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Characterization of 4-methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate as an effective antiplatelet agent

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ABSTRACT

We have studied earlier a membrane bound novel enzyme Acetoxy Drug: protein transacetylase identified as Calreticulin Transacetylase (CRTAase) that catalyzes the transfer of acetyl groups from polyphenolic acetates (PAs) to the receptor proteins and thus modulating their biological activities. In this communication, we have reported for the first time that acetoxy quinolones are endowed with antiplatelet action by virtue of causing CRTAase catalyzed activation of platelet Nitric Oxide Synthase (NOS) by way of acetylation leading to the inhibition of ADP/Arachidonic acid (AA)-dependent platelet aggregation. The correlation of specificity of platelet CRTAase to various analogues of acetoxy quinolones with intracellular NO and consequent effect on inhibition of platelet aggregation was considered crucial. Among acetoxy quinolones screened, 6-AQ (4-methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate/6-acetoxyquinolin-2-one, **22**) was found to be the superior substrate to platelet CRTAase and emerged as the most active entity to produce antiplatelet action both in vitro and in vivo. 6-AQ caused the inhibition of cyclooxygenase-1 (Cox-1) resulting in the down regulation of thromboxane A2 (TxA2) and the inhibition of platelet aggregation. Structural modification of acetoxy quinolones positively correlated with enhancement of intracellular NO and antiplatelet action.

1. Introduction

Platelets are involved in the cellular mechanisms of primary homeostasis leading to the formation of blood clots. Platelets are activated when brought into contact with agents such as collagen, thrombin, and ADP. The damage to the blood vessel walls exposes the sub endothelium proteins, most notably collagen. The circulating platelets bind collagen with collagen-specific glycoprotein Ia/IIa receptors. The adhesion is strengthened further by the large, multimeric circulating protein like von Willebrand factor (vWF), which forms links between the platelets glycoprotein Ib/IX/V and the collagen fibrils. This adhesion activates the platelets. ADP receptors P2Y1 and P2Y12, both belonging to the G-protein-coupled seven-transmembrane domain receptor family, expressed on platelets, extensively bind to ADP resulting in the release of dense granules containing PAF (Platelet Activating Factors) vWF, serotonin, thromboxane A2 (TxA2) which further activate the other circulating platelets.[1] Numerous antiplatelet agents were developed based on their ability to block the receptors responsible for platelet activation. Further, the agents causing the inhibition of cyclooxygenase catalyzed TxA2 synthesis would also lead to the inhibition of platelet aggregation.[2] Previous investigations carried out in our laboratory documented for the first time, the remarkable activation of endothelial NOS by a certain class of PAs by way of acetylation of NOS mediated by CRTAase.[3] Accordingly, PAs were found to be effective in the inhibition of ADP induced platelet aggregation.

In the present investigation, efforts have been made to compare the specificities of acetoxy quinolones on CRTAase mediated activation of NOS and also to delineate the structure activity relationship (SAR) with reference to the effect of position of substitution of acetoxy group on benzenoid ring and pyridone ring, alkyl group at C-3 position of the quinolone moiety, and substitution at *N*- and *O*- of the pyridone ring of PAs. The results clearly demonstrated 6-acetoxy-4-methylquinolin-2-one (6-AQ, **22**) to be the best substrate to platelet CRTAase compared to the other acetoxy quinolones resulting in inhibition of ADP induced platelet aggregation.

Abbreviations: AA, arachidonic acid; AQ, acetoxy quinolone; CDNB, 1-cloro 2,4-dinitro benzene; Cox-1, cyclooxygenase-1; Cox-2, cyclooxygenase-2; CRTAase, calreticulin transacetylase; DAMC, 7,8-diacetoxy-4-methyl coumarin; DCFH-DA, dichlorofluorescin diacetate; NOS, nitric oxide Synthase; GSH, reduced glutathione; GST, glutathione-*S*-transferase; NO, nitric oxide; PAs, polyphenolic acetates; PPP, platelet poor plasma; PRP, platelet rich plasma; TxA2, thromboxane A2; TxB2, thromboxane B2.

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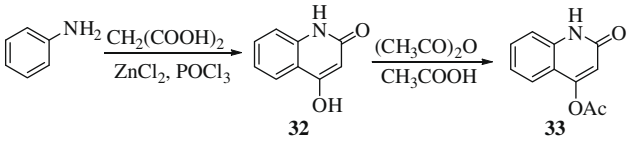
2. Results

In our earlier work we elucidated the role of acetoxy groups on the benzenoid ring of chromones, coumarins, xanthones, and flavones in facilitating the acetylation of receptor proteins catalyzed by CRTAase. In this regard we also studied the factors, such as the proximity of the acetoxy group to the oxygen heteroatom, the role of carbonyl group on the benzopyran nucleus, and the effect of substituents on the coumarin molecule in controlling the protein acetylation.[4,5]

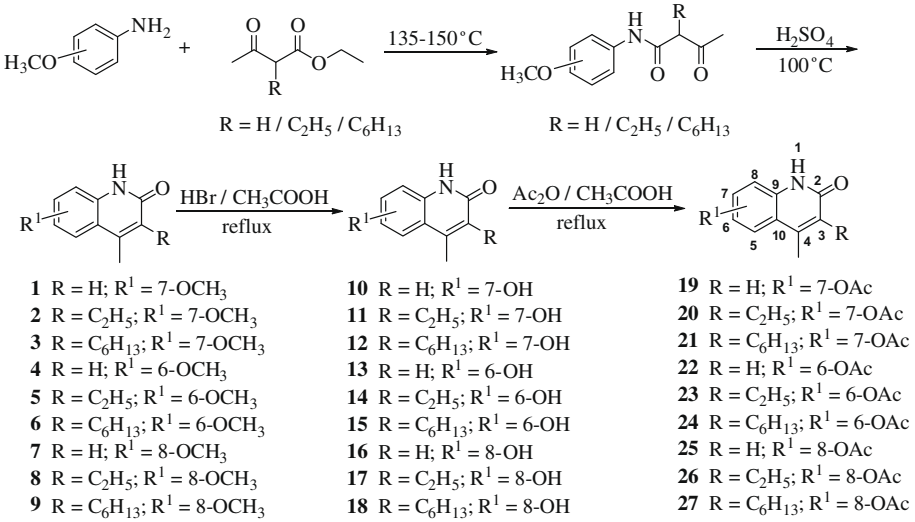
However, the action of CRTAase on acetoxy quinolones has not been studied so far. Herein, we have elucidated the action of CRTAase on a series of acetoxy quinolones and consequent effect on the enhancement of NO levels in platelets and inhibitory effect on ADP/AA induced platelet aggregation. We have compared the specificities of acetoxy quinolones by varying the position of acetoxy group on benzenoid ring, replacing 4-methyl group by acetoxy group, and by incorporating alkyl groups of varying size at C-3 position. Also, we have synthesized N- and O-substituted alkyl esters of acetoxy quinolin-2-ones to study the CRTAase substrate specificity. Such a study would allow us to observe the effect thereof on the rate of catalytic activity of CRTAase and the efficacy of these acetoxy quinolones to activate platelet NOS. The methoxy derivatives of C-3 alkyl quinolones (**1**–**9**) were synthesized via Knorr reaction of 2-alkyl ethyl acetoacetate with anisidines. Demethylation was carried out with a mixture of hydrobromic acid and acetic acid to yield corresponding hydroxy quinolones (**10**–**18**) which were then acetylated with acetic anhydride in acetic acid to yield acetoxy derivatives **19**–**27** (Scheme 1). The 6- and 7-acetoxy derivatives of quinolones were further derivatized into *N*- (**28** and **29**) and *O*-alkyl esters (**30** and **31**) by reaction with ethyl bromoacetate in the presence of potassium carbonate (Scheme 2). The formation of two isomers is due to the existence of tautomerism in the amidic bond. 4-Hydroxy quinolone (**32**) was prepared by reaction of aniline with malonic acid in the presence of zinc chloride and phosphorus oxychloride and further its acetylation gives 4-acetoxy quinolone (4-AQ, **33**), as shown in Scheme 3. All the compounds were fully characterized on the basis of their physical and spectral data, and of total 33 quinolone derivatives, 21, that is, **2**, **3**, **5**, **6**, **8**, **9**, **12**, **14**, **15**, **17**, **18**, **21**, and **23**–**31** are novel.

