# Organocatalyzed Synthesis, DNA Binding and Microbial Studies of Warfarin Analogues

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**Summary:** A noval chiral *sec*-amine/amidine-base hybrid catalyst, *N [S-carbonylprolyl] cyclohexyl Amine* is described, which is able to catalyze conjugate addition of 6-Methyl-4-hydroxypyran and 2-Hydroxy-naphthaquinone with various benzylideneacetones through Michael reactions that directly gives anticoagulant Warfarin analogues. These analogues were prepared in good yields (54–82%) and in good enantiomeric excess (50–75%). Identification of synthesized compounds was done by physio-chemical properties and spectral analysis ($^{1}$H-NMR & $^{13}$C-NMR).These compounds were further investigated for their antimicrobial (antibacterial & antifungal) activities and DNA-binding studies. Antimicrobial studies were carried out by Disc Diffusion while DNA-binding studies were carried out by Cyclic Voltammetry and UV-Visible spectroscopy. These studies showed that the compounds showed significant interaction with DNA. Some analogues also imparted prominent antimicrobial activities.

**Keywords:** Organocatalyst, Enantiomeric excess, Cyclic voltammetry, Warfarin.

## Introduction

Warfarin is the synthetic derivative of 4-Hydroxycoumarin (β di carbonyl system) which is used as an oral anticoagulant drug. Warfarin exists in acyclic (open chain keto form) and cyclic state in hemiketal tautomeric forms. In acyclic form warfarin exists in R-(+) - and S-(-)-enantiomers but in cyclic tautomer it exist in RR, SS, RS, and, SR enantiomeric form [1]. Among the different forms of warfarin, ring conformers are dominant in environments like nonpolar solvents [2] while the equilibrium shifts to the open side chain form in polar solvents. Therefore the open side chain forms are most common in the biological context. This is evidenced by the observation that all known structures of warfarin in complex with proteins (HSA, P450 CYP2C9) are in the open side chain form [3].

The important application of synthetic derivative of coumarin (Warfarin) is as anticoagulant drug by inhibiting vitamin K epoxide reductase complex. As metabolism and pharmaceutical applications of warfarin is stereo selective [2]) hence the development of a suitable method for chiral separation is of preliminary importance. Therefore the pharmacological properties and stereochemistry of warfarin encouraged the researchers to develop procedure for separation of its racemic mixture [4]. Warfarin enantiomers exist in nearly equal quantities as a racemate but vary in activity i.e. one enantiomer is highly effective than other [5]. (S)-warfarin has been reported to have a faster metabolism and an anticoagulant potency that is 2–5 times higher than that of (R)-warfarin Therefore separation of S- enantiomer of warfarin is of great importance [6]. Different approaches were made to synthesize warfarin in past [7-9]. Generally it is prepared by Michael addition reaction of 6-Hydroxycoumarin and α, β unsaturated ketones in the presence of suitable solvents [10]. For its stereo selective synthesis different approaches were made previously including Chiral Pool Synthesis [11]. Resolution of racemic mixture [12]) and Asymmetric Synthesis [13]. Use of Chiral organocatalyst is an important tool in asymmetric synthesis because of its less toxic effects as well as an easy reaction protocols [14]. Chiral ammines have played a vital role in Asymmetric Synthesis because of their interaction through hydrogen bonding which worked along with a Lewis acid functional group to achieve the activation of both the nucleophile and the electrophile [15-16].

Apart from Warfarin, other asymmetric Michael products are very important compounds. Added them anticoagulant activities, different Michael adducts obtained by addition of cyclic β-diketones and α,β unsaturated compounds also possess other biological properties. Michael adducts of quinones called as α-lapacholos and β-lapacholos possess antibacterial, fungicidal, antimalarial, trypanocidal, antiparasitic,and antitumor agents [17]. Their trypanocidal activity could be used to cure Chagas, one of the most important endemic diseases caused by Trypanosoma cruzi, which affects16–18 million people in large areas of Latin America and Africa [18]. Furthermore, lapachone analogues may possess potential inhibitive ability of

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DNA to poly isomerase II, and by this way, they have great cancer-preventing potential [19].

DNA binding studies of any drug is a center of attention to find new diagnostic tools for clinical studies [20]. These studies are normally carried out by UV-Visible Spectroscopy and Cyclic Voltammetric studies [21-22].

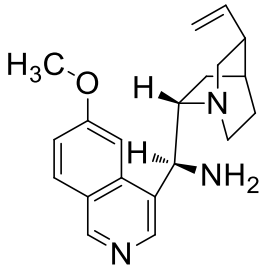
Inspiring from these results, two chiral amine catalysts were used for asymmetric synthesis of Warfarin Analogues. *9-Amino-9-deoxyepiquinine* (catalyst A), a natural organocatalyst and a chiral organocatalyst *N [(S)-prolyl] cyclohexyl Amine* (Catalyst B) which was prepared by simple protocol. In the current study Catalyst B is further used to synthesize analogues of Warfarin using two β-diketone systems, *4-Hydroxypyran* and *2,-Hydroxy-1,4-naphthaquinone*. The interaction of these analogues with DNA using UV-Visible spectroscopy and Cyclic Voltammetry has also been carried out. The synthesized compounds were further screened for their antimicrobial activities.

## Experimental

### *Materials and Methods*

All reagents and materials used are of analytical grade without further purification. 4-bromobenzaldehyde, 4-nitrobenzaldehyde and 4-N,N Dimethyl-benzaldehyde were purchased from Acros Organics while 6-Methyl-4-hydroxypyrane, 2-Hydroxy-1,4-naphtaquinone, Ethyl chloroformate, Palladium on activated charcoal, Sabouraud dextrose agar and Nutrient agar were purchased from Sigma Aldrich. and N-(S)-benzyloxycarbonyl proline was purchased from Alfa Aeser.

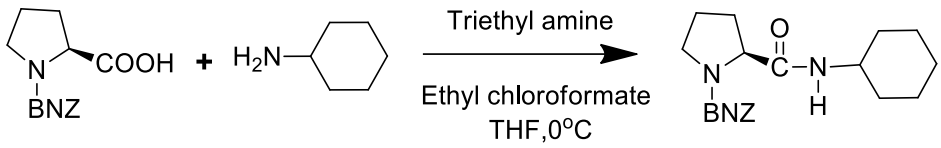
Melting points were resolute from a Gallen Kamp instrument. Thin Layer Chromatography was performed by pre coated silica gel plates (aluminum sheets, layer thickness 0.2mm, HF-254, Riedel-de-Haen using UV light (254 and 360 nm) and vanillin spray. For column chromatography, columns were packed in silica gel in n-hexane or pet ether. $^{1}H$ and $^{13}C$ NMR spectra were recorded in DMSO-$d_6$ or $CDCl_3$ solvent on Jeol ECS-300/ Bruker 400 MHz spectrophotometers using TMS as an internal reference. The enantiomeric excess was determined by using chiral columns, Chiralpak AD-H (phenomenix), Daciel chiralpak AD. The instrument used for this technique was HPLC PerkinElmer. EIMS were recorded on Agilent technologies (6890N-GC) and an inert mass selective detector 5973 mass spectrometer. DNA binding studies using absorption spectra were recorded on Shimadzu 1800 UV-Vis spectrophotometer and voltamograms were recorded on Eco Chemie Auto lab PGSTAT 12 potentiostat/galvanostat (Utrecht, The Netherlands) equipped with the electrochemical software package GPES 4.9.



