotton effect at maximum absorption at 206nm that confirms its absolute configuration (*S*) as compared to reported warfarin CD spectrum. b) Chiral HPLC chromatogram of most potent compound **5b** showing separation of two enantiomer having enantiomeric excess with a ratio of 57.73:42.10 (*S*) and (*R*) respectively. Small peaks are tautomers of respective enantiomers

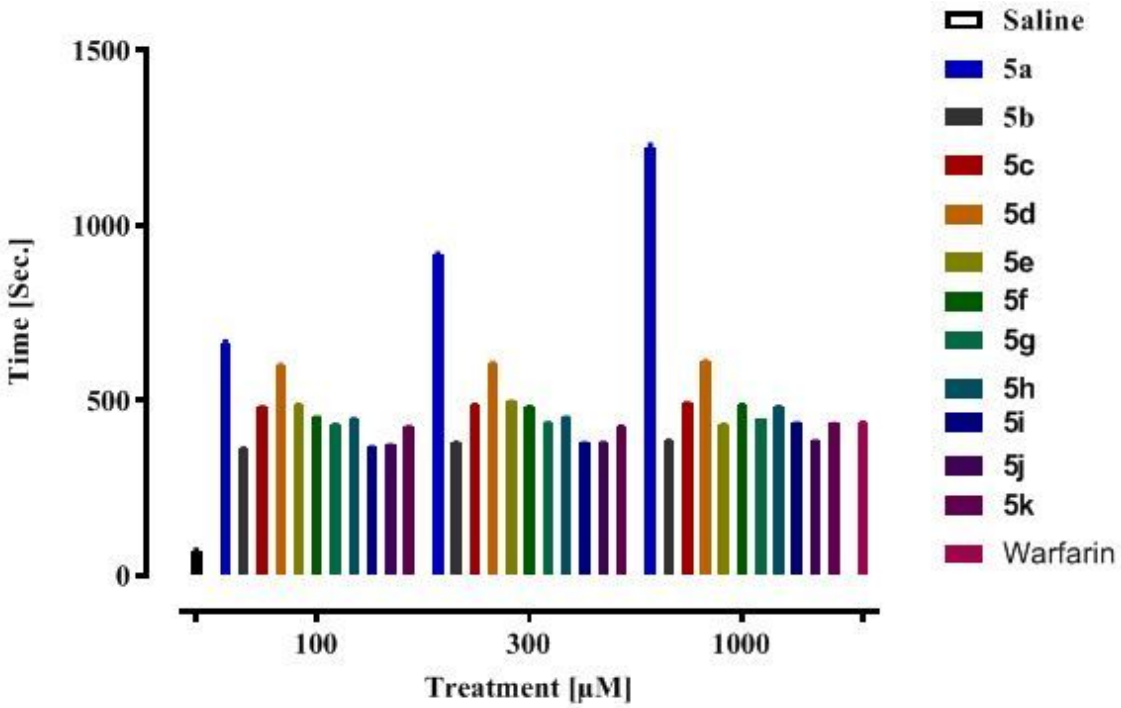

Figure 4

Bar chart showing increase/decrease in plasma recalcification time (PRT) caused by different concentrations of compound **5a, 5b, 5c, 5d, 5e, 5f, 5g, 5h, 5i, 5j, 5k** and warfarin. Data expressed as mean ± SEM, n=5, ^{T}P< 0.001 vs. saline group, one way ANOVA with post-hoc Tukey's test.