2.1. CRTAase activity

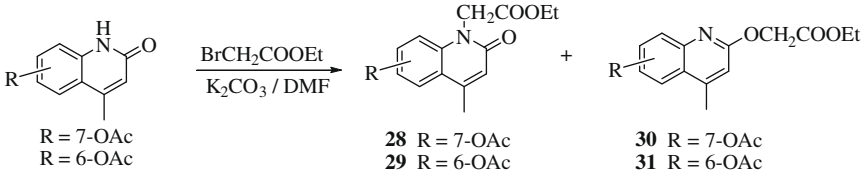
The results documented in Table 1 revealed the deferential specificity of platelet CRTAase to a number of acetoxy quinolones and the position of acetoxy group played a key role in deciding specificity of platelet CRTAase and was found to be in the order: 6-AQ > 7-AQ∼8-AQ ⋙ 4-AQ. It is evident from the results (Table 1) that substitution of alkyl group at C-3 position resulted in drastic reduction of CRTAase activity and longer the alkyl chain greater was the inhibition. The substitution of ester group at *N*-, that is, in compounds **28** and **29** resulted in the marginal decline of CRTAase activity of platelets, while O-substitution in compounds **30** and **31** hardly affected the activity. Moreover, substitution of 4-methyl group with acetoxy group (compound **33**) showed poor inhibition of platelet aggregation.



Scheme 3. Synthesis of 4-acetoxy derivative of quinolin-2-one.



Scheme 1. Synthesis of 3-alkyl derivatives of quinolin-2-ones.



Scheme 2. Synthesis of N- and O-substituted alkyl esters of quinolin-2-ones.

Table 1
Assay of CRTAase activity of platelets using acetoxy quinolones as the acetyl group donor

Acetoxy quinolones	CRTAase activity (units)
19	13.25 ± 0.085
20	13.0 ± 0.073
21	2.0 ± 0.052
28	10.0 ± 0.044
30	13.2 ± 0.081
22	31.0 ± 0.095
23	26.0 ± 0.071
24	7.0 ± 0.067
13	Nil
29	23.0 ± 0.077
31	28.6 ± 0.268
25	12 ± 0.052
26	11.5 ± 0.045
27	Nil
33	Nil

CRTAase activity was assayed in platelet lysate as described in Section 4.1. The unit of CRTAase was expressed in terms of % inhibition of GST under the experimental conditions. Values are mean ± SEM of five observations (p <0.01).

2.2. Enhancement of intracellular nitric oxide (NO) levels

2.2.1. Measurement of NO level by flow cytometry

The influence of acetoxy quinolones on the NO level in platelets has been clearly brought out in Figure 1. Platelets were incubated with acetoxy quinolones followed by the measurement of NO levels by flow cytometry. It is clear from the results that 6-AQ (**22**) profoundly enhanced NO level in platelets as compared to 7-acetoxy- (**19**) and 8-acetoxyquinolone (**25**) derivatives. The structural modification of acetoxy quinolones was found to influence their effect on NO production in platelets in tune with the specificity of platelet CRTAase to these compounds (Table 1).

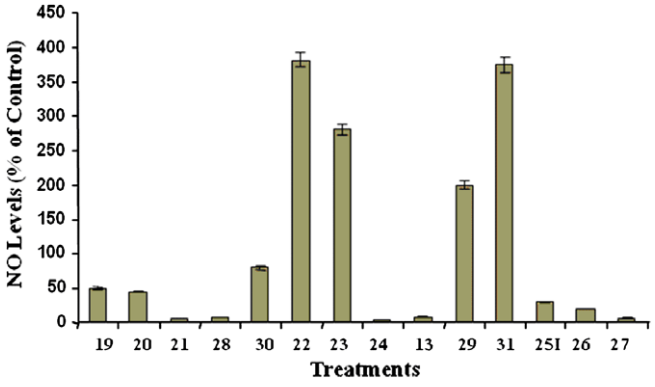


Figure 1. Influence of acetoxy quinolones on NO levels in washed platelets.

2.2.2. Effect of acetoxy quinolones on platelet aggregation

2.2.2.1. Platelet aggregation in vitro. The effect of acetoxy quinolones on the ADP and AA induced platelet aggregation in vitro is shown in Figure 2. PRP was incubated with acetoxy quinolones followed by the measurement of platelet aggregation by the addition of ADP/AA. 6-AQ (**22**) inhibited the ADP induced platelet aggregation to a greater extent as compared to 7-AQ (**19**) and 8-AQ (**25**) derivatives. The modification of carbonyl group of the quinolone moiety (**30** and **31**) hardly affected the platelet aggregation while the presence of alkyl ester at *N*- (**28** and **29**) resulted in appreciable reduction of platelet aggregation. 6-AQ has profound effect on AA induced platelet aggregation as compared to that induced by ADP. The trend of activities followed the same manner, higher in case of 6-acetoxy as compared to 7- and 8-acetoxy derivatives. These observations have amply revealed that the structural modifications of acetoxy quinolones alter their effectiveness in the manner dependent on their specificities to CRTAase as the substrate. The 6-hydroxyquinolone (**13**) was devoid of antiplatelet activity.