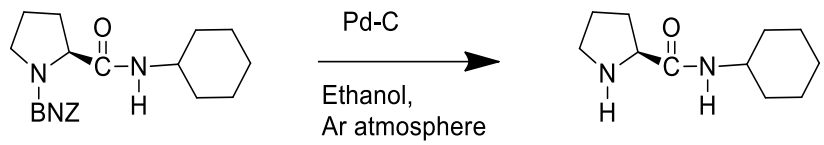

*9-Amino-9-deoxyepiquinine* (catalyst A)

### *Synthesis of Catalyst B*

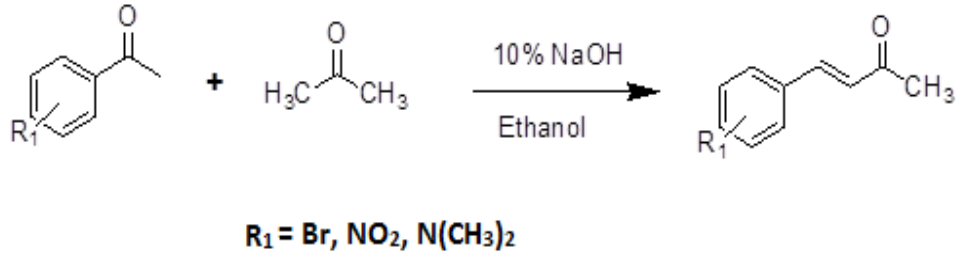
The synthesis of chiral amide of l-proline from cyclohexylamine was carried out by literature procedure [23] with some modifications. In first step synthesis of chiral amide was carried out by dropwise addition of ethyl chloroformate (14.8 mmol) in mixture of *(S)-N-benzyloxycarbonyl proline* (14.8 mmol) and triethyl amine (14.8 mmol) using vigorous stirring in THF (80 ml) previously ice cooled. After stirring for 30 minutes, cyclohexyl amine (14.8 mmol) was added drop wise for 15 minutes and the resultant mixture was stirred for 1h at 0°C. The solvent was evaporated and the residue was extracted with ethyl acetate and washed successively with water, aqueous $NaHCO_3$ and brine. The organic layer was dried over magnesium sulphate and evaporated in vacuum.



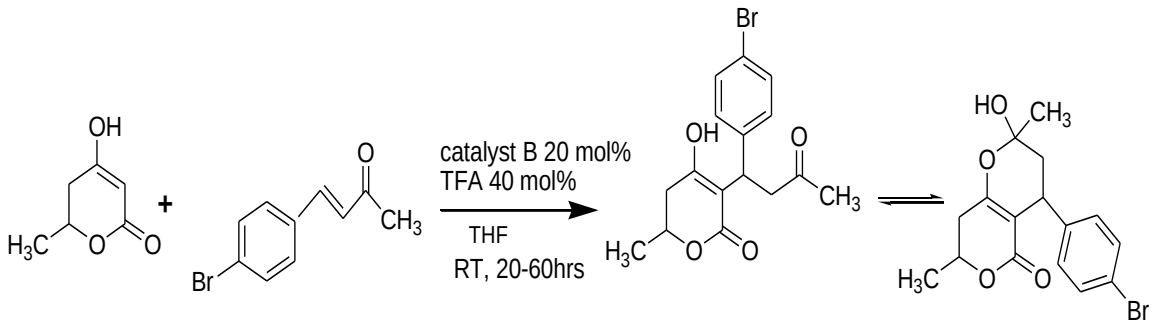

Scheme 1: Synthesis of *N [(S)-benzyloxycarbonylprolyl] cyclohexyl Amine.*




Scheme-2: Synthesis of *N [S- carbonylprolyl] cyclohexyl Amine* (Catalyst B).




Scheme-3: Synthesis of α,β unsaturated ketones.




Scheme-4: Synthesis of Warfarin Analogues (5c) using 4-Hydroxypyran and Catalyst B.

*Synthesis of N-[(S)-carbonyl prolyl cyclohexyl] Amine*

In second step deprotection of *N [(S)-benzyloxycarbonylprolyl] cyclohexyl Amine* was carried out by refluxing a mixture of *N [(S)-benzyloxycarbonylprolyl] cyclohexyl Amine* (1.9 mmol), cyclohexyl amine (3.8 mmol) and 180 mg of palladium charcoal in 20ml of ethanol for 3h under argon atmosphere and filtered. The catalyst was washed with ethanol and the filtrate was evaporated under reduced pressure to get cyclohexyl amide of l-proline

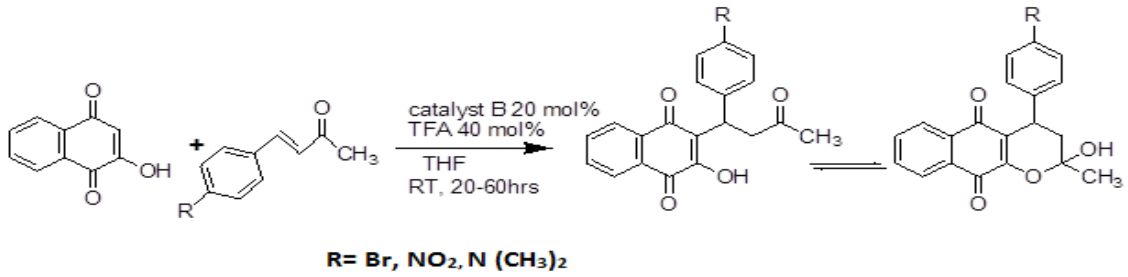
*Synthesis of α, β unsaturated ketones (bezaledeneacetone)*

α,β unsaturated ketones were prepared by addition of aromatic aldehyde (0.5 mol) to acetophenone (0.5 ml) in catalytic amount of NaOH and ethanol as a solvent and the reaction mixture was stirred for 30 min at 0°C and the reaction completion was monitored by TLC (α,β unsaturated ketones developed pink color in vanillin spray). The reaction mixture was neutralized with HCl and obtained precipitates were washed with water and recrystallized with ethanol.

*Synthesis of Michael adducts from 6-Methyl 4-hydroxypyran using Catalyst B*

Dissolve 6-Methyl-4-hydroxypyran (0.16 mmol), α, β unsaturated ketones (0.1 mmol) and catalyst B (20 mol%) to 20 ml of dry tetrahydrofuran in round bottom flask. Add TFA (40 mol%) to this reaction mixture. This reaction mixture was stirred at room temperature and progress of the reaction was monitored the by TLC under UV lamp developed in vanillin spray. The product developed blue spot in vanillin. The final product was purified by column chromatography.

*Synthesis of Michael adducts from 2-Hydroxy-1,4-naphthaquinone using Proline Amids*




Scheme-5: Synthesis of Warfarin Analogues (6c-6e) using *2-Hydroxynaphthaquinone* and Catalyst B.

0.16 mmol of *2-Hydroxy-1,4-naphthaquinone* and 0.1 mmol of α,β unsaturated ketones, 20 mol% of catalyst B were dissolved in 20 ml of dry tetrahydrofuran in round bottom flask. 40 mol% of TFA was also added in the reaction mixture. This reaction mixture was stirred at room temperature for different time intervals. The progress of the reaction was monitored by TLC visualized under UV lamp and developed in vanillin spray. The product gave blue spot in vanillin with α,β unsaturated ketones. When there was not further significant increase in concentration of product, the reaction was stopped. The final product was purified by column chromatography.

*Antimicrobial Activities*

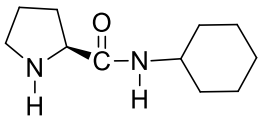
Preliminary in vitro tests for antimicrobial activity of Warfarin analogues had been carried out by disk diffusion method [24-26].