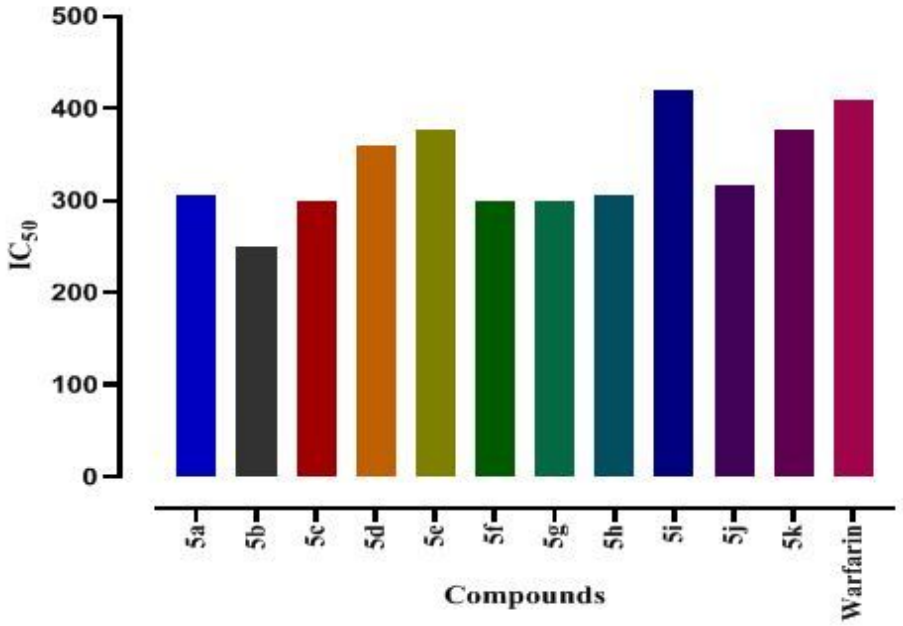

Figure 5

The IC_{50} values of synthesized compounds ***5***a-***5***k and standard drug warfarin.

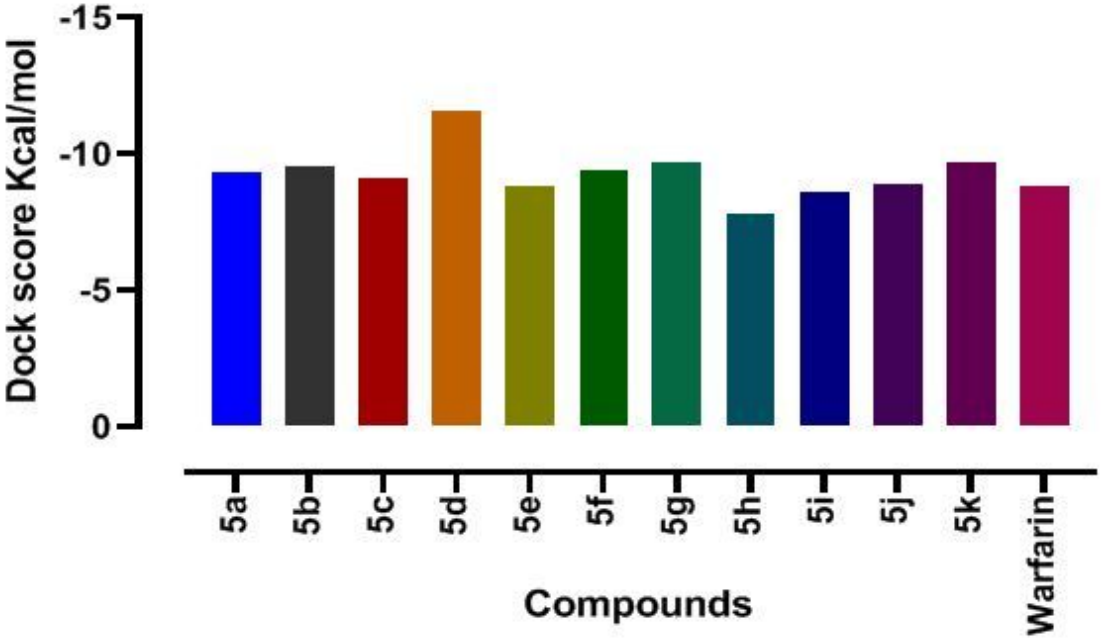

Figure 6

Summary of docking results of compounds ***5***a-***5***k.

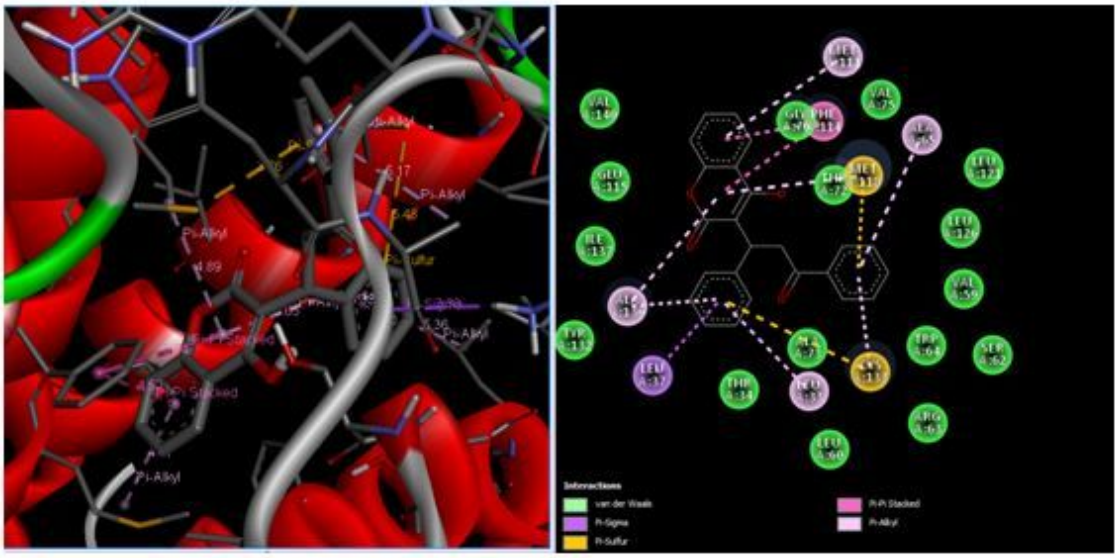

Figure 7

2D interactions of most potent derivative **5b** with target protein PDBID 3kp9.