2.2.2.2. Platelet aggregation in vivo. The effect of 6-AQ (**22**) on the ADP/AA induced platelet aggregation in vivo is shown in Figure 3. Rats were treated with 6-AQ p.o., sacrificed after 24 h and the platelet aggregation phenomenon was assessed by aggregometry. 6-AQ was also found to effectively inhibit both ADP as well as AA induced platelet aggregation in vivo. Like aspirin, 6-AQ has also effectively inhibited AA induced platelet aggregation much more than induced by ADP. Accordingly, 6-AQ exhibited greater antiplatelet activity in rats while the 6-hydroxy derivative (**13**) had no ability to inhibit platelet aggregation.

2.3. Cox-1 activity assay

The effect of 6-AQ (**22**) on the Cox-1 activity in vivo is shown in Figure 4. Rats were treated with 6-AQ p.o., sacrificed after 24 h and the Cox-1 activity was measured by ELISA. 6-AQ was found to effectively inhibit Cox-1 activity like aspirin by approximately 3.5-fold. Accordingly, 6-AQ exhibited greater inhibition of Cox-1 activity in rats while the 6-hydroxy derivative (**13**) could not.

3. Discussion

Cardiovascular diseases such as myocardial infarction, unstable angina, and deep vein thrombosis greatly contribute to the mortality in the developed world. For the treatment of such heart condi-

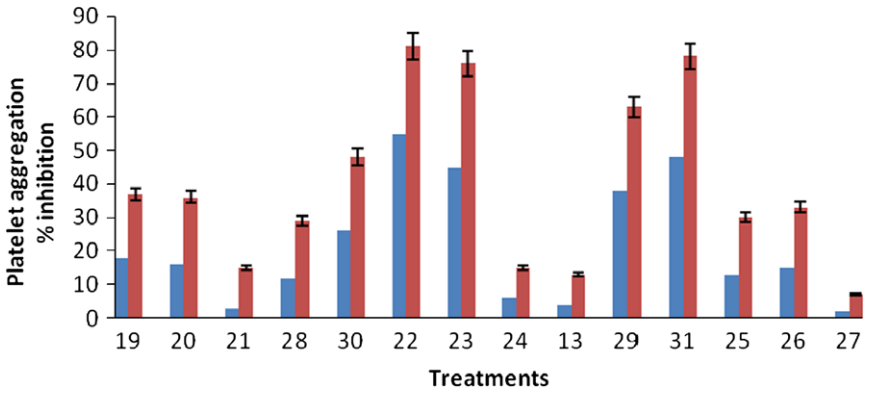


Figure 2. Screening of acetoxy quinolones for antiplatelet activity in vitro.

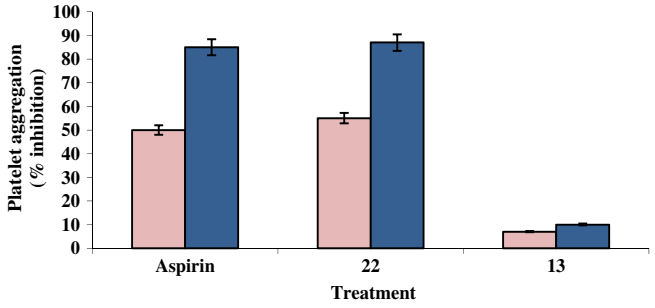


Figure 3. Antiplatelet activity of 6-AQ in vivo.

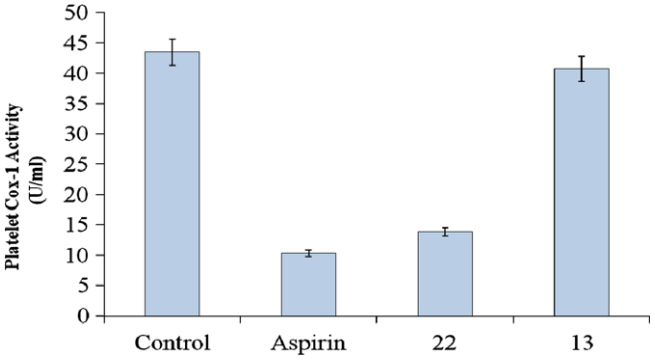


Figure 4. Assay for Cox-1 activity.

tions there is a greater need for the application of antiplatelet drugs.[6] Intensive search for the newer antiplatelet drugs is going on worldwide. We have in this report characterized for the first time acetoxy quinolones as the effective antiplatelet agents. The rationale for viewing these compounds as the possible antiplatelet targets had its origin with our novel finding that PAs such as acetoxy coumarins were the enhancers of intracellular NO by virtue of causing acetylation of NOS.[7] Recently, it was also observed that aspirin acetylates NOS-3 in platelets that increases the activity of this enzyme which would give rise to an increase in platelet NO biosynthesis and thus contribute to the antiplatelet effect of aspirin. Phosphorylation of NOS-3 has been shown to occur on serine residues 114, 615, 633, and 1177 as well as on threonine-495 and can have important modulatory effects on their activity independent of any changes in Ca^{2+} concentration.[8] However, our earlier work convincingly established that Calreticulin, an important Ca^{2+} binding protein of lumen of endoplasmic reticulum, mediate the transfer of acetyl group from PAs to target protein such as NOS.[7,9,10] The protein acetyl transferase function of Calreticulin utilizing PAs as the acetyl group donors was termed CRTAase.[11] PAs by virtue of enhancing NO levels in platelets were considered to merit as antiplatelet agents. Accordingly, 7,8-diacetoxy 4-methylcoumarin (DAMC), a model PA, was found to significantly inhibit ADP induced platelet aggregation.[12] We have in this paper sought to correlate the ability of acetoxy quinolones to inhibit platelet aggregation with special reference to the specificity of platelet CRTAase to acetoxy quinolones and NOS activation. The results presented in the Table 1 revealed that various acetoxy quinolones are the substrates for platelet CRTAase to varying degrees. Accordingly, among the acetoxy quinolones screened, 6-AQ (**22**) was found to be the most suitable substrate to platelet CRTAase that activated platelet NOS to a greater extent compared to the other acetoxy quinolones resulting in inhibition of ADP/AA induced platelet aggregation. The kinetic analysis depicts a clear picture of structural activity relation where the affinity for various quinolones is in the order: 6 AQ > 7AQ∼8 AQ (Table 2). The results documented in Tables 1 and 2 reveal the deferential specificity of

Table 2
Kinetic analysis of PAs to CRTAase catalyzed transacetylase activity

Compound	K_m	v_{max}
DAMC	980	352
7-AQ (**19**)	1528	258
6-AQ (**22**)	1050	327
8-AQ (**25**)	1558	240

Substrates were separately preincubated (37 °C, 10 min) with CRTAase and GST in potassium phosphate buffer (pH 6.5) followed by addition of GSH and CDNB. The absorbance was measured at 340 nm. Initial reaction velocities of CRTAase were determined at varying substrates concentrations.

platelet CRTAase to a number of acetoxy quinolones and shows that the position of acetoxy group on the phenyl ring of quinolones plays an important role in deciding specificity of platelet CRTAase. It is evident from the results (Table 1) that the addition of alkyl group at C-3 results in drastic reduction of CRTAase activity, that is, longer the alkyl chain greater is the inhibition. The addition of ester group at *N*- results in the marginal decline of CRTAase activity of platelets, while the substitution of ethyl ester group at *O*- hardly affects the activity of platelet CRTAase when used as substrate.