For antibacterial assay *Escherichia coli, Acetobacter aceti, Staphylococcus aureus, Klebsilla pneumonia and Pseudomonas aeruginosa* strains were selected. Nutrient agar was used to grow bacteria for inoculums preparations and for this purpose bacteria were maintained on nutrient agar medium at 4°C. Media was prepared by dissolving nutrient agar 2.9 g/100ml in distilled water and autoclaved at 121°C for 20 minutes. After sterilization media was poured on sterile plates under LFC and allowed to solidify followed by pre-incubated at 37°C for 24 hours to confirm sterility. The plates showing no growth were then used for anti-bacterial activity testing. The stock solutions of synthesized compounds (500 µg/mol and 1000 µg/mol) in DMSO were prepared and then a DMSO solution of the test sample was added to the respective wells. Diluted DMSO was used as negative control while Levofloxacin was used as positive control for antibacterial activities and Fluconazole for antifungal activity. Triplicate plates of each microbial strain was prepared and activity was determined by measuring % inhibition.

*Drug Compound Interaction Study*

For interactions of synthesized compounds with DNA, DNA solution was prepared as per procedure available in literature [27], it was ensured that the DNA was sufficiently free of protein. The UV absorption titrations were performed by keeping the concentration of the compound fixed while varying the DNA concentration. Equivalent solutions of DNA were added to the compound and reference solutions to eliminate the absorbance of DNA itself. Compound-DNA solutions were allowed to incubate for about 10 min at room temperature before measurements were made. Absorption spectra were recorded using cuvettes of 1 cm path length at room temperature (25 ± $1^0$C).

**Results and Discussion**



*Structure of (Catalyst B)*

*N [(S)- carbonylprolyl] cyclohexyl Amine*

Molecular formula: $C_{11}H_{20}N_2O$, Molecular weight: 196.29, Yield %: 49

$R_f$ value: 0.32 (n-hexane: Ethyl acetate) (1:1),

IR data: IR; (KBr, $cm^{-1}$): (C=O) 1687, (N-H) 3195, (NH-C=O) 3645.

Table-1: Physical Data of Synthesized Compounds.

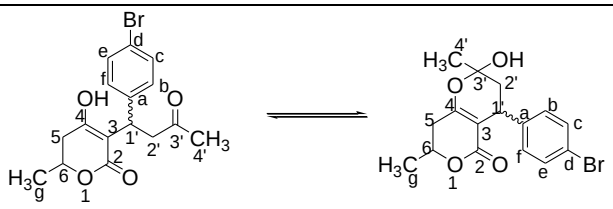
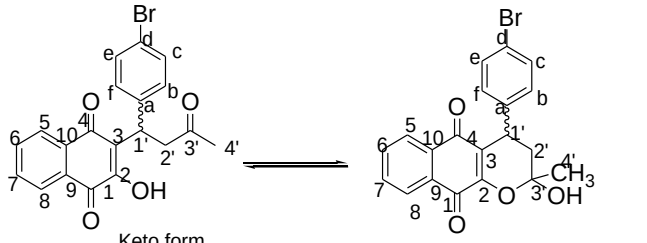
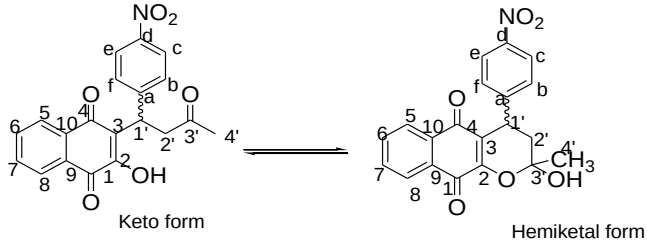
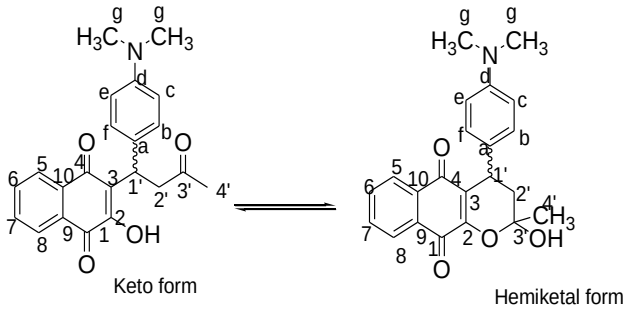
| Compound | Code | Structure (Name) | %Yield | Melting Point (°C) | ee |
|---|---|---|---|---|---|
| **1** | **5c** |  **4-Hydroxy-3-[1-(4-bromophenyl)-3-oxo-3-phenylpropyl]-6-methyl-2*H*-pyran-2-one** | **82** | **164.5** | **50** |
| **2** | **6c** |  **2-Hydroxy-3-[1-(4-bromophenyl)-3-oxobutyl]naphthalene-1,4-dione** | **65** | **188** | **74** |
| **3** | **6d** |  **2-Hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl] naphthalene-1,4-dione** | **61** | **172-175** | **63** |
| **4** | **6e** |  **2-Hydroxy-3-[4-(dimethylamino)phenyl]-3-oxobutyl] naphthalene-1,4-dione** | **54** | **157-159** | **75** |

Table-2: Spectral Data of Synthesized Compounds.

| Compound (Code) | $^{1}$H -NMR | $^{13}$C-NMR | EI-MIS (Molecular Ion Peak) |
|---|---|---|---|
| **1 (5c)** | **(300MHz, $CDCl_3$) δ (ppm): 7.82 (d, *J*=8.60Hz, 1H), 7.71 (d, *J*=8.6Hz, 1H), 4.62 (dd, $J_1$=4.3Hz, $J_2$=9.0Hz, 1H) 4.12 (dd, $J_1$=6.5Hz, $J_2$=10.4Hz, 1H), 4.00 (dd, $J_1$=6.12Hz, $J_2$=10.8Hz, 2H), 3.74 (m, 1H)3.72 (dd, $J_1$=3.24Hz, $J_2$=8.8Hz, 2H), 3.50 (d, *J*=6.84Hz, 2H), 3.14 (m, 2H), 2.20 (m, 2H), 2.17 (s, 3H), 1.84 (t, 3H), 1.68 (d, *J*=5.8Hz, 3H).** | **(400MHz, $CDCl_3$) δ (ppm): 200.1, 182.3, 178.3, 142.5, 131.5, 129.9, 123.0, 121.2, 102.3, 100.58, 95.8, 77.35, 40, 42.49, 49, 38.2, 30.2, 28.79, 29.69, 22.4, 29.7.** | **414.9 & 416.9** |
| **2 (6c)** | **(300MHz, $CDCl_3$, δ ppm): 8.15 (d, *J*=7.8Hz, 2H), 8.05 (dd, $J_1$=11.8Hz, $J_2$=7.2Hz, 1H), 7.5 (d, *J*=7.3Hz, 2H), 7.66 (m, 1H), 4.90 (dd, $J_1$=9.6Hz, $J_2$=5.7Hz, 1H), 5.12 (dd, $J_1$=5.7Hz, $J_2$=6.9Hz, 1H), 3.80 (bs, 1H), 3.54 (dd, $J_1$=8.4Hz, $J_2$=16.4Hz, 2H), 2.35 (dd, $J_1$=16.4Hz, $J_2$=16.1Hz, 2H), 2.20 (s, 1H).** | **(400 MHz, $CDCl_3$, δ ppm): 206.5, 199, 196, 173.6, 160.2, 165.5,158, 148.0, 137.5, 135.2, 133.0, 131.3, 127.5, 125.8, 121.3,115.6, 79.7, 55.8, 53.1, 47.8, 38.8, 30.1** | **400.1 & 402.12** |
| **3 (6d)** | **(300MHz, $CDCl_3$, δ ppm): 8.31(d, *J*=7.3Hz, 2H), 7.66-7.8 (m, 4H), 7.5 (d, *J*=7.2Hz, 2H), 7.80 (d, *J*=6.8Hz, 2H), 7.66 (m, 2H), 5.6 (dd, $J_1$=5.7Hz and $J_2$=16.2Hz,2H),5.2 (dd, $J_1$=6.5Hz, $J_2$=17.8Hz,1H), 3.78 (dd, $J_1$=7.9Hz and $J_2$=8.2Hz, 1H), 3.45(dd, $J_1$=7.3Hz, $J_2$=6.4Hz, 1H), 3.75 (bs, 1H), 3.41(dd, $J_1$=17.3Hz, $J_2$=5.8Hz, 1H), 2.32 (s, 1H).** | **(400 MHz, $CDCl_3$, δ ppm): 208.5, 199, 196, 183.5, 181.2, 173.6, 170.2, 169.2, 148.0, 145.2, 135.0, 133.2, 121.3, 129.0, 124.0, 115.3, 79.7, 50.1, 47.2, 45.2, 24.6** | **365.12** |
| **4 (6e)** | **(300MHz, $CDCl_3$, δ ppm):8.40(d,*J*=7.8Hz, 1H), 8.23 (d,*J*=8.2Hz, 2H), 8.11 (dd, $J_1$=14.6Hz, $J_2$=7.8Hz, 1H), 8.00 (d, *J*=7.2Hz, 2H), 7.66 (m, 2H), 5.22 (dd, $J_1$=17.8Hz, $J_2$=6.5Hz, 1H), 3.78 (dd, $J_1$=16.7Hz, $J_2$=7.9Hz, 1H), 3.70 (bs, 1H), 3.45 (dd, $J_1$=7.3Hz, $J_2$=6.4Hz, 1H), 3.07 (s, 6H) 2.20 (s, 1H).** | **(400 MHz, $CDCl_3$, δ ppm): 207.3, 184, 196, 175.2, 172.6, 170.4, 163, 148.4, 135.2, 133.0, 131.4, 125.8, 114.0, 79.7, 55.8, 53.1, 47.8, 42.7, 29.3** | **363.11** |