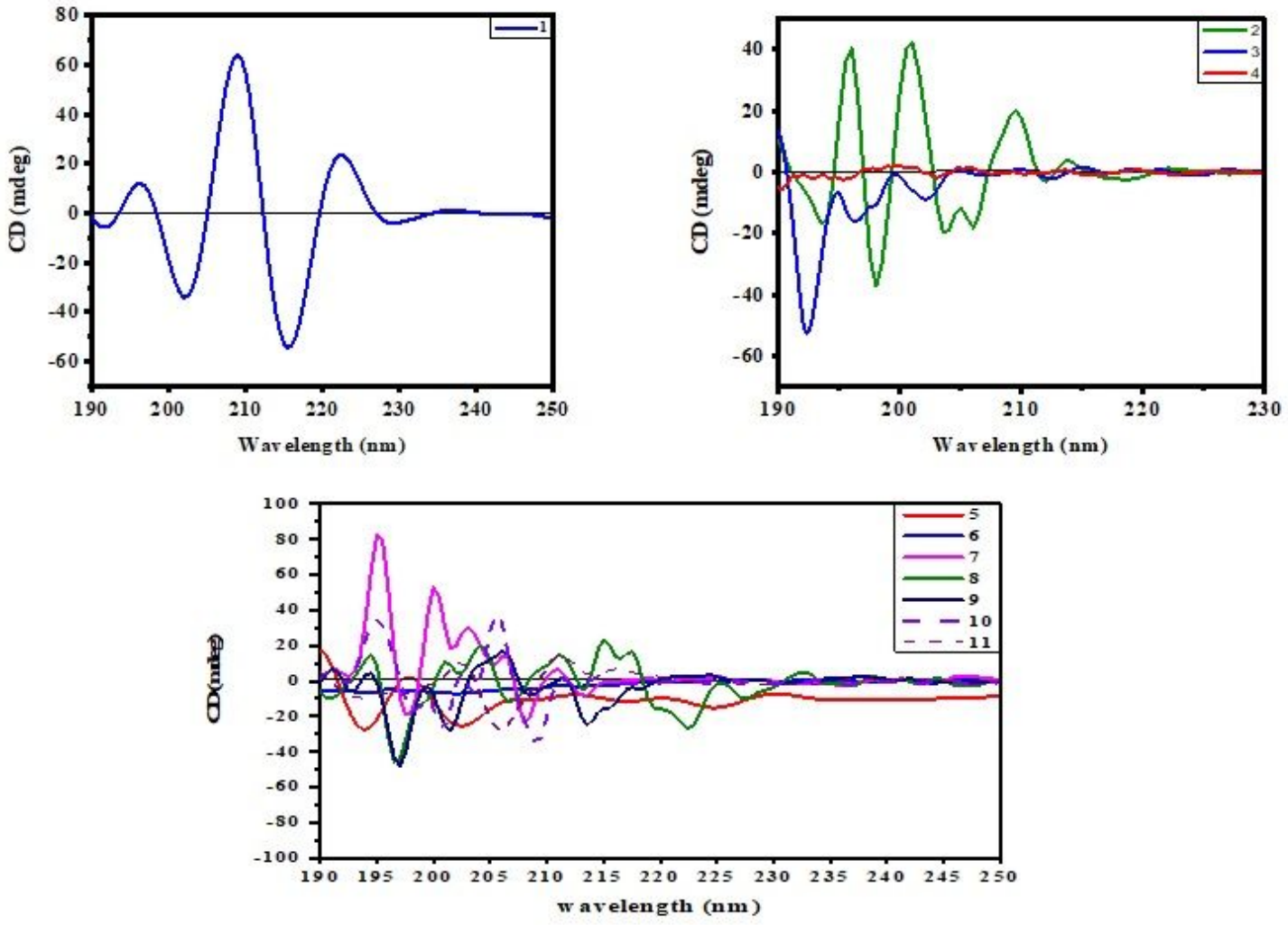

Figure 8

UV CD spectrum of all synthesized compounds

Supplementary Files

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- Scheme1..jpg
- Supportinginformation.docx
- GraphicalAbstract.jpg