It is hypothesized that the enzyme catalyzed acetylation led to the generation of phenoxide ion, the partial charge calculated using ChemBio3D Ultra 11.0 (2008) indicates the amidic nitrogen to have substantial positive charge (+0.6824) and to partially offset this charge lone pair of electrons on oxygen attached to the benzenoid ring may undergo partial conjugation with amidic nitrogen (Fig. 5). This conjugation seems to be more effective in case of 6-acetoxy quinolones as compared to its 7-/8-acetoxy analogues.

It is pertinent to point out that the ability of 6-AQ to inhibit platelet aggregation by the enhancement of NO levels in platelets. The enhanced NO formation in blood vessels is known to regulate the vascular functions.[13] 6-AQ effectively inhibited the Cox-1 activity like aspirin through the blunting of Cox-1 activity and eventually TxA2, an AA metabolite acting as an endogenous platelet activator, intensifies the extent of platelet aggregation. It is noteworthy that 6-hydroxyquinolone (**13**), the deacetylated product of 6-AQ was totally ineffective for the inhibition of platelet aggregation and the similar pattern was observed for Cox-1 activity also. This observation highlighted the acetyl group of quinolone as the active moiety responsible for activation of platelet NOS through acetylation leading to the antiplatelet action of acetoxy quinolones. Fluoroquinolones are important and effective antibacterial agents. They are recommended for a number of serious bacterial infections and in some cases of life threatening infections. Quinolones in general are well tolerated drugs. However, the toxicity of 6-AQ if any, has to be ascertained before they are chosen for antiplatelet therapy.[14] The results documented in this paper have projected for the first time the antiplatelet action of a PA, 6-AQ implicating the cardinal role of CRTAase in the mechanism of action.

4. Experimental

4.1. Materials and methods

4.1.1. Chemicals

The organic solvents (acetone, acetic anhydride, chloroform, tetrahydrofuran, DMF, petroleum ether, and ethyl acetate) were dried and distilled prior to their use. Reactions were monitored

Figure 5. Conjugation in case of 6-substituted quinolone.

by precoated TLC plates (Merck Silica Gel 60F254); the spots were visualized either by UV light, or by spraying with 5% alcoholic $FeCl_3$ solution. Silica gel (100–200 mesh) was used for column chromatography. Sodium hydride (60% dispersed in mineral oil) was supplied from Spectrochem. Pvt. Ltd, India. Melting points were recorded in capillaries in sulfuric acid bath and are uncorrected. Infrared spectra were recorded on Perkin–Elmer FT-IR model BXspectrophotometer. The ^1H NMR and ^{13}C NMR spectra were recorded on Bruker AC-400 (400 MHz, 100 MHz) NMR spectrometer and Avance-300 spectrometer using TMS as internal standard. The chemical shift values are on δ scale and the coupling constant values (J) are in Hertz. The EI/HR mass spectra were recorded on Agilent-6210 ES-TOF. Ethyl 2-ethyl-3-oxobutanoate, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), dichlorofluorescin diacetate (DCFH-DA), L-arginine, adenosine diphosphate (ADP), were obtained from M/S Sigma Chemical Co. St. Louis. Mo. USA. All other chemicals used were of high purity and were obtained from local suppliers.

4.1.2. General procedure for the synthesis of 3-alkyl-methoxy-4-methylquinolin-2-ones (1–9)

Anisidine (20 g, 164 mmol) was added drop wise to alkylated ethyl acetoacetate[15] (277 mmol) and the reaction mixture was refluxed for 20 h. After the completion of reaction, the contents were cooled and then poured on sodium carbonate solution. The compound was extracted with chloroform and the solvent was evaporated in vacuo. 70% Sulfuric acid (40 mL) was added and the reaction mixture was stirred at 95 °C. The progress of reaction was monitored on TLC (5% methanol–chloroform). On completion of the reaction, the solution was cooled and poured on crushed ice (500 g). The resulting precipitate was filtered and washed with water and petroleum ether. The crude product was recrystallized from ethanol to give methoxy quinolin-2-ones **1**–**9**.[16]

4.1.2.1. 7-Methoxy-4-methylquinolin-2(1*H*)-one (1). It was obtained as white solid (83%); mp: 195 °C, (Literature value = 196 °C);[17] ^1H NMR (acetone-d_6, 300 MHz): δ 2.45 (s, 3H, C-4 CH_3), 3.88 (s, 3H, OCH_3), 6.30 (s, 1H, H-3), 6.84 (d, 1H, J = 8.7 Hz, H-6), 6.94 (s, 1H, H-8), 7.66 (d, 1H, J = 8.9 Hz, H-5), 11.00 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 19.36 (C-4 CH_3), 56.14 (OCH_3), 99.12, 111.12 (C-6 and C-8), 114.68 (C-3), 118.56 (C-10), 127.02 (C-5), 141.26 (C-9), 148.85 (C-4), 161.80 (C-7), 162.89 (C-2); IR (KBr) ν_{max}: 2957.41, 1658.97, 1629.05, 1549.71, 1474.57, 1417.53, 1261.70, 1217.14, 1177.11, 1023.59, 856.63, 808.71, 710.74 cm^{-1}; UV (methanol) λ_{max}: 323 and 337 nm; HRMS: $C_{11}H_{11}O_2N$ $[M]^+$: 189.9665.