*DNA-binding studies (UV-visible spectroscopy)*

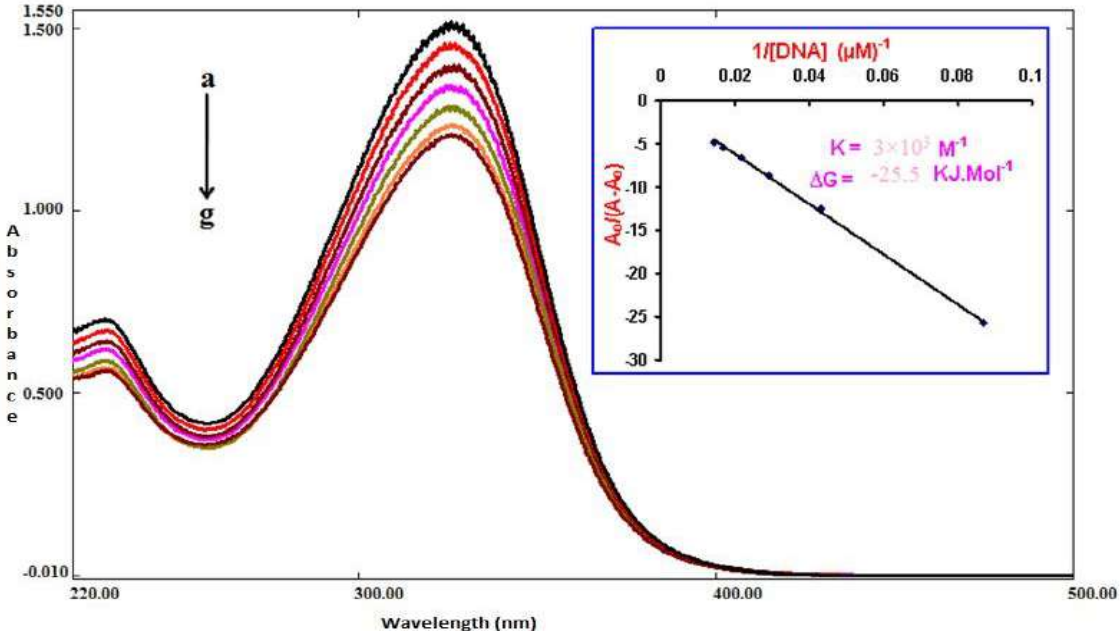



Fig. 1: UV Spectrum of compound 1 in the Presence and Absence of DNA.

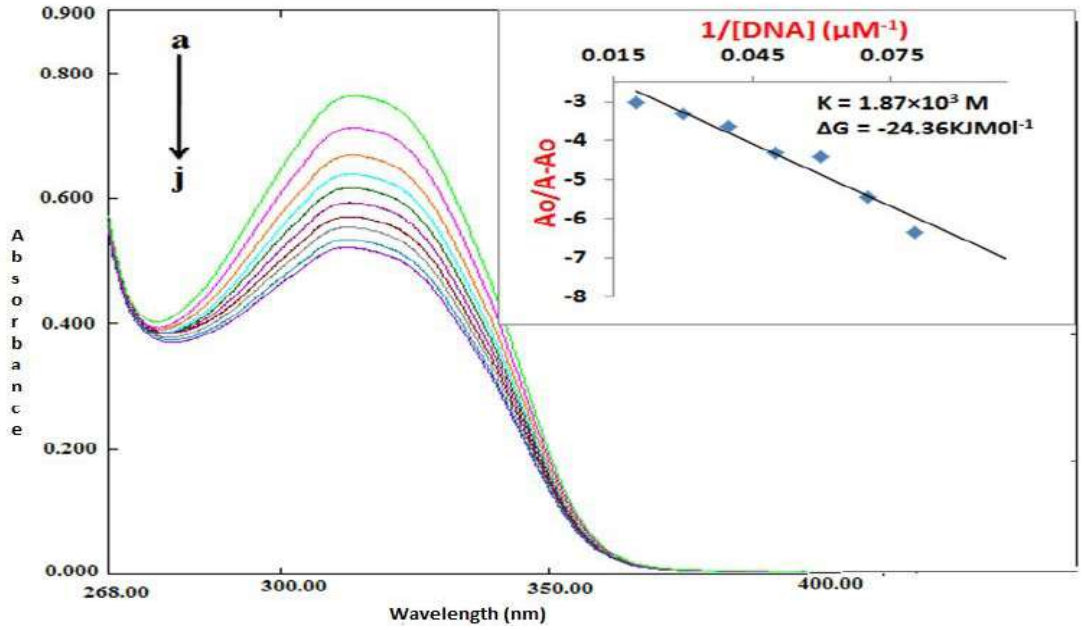



Fig. 2: UV Spectrum of compound 2 in the Presence and Absence of DNA.