4.1.2.2. 3-Ethyl-7-methoxy-4-methylquinolin-2(1*H*)-one (2). It was obtained as yellow solid (65%); mp: 126 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.17 (t, 3H, J = 7.4 Hz, CH_2CH_3), 2.46 (s, 3H, C-4 CH_3), 2.81 (q, 2H, J = 7.5 Hz, CH_2CH_3), 3.89 (s, 3H, –OCH_3), 6.78 (s, 1H, H-8), 6.81 (d, 1H, J = 9.2 Hz, H-6), 7.59 (d, 1H, J = 8.7 Hz, H-5), 11.95 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.50 (CH_2CH_3), 14.84 (C-4 CH_3), 23.74 (CH_2CH_3), 55.51 (OCH_3), 107.09, 119.39 (C-6 and C-8), 122.56 (C-10), 124.94 (C-3), 131.05 (C-5), 143.97 (C-9), 147.56 (C-4), 152.31 (C-7), 160.38 (C-2); IR (KBr) ν_{max}: 2932.78, 1660.95, 1621.90, 1560.80, 1512.79, 1461.85, 1396.94, 1255.88, 1223.07, 1179.61, 1141.37, 1029.98, 925.23, 842.09, 763.03 cm^{-1}; UV (methanol) λ_{max}: 324 and 339 nm; HRMS: $C_{13}H_{15}O_2N$ $[M+H]^+$: 218.3962.

4.1.2.3. 3-Hexyl-7-methoxy-4-methylquinolin-2(1*H*)-one (3). It was obtained as yellow solid (75%); mp: 140 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.91 (br s, 3H, CH_2CH_3), 1.26–1.55 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.47 (s, 3H, C-4 CH_3), 2.76 (t, 2H, J = 7.3 Hz, CH_2CH_2), 3.89 (s, 3H, OCH_3), 6.81–6.86 (m, 2H, H-6 and H-8), 7.60 (d, 1H, J = 8.9 Hz, H-5), 12.29 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 11.80 (CH_2CH_3), 14.48 (C-4 CH_3), 23.09, 27.27, 29.57, 30.01, 32.21 ($(CH_2)_5CH_3$), 55.79 (OCH_3), 98.72, 111.94 (C-6 and C-8), 115.71 (C-10), 125.96 (C-3), 129.05 (C-5), 138.92 (C-9), 143.51 (C-4), 160.97 (C-7), 164.76 (C-2); IR (KBr) ν_{max}: 1660.22, 1612.15, 1562.22, 1514.39, 1462.82, 1398.69, 1260.73, 1225.71, 1173.39, 1028.91, 940.95, 927.99, 798.50, 607.85 cm^{-1}; UV (methanol) λ_{max}: 325 and 339 nm; HRMS: $C_{17}H_{23}O_2N$ $[M]^+$: 273.1893.

4.1.2.4. 6-Methoxy-4-methylquinolin-2(1*H*)-one (4). It was obtained as white solid (75%); mp: 260–262 °C, (Literature value = 260–262 °C);[18] ^1H NMR (DMSO-d_6, 300 MHz): δ 2.42 (s, 3H, C-4 CH_3), 3.82 (s, 3H, OCH_3), 6.39 (s, 1H, H-3), 7.13–7.18 (m, 2H, H-5 and H-7), 7.26 (dd, 1H, J = 1.8 and 5.3 Hz, H-8), 11.46 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 19.45 (C-4 CH_3), 56.37 (OCH_3), 107.69, 117.52 (C-5 and C-7), 119.87 (C-10), 121.06 (C-8), 122.11 (C-9), 131.38 (C-3), 148.27 (C-4), 154.96 (C-6), 162.03 (C-2); IR (KBr) ν_{max}: 3433.06, 2821.76, 1653.70, 1621.98, 1503.71, 1421.31, 1275.63, 1240.22, 1202.18, 1179.30, 1044.05, 835.98, 628.40 cm^{-1}; UV (methanol) λ_{max}: 269 and 350 nm; HRMS: $C_{11}H_{11}O_2N$ $[M]^+$: 189.8188.

4.1.2.5. 3-Ethyl-6-methoxy-4-methylquinolin-2(1*H*)-one (5). It was obtained as white solid (65%); mp: 204–206 °C; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.01 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.39 (s, 3H, C-4 CH_3), 2.63 (q, 2H, J = 7.2 Hz, 2H, CH_2CH_3), 3.79 (s, 3H, OCH_3), 7.06–7.14 (m, 2H, H-5 and H-7), 7.21 (d, 1H, J = 8.7 Hz, H-8), 11.49 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.21 (CH_2CH_3), 14.52 (C-4 CH_3), 19.71, (CH_2CH_3), 55.40 (OCH_3), 106.83, 116.19 (C-5 and C-7), 117.65 (C-8), 120.64 (C-10), 131.45 (C-3), 132.90 (C-9), 140.99 (C-4), 154.02 (C-6), 160.85 (C-2); IR (KBr) ν_{max}: 3434.17, 2965.75, 1642.07, 1502.12, 1462.63, 1414.19, 1371.42, 1271.48, 1218.23, 1131.27, 1038.23, 927.21, 836.80, 633.80 cm^{-1}; UV (methanol) λ_{max}: 272 and 349 nm; HRMS: $C_{13}H_{15}O_2N$ $[M]^+$: 217.7774.

4.1.2.6. 3-Hexyl-6-methoxy-4-methylquinolin-2(1*H*)-one (6). It was obtained as white solid (70%); mp: 146–148 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.91 (br s, 3H, CH_2CH_3), 1.26–1.57 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.47 (s, 3H, C-4 CH_3), 2.82 (t, 2H, J = 7.1 Hz, CH_2CH_2), 3.87 (s, 3H, OCH_3), 7.08–7.11 (m, 2H, H-5 and H-7), 7.35 (d, 1H, J = 8.4 Hz, H-8), 12.35 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.18 (CH_2CH_3), 15.21 (C-4 CH_3), 22.67, 27.13, 29.08, 29.56, 31.81 ($(CH_2)_5CH_3$), 55.71 (OCH_3), 106.57, 117.34 (C-5 and C-7), 117.73 (C-8), 121.72 (C-10), 131.51 (C-3), 132.18 (C-9), 142.28 (C-4), 154.83 (C-6), 163.52 (C-2); IR (KBr) ν_{max}: 3148.91, 2928.00, 2850.90, 1656.22, 1624.10, 1560.71, 1506.61, 1462.53, 1418.38, 1382.73, 1279.60, 1209.01, 1173.66, 1039.55, 855.83. 804.15, 725.86, 643.24 cm^{-1}; UV (methanol) λ_{max}: 274 and 348 nm; HRMS: $C_{17}H_{23}O_2N$ $[M]^+$: 273.4577.

4.1.2.7. 8-Methoxy-4-methylquinolin-2(1*H*)-one (7). It was obtained as white solid (30%); mp: 192–194 °C, (Literature value = 188–190 °C);[19] ^1H NMR (DMSO-d_6, 300 MHz): δ 2.41 (s, 3H, C-4 CH_3), 3.89 (s, 3H, OCH_3), 6.42 (s, 1H, H-3), 7.14–7.15 (m, 2H, H-5 and H-7), 7.27–7.30 (m, 1H, H-6), 10.58 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 18.74 (C-4 CH_3), 56.07 (OCH_3), 110.89, 116.36 (C-5 and C-7), 120.08 (C-9), 121.36, 121.57 (C-3 and C-6), 128.57 (C-10), 145.80 (C-4), 148.15 (C-8), 161.14 (C-2); IR (KBr) ν_{max}: 3163.95, 2933.15, 1648.26, 1605.82, 1462.84, 1389.90, 1265.97, 1154.88, 1049.33, 860.83, 791.04, 739.93, 726.00, 636.77 cm^{-1}; UV (methanol) λ_{max}: 278 and 335 nm; HRMS: $C_{11}H_{11}O_2N$ $[M]^+$: 189.7739.

4.1.2.8. 3-Ethyl-8-methoxy-4-methylquinolin-2(1*H*)-one (8). It was obtained as white solid (20%); mp: 186–188 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.03 (t, 3H, J = 7.5 Hz, CH_2CH_3), 2.41 (s, 3H, C-4 CH_3), 2.65 (q, 2H, J = 7.5 Hz, CH_2CH_3), 3.89 (s, 3H, OCH_3), 7.07–7.16 (m, 2H, H-5 and H-6), 7.32 (dd, 1H, J = 2.1 and 7.5 Hz, H-7), 10.48 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.12 (CH_2CH_3), 14.69 (C-4 CH_3), 19.69 (CH_2CH_3), 56.02 (OCH_3), 109.83, 116.28 (C-5 and C-7), 120.47 (C-3), 121.33 (C-6), 126.93 (C-9), 132.98 (C-10), 141.66 (C-4), 145.47 (C-8), 160.75 (C-2); IR (KBr) ν_{max}: 3148.45, 2927.25, 1638.11, 1485.25, 1390.38, 1259.63, 1214.22, 1051.94, 866.49, 766.44, 727.12, 677.02 cm^{-1}; UV (methanol) λ_{max}: 256, 281 and 333 nm; HRMS: $C_{13}H_{15}O_2N$ [M]$^+$: 217.1143.

4.1.2.9. 3-Hexyl-8-methoxy-4-methylquinolin-2(1*H*)-one (9). It was obtained as white solid (25%); mp: 112–114 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.89 (br s, 3H, CH_2CH_3), 1.31–1.52 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.46 (s, 3H, C-4 CH_3), 2.74 (t, 2H, J = 7.2 Hz, CH_2CH_2), 3.96 (s, 3H, OCH_3), 6.93 (d, 1H, J = 7.8 Hz, H-5), 7.12 (t, 1H, J = 8.1 Hz, H-6), 7.29 (d, 1H, J = 8.4 Hz, H-7), 9.10 (br s, 1H, NH); ^{13}C NMR (DMSO, 75 MHz): δ 14.14 (CH_2CH_3), 15.36 (C-4 CH_3), 22.70, 27.22, 29.00, 29.60, 31.84 ($(CH_2)_5CH_3$), 55.98 (OCH_3), 108.75, 116.36 (C-5 and C-7), 121.37 (C-3), 121.46 (C-6), 126.84 (C-9), 132.73 (C-10), 142.63 (C-4), 145.39 (C-8), 161.64 (C-2); IR (KBr) ν_{max}: 2924.14, 1654.17, 1647.83, 1459.82, 1271.95, 1217.55, 1051.29, 862.42, 765.87, 727.19, 618.33 cm^{-1}; UV (methanol) λ_{max}: 281 and 334 nm; HRMS: $C_{17}H_{23}O_2N$ [M]$^+$: 273.2189.

4.1.3. General procedure for the synthesis of 3-alkyl-hydroxy-4-methylquinolin-2-ones (10–18)

A mixture of 10 mL hydrobromic acid and acetic acid (7:3) was added to 1 g of 3-alkyl-methoxy-4-methyl-1*H*-quinolin-2-ones (**1**–**9**). The reaction mixture was refluxed for 75 h and then poured on crushed ice. The resulting precipitate was filtered and washed with water to yield 3-alkyl-7-hydroxyquinolin-2-ones (**10**–**18**).[20]

4.1.3.1. 7-Hydroxy-4-methylquinolin-2(1*H*)-one (10). It was obtained as white solid (85%); mp: >300 °C, (Literature value = 306–307 °C);[21] ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.28 (s, 3H, C-4 CH_3), 6.08 (s, 1H, H-3), 6.59 (d, 1H, J = 8.8 Hz, H-6), 6.65 (d, 1H, J = 2.4 Hz, H-8), 7.45 (d, 1H, J = 8.8 Hz, H-5), 10.02 (br s, 1H, OH), 11.33 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 19.03 (C-4 CH_3), 100.61, 111.80 (C-6 and C-8), 113.32 (C-3), 117.50 (C-10), 126.68 (C-5), 141.04 (C-9), 148.44 (C-4), 159.95 (C-7), 162.69 (C-2); IR (KBr) ν_{max}: 3425.24, 2927.52, 2364.39, 1652.89, 1541.56 (amide-II), 1474.84, 1406.35, 1255.85, 1220.96, 1074.07, 905.44, 817.60, 689.94 cm^{-1}; UV (methanol) λ_{max}: 324 and 337 nm; HRMS: $C_{10}H_9O_2N$ [M+H]$^+$: 176.2008.

4.1.3.2. 3-Ethyl-7-hydroxy-4-methylquinolin-2(1*H*)-one (11). It was obtained as yellow solid (70%); mp: 250–252 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.01 (t, 3H, J = 7.3 Hz, CH_2CH_3), 2.35 (s, 3H, C-4 CH_3), 2.59 (q, 2H, J = 7.2 Hz, CH_2CH_3), 6.56 (d, 1H, J = 7.6 Hz, H-6), 6.63 (s, 1H, H-8), 7.53 (d, 1H, J = 8.7 Hz, H-5), 9.87 (br s, 1H, OH), 11.34 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.89 (CH_2CH_3), 14.82 (C-4 CH_3), 19.82 (CH_2CH_3), 100.21, 111.56 (C-6 and C-8), 113.67 (C-10), 126.39 (C-3), 128.92 (C-5), 139.23 (C-9), 142.07 (C-4), 158.85 (C-7), 162.30 (C-2); IR (KBr) ν_{max}: 3291.86, 3229.49, 2966.43, 1629.42, 1573.05, 1514.05, 1417.29, 1319.53, 1256.84, 1191.89, 1066.27, 972.79, 862.40, 791.89, 690.01, 657.09 cm^{-1}; UV (methanol) λ_{max}: 325 and 339 nm; HRMS: $C_{12}H_{13}O_2N$ [M+H]$^+$: 204.3963.