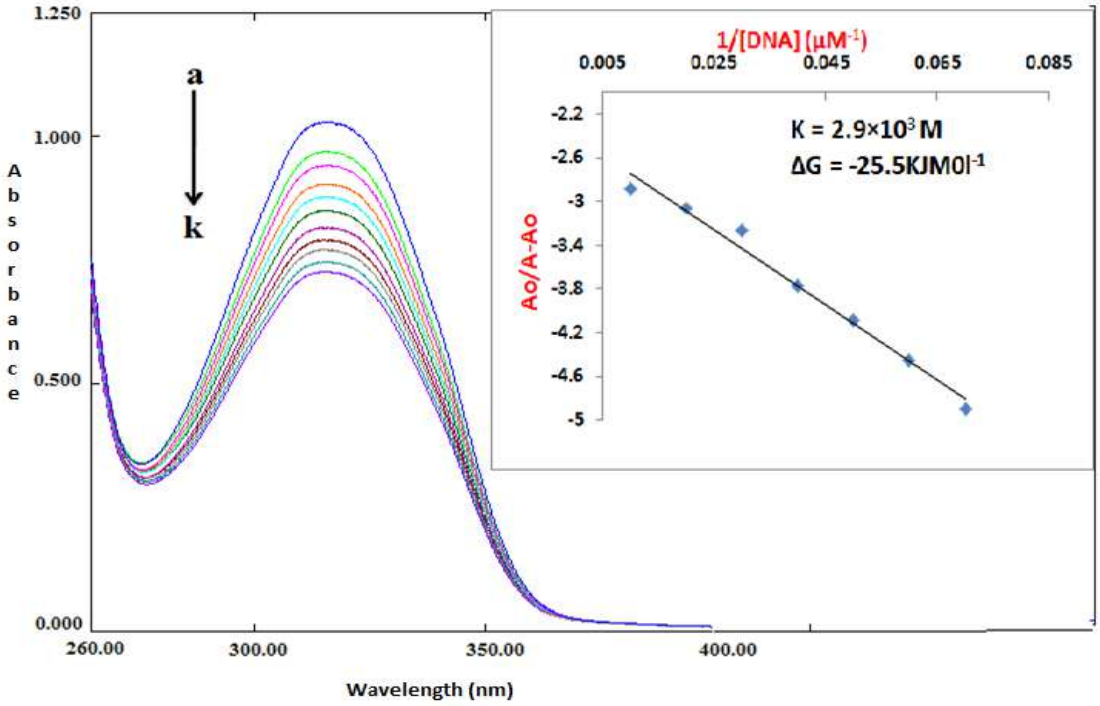



Fig. 3: UV Spectrum of compound 3 in the Presence and Absence of DNA.

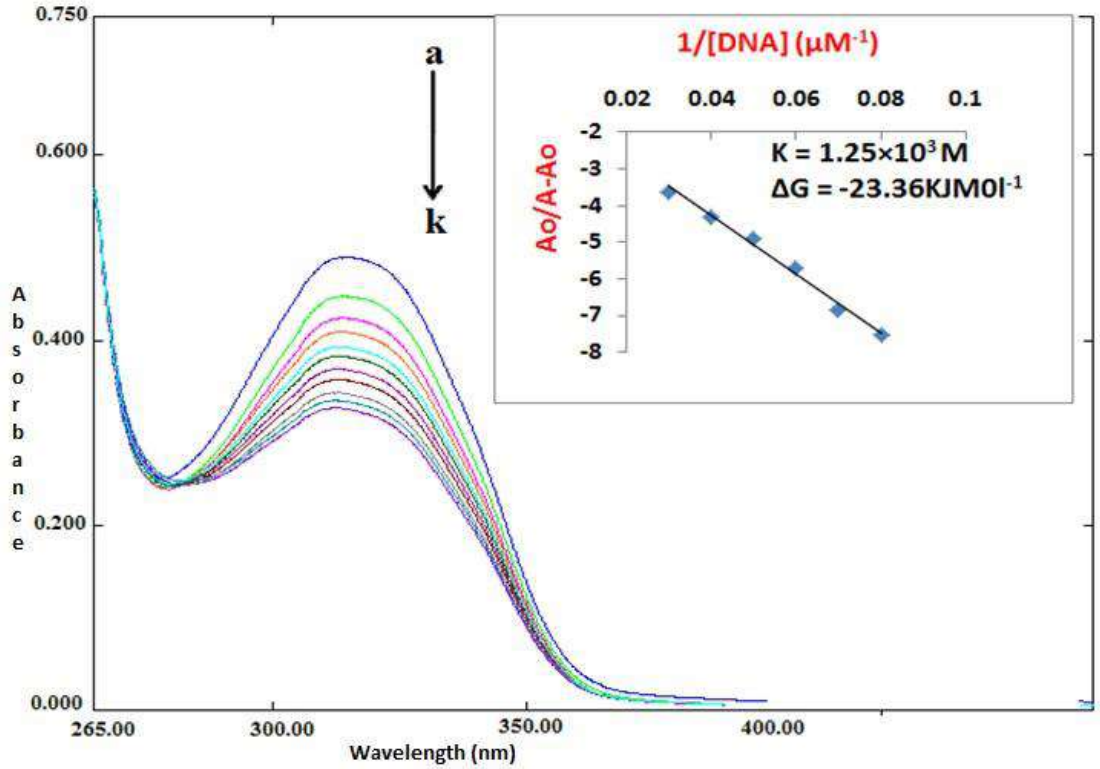



Fig. 4: UV Spectrum of compound 4 in the Presence and Absence of DNA.

*Dna-binding studies (CV-Studies)*

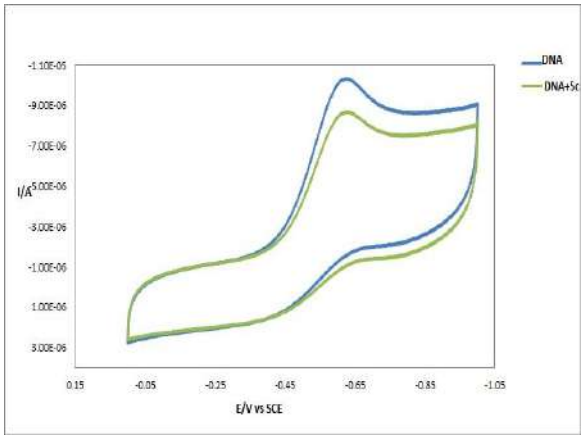



Fig. 5: Cyclic Voltammogram for (0.1M) compound 1 before and after addition of DNA at Scan Rate of 0.2V/s in Ethanol Water Mixture (8:2) at pH 7.4.

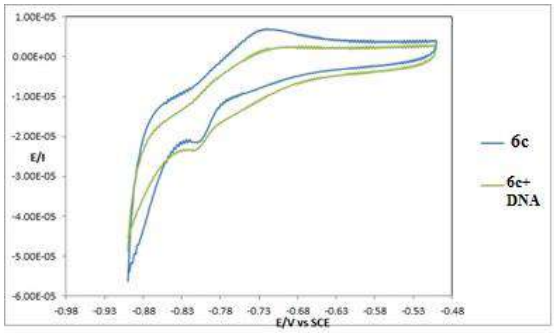



Fig. 6: Cyclic Voltammogram for (0.1M) compound 2 before and after addition of DNA at Scan Rate of 0.2V/s in Ethanol Water Mixture (8:2) at pH 7.4.

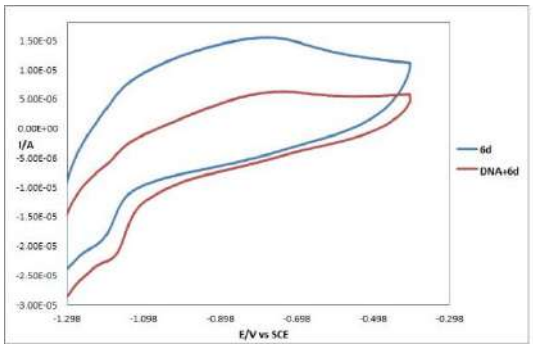



Fig. 7: Cyclic Voltammogram for (0.1M) compound 3 before and after addition of DNA at Scan Rate of 0.2V/s in Ethanol Water Mixture (8:2) at pH 7.4.

Table-3: Free- Energies and Binding Constants for Compounds-DNA Complexes.

| Compound | Binding Constant "$K_b$ $M^{-1}$" | Free Energy (ΔG (kJ/mol)) |
|---|---|---|
| 1 | $3.0X10^3$ | -25.52 |
| 2 | $1.87X10^3$ | -24.36 |
| 3 | $2.90X10^3$ | -25.50 |
| 4 | $1.25X10^3$ | -23.36 |

Table-4: Values of Voltage and Current in CV Measurements.

| ompound | nly Compound | | ompound + DNA | | hift in potential |
|---|---|---|---|---|---|
| | urrent | oltage | urrent | oltage | |
| | $5.5\times10^{-5}$ | .180 | $.9\times10^{-5}$ | .186 | o negative |
| | $.55\times10^{-5}$ | .762 | $.62\times10^{-6}$ | .746 | o Positive |
| | $.48\times10^{-6}$ | .730 | $.7\times10^{-6}$ | .726 | o Positive |

Table-5: Calculated Binding parameters by Cyclic Voltammetric Studies.

| Compound | K (Binding constant) | -ΔG(Binding energy) |
|---|---|---|
| 1 | $2.98\times10^3$ | 25.54 |
| 2 | $1.81\times10^3$ | 24.51 |
| 3 | $2.80\times10^3$ | 25.71 |

*Antimicrobial Screening*

Table-6: % Inhibition of Synthesized compounds for Antibacterial Screening.

| Compound | % Inhibition | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *E. Coli* (µg/ml) | | *A.Acceti* (µg/ml) | | *S.Aureus* (µg/ml) | | *K.Pneumoniae* (µg/ml) | | *P. Aeuroginosa* (µg/ml) | |
| | 500 | 1000 | 500 | 1000 | 500 | 1000 | 500 | 1000 | 500 | 1000 |
| 1 | 59 | 54 | 29 | 58 | 19 | 39 | 26 | 45 | 20 | 43 |
| 2 | 18 | 67 | 33 | 13 | 35 | 55 | 29 | 8 | 11 | 21 |
| 3 | 44 | 69 | 24 | 78 | 5 | 17 | 22 | 32 | 24 | 59 |
| 4 | 32 | 43 | 25 | 41 | 11 | 65 | - | - | 26 | 46 |
| Levofloxacin | 64 | 79 | 71 | 83 | 65 | 85 | 58 | 67 | 43 | 68 |

Table-7: % Inhibition of synthesized compounds for Antifungal Screening.

| Compound | % Inhibition | | | |
|---|---|---|---|---|
| | A. flavus (µg/ml) | | C. albicans (µg/ml) | |
| | 500 | 1000 | 500 | 1000 |
| 1 | 59 | 71 | 60 | 72 |
| 2 | 34 | 64 | 60 | 72 |
| 3 | 37 | 70 | 49 | 69 |
| 4 | 23 | 52 | 34 | 63 |
| Fluconazole | 64 | 78 | 72 | 94 |

*Synthesis of Warfarin Analogues using 6-Methyl-4-hydroxypyran and Catalyst B*

The results show that Catalyst B was found to be an active catalyst in asymmetric synthesis. Compound 5c was prepared in maximum yield of 82% while its enantioselectivity was 50%ee. Maximum enantiomeric excess was obtained for compound **6e** which is 75% while its obtained yield was only 54% .Next compound 6d was prepared in 61% yield and 63%ee. Compound 6c was prepared in 65% yield and 74% ee.

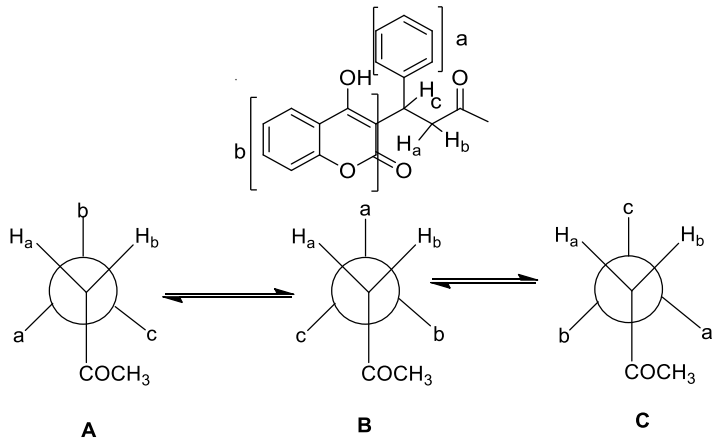
*NMR*

NMR study of Warfarin suggests that the structure of warfarin contains a chiral centre carbon-1' and methylene carbon-2' attached to a chiral center [28]. The protons of carbon-2' are diastereotopic protons and do not give a normal n+1 splitting pattern in NMR spectroscopy. The $^1$H-NMR of a typical warfarin suggests that the rapid equilibrium exists between its diasteromeric hemiketal (cyclic form) and keto forms (open chain form). The diastereotopic protons show phenomenon of anisochrony [29]. The origin of anisochrony in warfarin and its analogues can be explained on the basis of scheme 5 three different conformations of Warfarin are considered.

*$^1$H-NMR*

All the spectral assignments are given in Table-2. The first compound 1 (4-hydroxy-6-methyl-3-(3-oxo-1-(4-bromophenyl)butyl)-5,6-dihydro-2*H*-pyran-2-one) was obtained as a Michael product of 6-methyl-4-hydroxypyran and benzylidene acetone. The first doublet of doublet for methine proton of carbon-1' was appeared at 4.12ppm with coupling constants 6.5Hz and 10.4Hz. Another doublet of doublet was obtained at 4.62ppm. Its coupling constants are 4.3Hz and 9.0Hz. These two doublet of doublet were assigned to proton of hemiketal of carbon-1' and carbon-1''. The proton for keto tautomer was resonated as a triplet at chemical shift of 5.80ppm with coupling constant of 14.2Hz. For methylene proton of carbon-2' two multiplets and two doublet of doublets were obtained. The up field multiplets were assigned to hemiketal tautomer while the other to keto tautomer. There are present two types of methyl groups. One of carbon-4' that have resonated in the form of pairs at 1.84ppm. The protons of methyl **g** appeared at 2.14ppm. Two doublets for aromatic region at 7.82ppm and 7.71ppm are obtained. The methylene proton of carbon-5 appeared as a doublet at 3.50ppm. The methylene proton of carbon-6 appeared as multiplet at 3.74ppm suggesting that the compound is an analogue of Warfarin which exists in keto-enol tautomerism in solution form.

The NMR results of compound **2** agreed with literature [30]. It has been observed that most of the peaks appeared at same position. Methine carbon-1' appeared as a doublets of doublet at 4.90 and 5.12ppm with coupling constants $J_1$=6.2Hz, $J_2$=9.15Hz, $J_1$=5.7Hz and $J_2$=9.6Hz. These values were due to the presence of non-equivalent protons on carbon- 2'. The two methylene protons appeared as doublet of doublets at 2.35ppm and 3.54ppm. The coupling constants for these were $J_1$=5.9Hz, $J_2$ =16.4Hz and $J_1$=8.4Hz, $J_2$ =16.1Hz respectively. Methyl protons gave a sharp singlet at 2.0ppm. The broad singlet of hydroxyl group appeared at 2.20ppm. The rest of protons appeared in aromatic region starting from 7.12ppm to 8.15ppm. Aromatic proton **6**, **7** resonate at 7.66ppm with a multiplet peaks Proton-5 appeared as doublet peak at 8.10ppm ($J$= 7.9Hz) and a dd is obtained for proton-8 at 8.05ppm ($J_1$=7.2Hz, $J_2$ =11.8Hz). Other set of aromatic protons **b,f** appeared as doublet at 7.98ppm ($J$= 7.3Hz). **c,e** also showed a doublet at 8.15ppm ($J$= 7.8Hz)




Scheme-5: Anisochronous Carbon of Warfarin in Mobile System.