4.1.3.3. 3-Hexyl-7-hydroxy-4-methylquinolin-2(1*H*)-one (12). It was obtained as yellow solid (75%); mp: 202 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.85–0.87 (m, 3H, CH_2CH_3), 1.23–1.38 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.34 (s, 3H, C-4 CH_3), 2.50–2.56 (m, 2H, CH_2CH_2), 6.60–6.67 (m, 2H, H-6 and H-8), 7.51–7.54 (m, 1H, H-5), 9.86 (br s, 1H, OH), 11.35 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.82 (CH_2CH_3), 15.54 (C-4 CH_3), 22.98, 27.02, 29.53, 29.78, 32.09 ($(CH_2)_5CH_3$), 100.61, 111.93 (C-6 and C-8), 114.10 (C-10), 126.78 (C-3), 128.13 (C-5), 139.64 (C-9), 142.67 (C-4), 159.22 (C-7), 162.86 (C-2); IR (KBr) ν_{max}: 3218.05, 2955.52, 2923.83, 1620.25, 1564.06, 1510.64, 1405.93, 1325.26, 1257.31, 1191.41, 1109.40, 807.53, 721.67, 692.46 cm^{-1}; UV (methanol) λ_{max}: 326 and 340 nm; HRMS: $C_{16}H_{21}O_2N$ [M]$^+$: 260.0510.

4.1.3.4. 6-Hydroxy-4-methylquinolin-2(1*H*)-one (13). It was obtained as white solid (80%); mp: 324–326 °C, (Literature value = 326–330 °C);[22] ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.34 (s, 3H, C-4 CH_3), 6.36 (s, 1H, H-3), 7.00 (br s, 2H, H-5 and H-7), 7.17 (br s, 1H, H-8), 9.40 (br s, 1H, OH), 11.42 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 19.36 (C-4 CH_3), 109.41, 117.43 (C-5 and C-7), 120.39 (C-10), 121.09 (C-8), 121.80 (C-9), 132.74 (C-3), 148.01 (C-4), 152.93 (C-6), 161.99 (C-2); IR (KBr) ν_{max}: 3489.59, 3187.46, 1661.73, 1634.87, 1608.22, 1513.42, 1433.01, 1294.34, 1191.88, 866.12, 853.10, 810.02, 642.28 cm^{-1}; UV (methanol) λ_{max}: 269 and 352 nm; HR MS: $C_{10}H_9O_2N$ [M]$^+$: 175.7805.

4.1.3.5. 3-Ethyl-6-hydroxy-4-methylquinolin-2(1*H*)-one (14). It was obtained as white solid (75%); mp: 262–264 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.98 (t, 3H, J = 7.2 Hz, CH_2CH_3), δ 2.31 (s, 3H, C-4 CH_3), 2.59 (q, 2H, J = 7.05 Hz, CH_2CH_3), 6.91 (d, 1H, J = 8.4 Hz, H-7), 7.01 (d, 1H, J = 1.8 Hz, H-5), 7.10 (d, 1H, J = 8.7 Hz, H-8), 9.32 (br s, 1H, OH), 11.39 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.24 (CH_2CH_3), 14.42 (C-4 CH_3), 19.68 (CH_2CH_3), 108.49, 116.05 (C-5 and C-7), 118.24 (C-8), 120.91 (C-10), 130.33 (C-9), 132.60 (C-3), 140.63 (C-4), 151.92 (C-6), 160.78 (C-2); IR (KBr) ν_{max}: 3231.81, 2973.40, 1641.50, 1620.48, 1505.72, 1480.33, 1431.71, 1392.90, 1319.33, 1279.99, 1207.53, 1170.02, 939.73, 880.82, 859.94, 700.63, 642.18 cm^{-1}; UV (methanol) λ_{max}: 274 and 350 nm; HRMS: $C_{12}H_{13}O_2N$ [M]$^+$: 203.7640.

4.1.3.6. 3-Hexyl-6-hydroxy-4-methylquinolin-2(1*H*)-one (15). It was obtained as white solid (80%); mp: 221–223 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.80 (br s, 3H, CH_2CH_3), 1.23 (br s, 8H, $CH_2(CH_2)_4CH_3$), 2.27 (s, 3H, C-4 CH_3), 2.55 (br s, 2H, CH_2CH_2), 6.88 (d, 1H, J = 8.1 Hz, H-7), 6.97 (s, 1H, H-5), 7.07 (d, 1H, J = 8.7 Hz, H-8), 9.24 (br s, 1H, OH), 11.34 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.89 (CH_2CH_3), 14.70 (C-4 CH_3), 22.06, 26.45, 28.51, 28.89, 31.17 ($(CH_2)_5CH_3$), 108.48, 116.04 (C-5 and C-7), 118.21 (C-8), 120.91 (C-10), 130.32 (C-9), 131.38 (C-3), 140.86 (C-4), 151.92 (C-6), 160.95 (C-2); IR (KBr) ν_{max}: 3565.89, 3143.50, 2952.85, 1647.12, 1624.99, 1504.90, 1422.73, 1317.46, 1288.39, 1208.76, 1165.02, 937.98, 826.24, 645.45 cm^{-1}; UV (methanol) λ_{max}: 274 and 350 nm; HRMS: $C_{16}H_{21}O_2N$ [M]$^+$: 259.7355.

4.1.3.7. 8-Hydroxy-4-methylquinolin-2(1*H*)-one (16). It was obtained as white solid (70%); mp: 250–254 °C, (Literature value = 248 °C);[23] ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.37 (s, 3H, C-4 CH_3), 6.34 (s, 1H, H-3), 6.89–6.98 (m, 2H, H-5 and H-6), 7.10 (d, 1H, J = 7.8 Hz, H-7), 10.25 (br s, 2H, NH and OH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 18.70 (C-4 CH_3), 114.36, 114.96 (C-5 and C-7), 120.48 (C-9), 121.04, 121.60 (C-3 and C-6), 127.84 (C-10), 143.67 (C-4), 148.19 (C-8), 160.91 (C-2); IR (KBr) ν_{max}: 3401.25, 1639.37, 1601.19, 1553.68, 1478.15, 1398.60, 1288.58, 1208.23, 1145.95, 924.47, 861.73, 792.88, 735.68, 600.31 cm^{-1}; UV (methanol) λ_{max}: 254, 279 and 336 nm; HRMS: $C_{10}H_9O_2N$ [M]$^+$: 175.9968.