In compound **2,** a nitro group is substituted at position-d of benzene ring of benzylidene acetone, Nitro group is electron withdrawing having two electronegative oxygen and one nitrogen atom. It de-shielded the proton more strongly [31]. The protons of chiral center-1' appeared at 5.12ppm and 5.6ppm as doublets of doublets ($J_1$=5.8Hz, $J_2$=13.1Hz and $J_1$=6.1Hz and $J_2$=12.7Hz). The protons of methylene C-2' appeared as doublet of doublets with repetition at downfield positions of 3.41ppm ($J_1$=7.9Hz, $J_2$=17.3Hz) and 3.99ppm ($J_1$=8.5Hz, $J_2$=17.5Hz). Methyl protons of C-4'gave a singlet at 2.32ppm. OH appeared as broad singlet at 3.24ppm. The most downfield doublet at 8.31ppm ($J$=7.3Hz) having two equivalent protons was assigned to **d** and **e**. Another doublet at 7.5ppm ($J$=7.5Hz) was assigned to protons **c** and **f.** A multiplet due to four protons appeared from 7.66ppm to 7.8ppm, it was assigned to aromatic protons **5, 6, 7** and **8**. The de-shielding extended in whole of the molecule due to induction effect and almost all of the signals appeared at downfield positions.

In case of compound **3** two methyl groups were present at nitro position in one of benzene ring which have electron donating effects. The structure had been elucidated with NMR spectroscopy. $^1$H-NMR spectra revels almost the same pattern of peaks as for compound **2** and **3** with small variations. Chiral methine protons of C-1'appeared as doublet of doublets at 5.22ppm and 5.66ppm ($J_1$=6.5Hz, $J_2$=17.8Hz and $J_1$=5.7Hz and $J_2$=16.2Hz). The methylene protons of C-2' appeared as two dd at 3.45ppm and 3.78ppm ($J_1$=7.3Hz, $J_2$=6.4Hz and $J_1$=7.9Hz and $J_2$=8.2Hz) showing the existence of keto and hemiketal two forms.

In compound **4**, a sharp singlet due to 6H appeared at 3.07ppm, which was assigned to methyl protons at **g**. The rest of peaks were almost same with little differences as of previous.

*$^{13}$C-NMR*

The detailed $^{13}$C-NMR spectral assignments of compound **1** showed the existence of the paired peaks of C-1', C-2' and C-4 which were methine protons, methylene and methyl carbons which were consistent with literature. The paired peaks of methine carbon-1' are present at 100.58ppm and 102.30ppm. The methylene carbon-2' appeared at 40.04ppm and 42.49ppm in both forms respectively, while that of methyl carbon-4' resonated at 28.79ppm and 29.69ppm. These paired peaks can be related to the synthesis of chiral compound and the existence of structure in diastereomeric forms with cyclic hemiketal and open chain keto tautomers. C=O, carbon had been assigned a peak at 200.1ppm.The aromatic carbons of benzene ring were present in the region of 128.10ppm to 163.21ppm. Here the most down field signal 163.21ppm was assigned to carbon-4. The methylene carbon-5 appeared at 77.35ppm, methine carbon-6 resonated at 129.10ppm and methyl carbon **g** appeared at 25.63ppm. Methine carbon-6 was present at much down field position then other carbons.

Compound **2**(2-Methyl-3-(3-oxo-1-(4-bromophenyl)butyl)naphthalene-1,4-dione) had been synthesized by the reaction of benzylideneacetone and 2-Hydroxy-1,4-naphthaquinone. In $^{13}$C-NMR three peaks were obtained for carbonyl-1, carbonyl-3 and carbonyl-4. The most down field of carbonyl carbon at 208.5ppm was assigned to carbon-1, because it was attached to carbon-2 on which an electronegative oxygen atom was present. The next down field carbonyl peak at 183.5ppm was assigned to carbon-4 and the peak at 181.2ppm was assigned to carbon-3'. The peaks for chiral carbon-1' and carbon-2' appeared at 114.3ppm, 121.1ppm and 46.3ppm, 45.2ppm with the same repetition showing the existence in two forms. Methyl carbon 4' resonate at 24.5ppm. The quaternary peaks of aromatic region were present between 158.5ppm to 173.6ppm. The peak at 173.6ppm was assigned to carbon-2 because of its hydroxyl group and 165.5ppm peak was assigned to C-3. The other set of aromatic carbon-**d** had been assigned a downfield peak at 169.5ppm which was due to the presence of electronegative bromo group at this position. While carbon **b, f** resonated at 121.3ppm and those of **c, e** resonated at 127. 4ppm.

In case of compound **3, $^{13}$**C-NMR spectra showed two peaks of chiral methyin C-1' at 115.3ppm and 121.5ppm. Two methylene carbons of keto and hemiketal forms of C-2'apperaed at 47.2ppm and 50.1ppm. Among three peaks 208.5ppm, 169.8ppm and 185.5ppm; the most downfield peak was assigned to carbon-1 because it was aromatic and was in neighbor of OH group, while second peak was assigned to Carbon-4 because in aromatic region and third peak to Carbon-3'which was the most up field. Methyl carbon-5' appeared at 24.6ppm. Among aromatic carbons, the most downfield peak at 178.5ppm was assigned to carbon-2 because it was attached with electronegative OH group. Next downfield peak of quaternary carbon-**d** resonated at 170.2ppm because of presence of nitro group which was electron withdrawing but less than oxygen of hydroxyl group. Carbon-**b**, **f** resonate at 129.6ppm while those of carbons **c**, electrons having same environment resonated at 131.5ppm. However the

next group of aromatic carbons resonated at their usual positions.

In compound **4,** $C^{13}$ NMR spectrum had shown almost the same peaks. Two peaks due to C-1' and C-2' appeared at their usual positions confirming keto and hemiketal forms for **6c**. Peaks of two methyl carbons at C-**g** appeared at 42.7ppm, a downfield peak because of attachment with nitrogen atom which was electron withdrawing.

*DNA Binding Studies*

The DNA binding studies of synthesized compounds showed quite different results. In case of compound **1.** In UV spectrum of compound, $\lambda_{max}$ was found at 310nm in Fig 1. With the addition of DNA ($0.1\times10^{-4}$ M to $1\times10^{-3}$M), decrease in absorption was observed with blue shift. The maximum hypsochromic shift of about 1nm (268-267) was observed for maximum concentration of DNA. The electronic spectra of other compounds **2-4**, are represented in Fig 2-4, showed a decreases in absorbance with increase of DNA concentration. The $\lambda_{max}$ depicted a typical π-π* transitions with the additions of DNA and hypochromic effect was observed in all cases. For compound **2** blue shift was observed at 315nm with a difference of 3nm (315-312). In compound **3** a decrease in absorbance had been observed but the shift of $\lambda_{max}$ to either lower or higher wavelength was not much prominent. Similar is the case for compound **4** as evident from spectral analysis. Decrease in absorbance at $\lambda_{max}$ of all the studied compounds reflected the interaction of these compounds with DNA. The observed phenomenon also indicated the interaction of the electronic states of the intercalating chromophores of the compounds and those of the stacked base pairs of CT-DNA [32].

*Determination of compound-DNA Binding Constant "$K_b$"*

It had been observed that the absorbance was varied after the addition of CT-DNA, so the binding constants "$K_b$" of compounds-DNA complex was determined from the variation in absorbance in UV-visible spectra. Binding constants can be determined spectrophotometrically by applying Benesi-Hildebrand equation.

$$\frac{Ao}{A-Ao} = \frac{\varepsilon o}{\varepsilon(H-G)-\varepsilon G} + \frac{\varepsilon}{\varepsilon(H-\varepsilon)-\varepsilon G}\,1/K\mathrm{b}[DNA]$$

where $A_o$ and A were the absorbance of compounds and complexes respectively. $\varepsilon_G$ and $\varepsilon_{H\text{-}G}$ are the molar extinction coefficients of compounds and complexes. From the plot of Ao/A-Ao to 1/[DNA], the ratio of intercept to the slop gave binding constants "$K_b$". Their plots are given along with their absorption spectra. The values for compounds are represented in table 3.

*Calculation of Free Energy "ΔG"*

The free energies ΔG (kJ/mol) of compounds-DNA complexes were calculated as.

$$\Delta G = -RTlnK$$

Where K is the binding constants, R is the General Gas constant and T is the temperature (298K). The values of these energies are also represented in table 2.

Binding constants (**$K_b$**) were the measure of stability of compound-DNA complexes while free energy "ΔG" indicated the spontaneity of the compounds-DNA complexes. The values of binding constants "$K_b$" of current study represented that how strongly compounds bound to DNA. The binding constants "$K_b$" in Table 1 suggested that compounds bound strongly to DNA. Among the studied compounds, compound **1** and **3** bound more strongly than other compounds having grater values of **$K_b$** which was $3.0\text{x}10^3$ and was $2.90\text{x}10^3$ for compound **2**. The values of free energies for compound **1** and **3** were minimum reflecting the more stability of these complexes as compared to others [33]. The basic nucleus in compound **1** was 4-Hydroxypyrane and the series of compounds **2-4** were 2-Hydroxy-1,4-napthaquinone. Compounds 2-4 could be considered as quinolones. The DNA binding studies of quinolone drugs were the center of attention by a number of researchers [34-35]. All these reports mentioned the quinolone based drugs interacted with DNA through the groove binding.

The cyclic voltammetric measurements of 0.1M strength solution of the compounds (**1-4**) were performed in the absence and presence of DNA and their voltammogarams were recorded at 298K. The scan rate was 0.2volt/sec at glassy carbon electrode. The electrochemical investigations of compound–DNA interactions supported the spectroscopic studies

The redox couple for each compound had been studied with addition of DNA and the corresponding potentials with their shifts are given in table 4. Generally positive shift in an electrochemical potential of a small molecule was observed when mode of interaction was intercalative, while negative shift was observed in the case of electrostatic interaction mode with DNA [36].

In case of present compounds, no new redox peaks were appeared after the addition of DNA to compound but the current of all the peaks changed significantly, suggesting the existence of an interaction between compound and DNA. In compound **1-4,** with addition of DNA, a gradual shift in the positive potential was observed in the voltammetric wave accompanied by decrease in peak current. The positive shift in peak potentials represented groove bindings as well as intercalative mode of bindings [37-38] while negative shifts were the indicative of electrostatic interactions [39-40]. Intercalative and groove bindings were dependent upon DNA double helix. But the electrostatic bindings took place outside the groove of DNA. The peak current decreased as the free compound molecules were used in the formation of compound-DNA adducts. Assignments in Table-3 showed that with increasing amounts of DNA, one of the cathodic (Epc) or the anodic (Epa) potentials of compounds showed a positive shift.

The voltammograms of compounds **1-4** seems to be reversible. The ΔE is given as;

$$\Delta E = E_{red} - E_{oxd}$$
$$= 0.10/n \text{ V}$$

If we consider to be single electron transfer process then n=1 and its value would be 0.10V which would not fulfil the condition of reversibility. For a reversible system its value should be equal to 0.059V. Also for a reversible systems the ratio of cathodic and anodic peak current should be equal to 1. Here ipr/ipf =1.0 so this system was not reversible. Mostly metal atom in a molecule showed reversible processes of oxidation and reductions. The voltammogram of compound **2** as represented in Fig 6 showed a positive shift in peak potential while its UV spectrum did not give a prominent shift to higher wavelengths**.** So it is suggested that compound **3** could interact with DNA through groove bindings. Table 5 showed that the binding constant of compound **3** with DNA was greater than other compounds. Moreover, data in Table-5 reflected that the compound-DNA adduct formation is a spontaneous process and both techniques spectroscopic and voltammetric assignments agree well with each other.

The binding constant "K" was calculated according to the following equation [41].

$$\log\left(\frac{1}{[DNA]}\right) = logK + \frac{\log(I(H-G))}{[IG-(I(H-G))]}$$

where K is the binding constant, $I_G$ and $I_{H-G}$ are the peak currents of the free guest (G) and the complex (H–G), respectively.

While standard Gibbs free energy was calculated by using binding constant. The value of "K" represents binding strength of compound with DNA while ΔG represents the spontaneity of adduct (compound +DNA complex). The maximum value of K was of compound **1** i.e $2.98*10^3$, suggesting stronger binding between DNA and compound. Almost same value of **1** had been calculated by UV studies i.e $3.0*10^3 M^{-1}$. While minimum value of ΔG was for compound **2** (-24.51kJ/mol) showing a stable adduct of DNA-compound. UV data had also calculated its value to be nearly -25.50 kJ/mol.

Both UV and CV studies showed a stronger interaction of compounds with DNA through intercalation. This could be proposed in terms of α,β unsaturated system which were responsible for reversible electron transfer and existence of different resonance structures and dimerization of the molecule. With the reaction of these centers, the resonance structures were disappeared thus making difficulties in electron transfers processes so giving irreversible peaks. The interactions in present case could be proposed to be due to the presence of substituents. In present study compound **3** showed stronger interactions among 2-Hydroxy-1,4-naphthaquinone derivatives. Compound **4** is 4-Nitrochalcone derivative of 2-Hydroxy-1,4-naphthaquinone moiety. It is suggested that as nitro being electron withdrawing group interacted with DNA through an intercalation as well as groove bindings.

*Antimicrobial activity*

Preliminary in vitro tests for antimicrobial activity of Warfarin Analogues had been carried out by disk diffusion method. For antibacterial assay *Escherichia coli, Acetobacter aceti, Staphylococcus aureus, Klebsilla pneumonia and Pseudomonas aeruginosa* strains were selected. In vitro biological screening tests of the synthesized compounds were carried out for antibacterial activity. The results are tabulated in Table 6. The experiment was performed in triplicate using the agar well-diffusion method. The antibacterial data demonstrated that all synthesized compounds showed antibacterial activity but lower than standard used. Among the synthesized drugs, compound **1** is more potent against *E.coli* and *K.Pneumoniae*. Similarly, compound **4** is more potent against *P.aeuroginosa* and showed no activity against *K.Pneumoniae*.

The synthesized compounds were also subjected to antifungal activity tests against two fungal strains (Aspergillus flavus and Candida Albicans) and results are tabulated in Table 7. Fluconazole was used as a standard drug in this assay. The criteria for activity was based on percent growth inhibition; more than 70% growth inhibition was considered as significant activity, 60–70% inhibition activity was good, 50–60% inhibition activity was moderate, while below 50% inhibition activity was considered as non-significant. The results showed that synthesized compound **1** had significant activity at 1000µg/ml against both types of strains. Similarly compound **3** had significant activity against *A. flavus* even at 500µg/ml.

Among synthesized compounds, compound **4** is least active against both strains of fungus and showed non-significant activity against both strains at 500µg/ml. The activity data showed that all synthesized compounds were less active than standard drug Fluconazole. The maximum activity of compound **1** may be due to the presence of bromine substitution position. It has been reported earlier that bromine substitution inhibit enzyme activity which is required for their activity.

## Conclusion

It has been concluded that catalyst **B** is an efficient chiral organocatalyst, which gave Michael adducts (**5c**, **6c-6e Compound1-4**) in good yields and enantioselectivity. Maximum yield of 85% was obtained for compound **1**(**5c**), while its enantioselectivity was low to 50%ee. The maximum ee value of 75% was obtained for compounds **4** (**6e**). Antifungal and antibacterial screening of these compounds suggests their effective use other than anticoagulant activities. The good antibacterial agent was found out to be compound **3** (**6d**) while the excellent antifungal compounds were compound **1** (**5c**) and **2** (**6c**). Furthermore DNA-binding studies also showed that chiral drugs can bind effectively with DNA. The anti-cancer activities of these compounds could be monitored and be beneficial in future.

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