4.1.3.8. 3-Ethyl-8-hydroxy-4-methylquinolin-2(1*H*)-one (17). It was obtained as white solid (65%); mp: 212–214 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.94 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.31 (s, 3H, C-4 CH_3), 2.56 (q, 2H, J = 7.2 Hz, CH_2CH_3), 6.83 (d, 1H, J = 7.5 Hz, H-5), 6.89–6.94 (m, 1H, H-6), 7.11 (d, 1H, J = 8.1 Hz, H-7), 10.11 (br s, 2H, NH and OH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.19 (CH_2CH_3), 14.68 (C-4 CH_3), 19.71 (CH_2CH_3), 113.31, 114.90 (C-5 and C-7), 120.94 (C-3), 121.47 (C-6), 126.24 (C-9), 132.63 (C-10), 141.84 (C-4), 143.31 (C-8), 160.62 (C-2); IR (KBr) ν_{max}: 3178.10, 2970.45, 1924.13, 1654.16, 1606.43, 1560.17, 1606.43, 1462.64, 1397.96, 1290.56, 1205.28, 1011.15, 831.96, 768.59, 732.46, 680.38, 639.97 cm^{-1}; UV (methanol) λ_{max}: 257, 282 and 334 nm; HRMS: $C_{12}H_{13}O_2N$ [M]$^+$: 203.6310.

4.1.3.9. 3-Hexyl-8-hydroxy-4-methylquinolin-2(1*H*)-one (18). It was obtained as white solid (65%); mp: 174–176 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88–0.90 (m, 3H, CH_2CH_3), 1.31–1.57 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.51 (s, 3H, C-4 CH_3), 2.80 (t, 2H, J = 6.9 Hz, CH_2CH_2), 7.09–7.26 (m, 3H, H-5, H-6 and H-7), 10.52 (br s, 2H, OH and NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.14 (CH_2CH_3), 15.50 (C-4 CH_3), 22.68, 27.14, 29.06, 29.58, 31.78 (($CH_2)_5CH_3$), 114.68, 115.18 (C-5 and C-7), 122.20 (C-3), 122.98 (C-6), 125.76 (C-9), 130.98 (C-10), 144.03 (C-4), 145.18 (C-8), 162.80 (C-2); IR (KBr) ν_{max}: 3377.54, 1638.80, 1624.56, 1600.44, 1552.36, 1394.62, 1278.70, 1229.22, 1200.55, 773.31, 710.18, 624.22 cm^{-1}; UV (methanol) λ_{max}: 255, 283 and 333 nm; HRMS: $C_{16}H_{21}O_2N$ [M]$^+$: 259.4653.

4.1.4. General procedure for the synthesis of 3-alkyl-4-methyl-2-oxo-1,2-dihydroquinolin-yl acetate (19–27)

A solution of 12 mL acetic anhydride and acetic acid (1:4) was added to 1 g of 3-alkyl-hydroxy-4-methylquinolin-2(1*H*)-ones **10**–**18**. The reaction mixture was refluxed for 6 h and then poured on crushed ice.[24] The resulting precipitate was filtered and washed with water to yield acetoxy-3-alkylquinolin-2-ones **19**–**27**.

4.1.4.1. 4-Methyl-2-oxo-1,2-dihydroquinolin-7-yl acetate (19). It was obtained as white solid (90%); mp: 258 °C, (Literature value = 257–258 °C);[21] ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.30 (s, 3H, C-4 CH_3), 2.42 (s, 3H, OCOCH_3), 6.39 (s, 1H, H-3), 6.98 (dd, 1H, J = 2.4 and J = 6.2 Hz, H-6), 7.05 (s, 1H, H-8), 7.73 (d, 1H, J = 6.1 Hz, H-5), 11.67 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 19.38 (C-4 CH_3), 21.76 (OCOCH_3), 108.77, 116.71 (C-6 and C-8), 118.36 (C-3), 121.21 (C-10), 126.98 (C-5), 140.43 (C-9), 148.52 (C-4), 152.59 (C-7), 162.63 (C-2), 169.82 (–O$COCH_3$); IR (KBr) ν_{max}: 2927.12, 2855.27, 1751.47, 1678.55, 1561.24, 1510.88, 1458.53, 1362.63, 1232.32, 116 6.29, 1025.06, 906.26, 856.71, 645.56 cm^{-1}; UV (methanol) λ_{max}: 323 and 335 nm; HRMS: $C_{12}H_{11}O_3N$ [M]$^+$: 217.2915.

4.1.4.2. 3-Ethyl-4-methyl-2-oxo-1,2-dihydroquinolin-7-yl acetate (20). It was obtained as yellow solid (75%); mp: 176–178 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.20 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.36 (s, 3H, C-4 CH_3), 2.50 (s, 3H, OCOCH_3), 2.84 (q, 2H, J = 7.3 Hz, CH_2CH_3), 6.84 (d, 1H, J = 7.8 Hz, H-6), 7.14 (s, 1H, H-8), 7.70 (d, 1H, J = 8.7 Hz, H-5), 12.12 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.32 (CH_2CH_3), 14.83 (C-4 CH_3), 20.09 (OCOCH_3), 21.09 (CH_2CH_3), 108.49, 116.18 (C-6 and C-8), 118.98 (C-10), 125.37 (C-3), 132.41 (C-5), 137.59 (C-9), 142.44 (C-4), 151.08 (C-7), 163.89 (C-2), 169.21 (–O$COCH_3$); IR (KBr) ν_{max}: 3448.86, 2931.73, 2851.67, 1770.34, 1658.08, 1562.63, 1510.84, 1370.03, 1207.92, 1157.16, 1012.24, 911.90 cm^{-1}; UV (methanol) λ_{max}: 323 and 336 nm; HRMS: $C_{14}H_{15}O_3N$ [M+H]$^+$: 246.7908.

4.1.4.3. 3-Hexyl-4-methyl-2-oxo-1,2-dihydroquinolin-7-yl acetate (21). It was obtained as yellow solid (80%); mp: 134–136 °C; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 0.93 (br s, 3H, CH_2CH_3), 1.38–1.58 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.35 (s, 3H, C-4 CH_3), 2.49 (s, 3H, OCOCH_3), 2.81 (br s, 2H, CH_2CH_2), 6.98 (d, 1H, J = 8.2 Hz, H-5), 7.14 (s, 1H, H-8), 7.70 (d, 1H, J = 8.4 Hz, H-6), 12.25 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 14.14 (CH_2CH_3), 15.23 (C-4 CH_3), 21.14 (OCOCH_3), 22.65, 26.92, 28.99, 29.48, 31.73 (($CH_2)_5CH_3$), 108.42, 116.25 (C-6 and C-8), 119.09 (C-10), 125.53 (C-3), 131.45 (C-5), 137.59 (C-9), 142.68 (C-4), 151.11 (C-7), 164.01 (C-2), 169.24 (O$COCH_3$); IR (KBr) ν_{max}: 3449.06, 2927.86 (C-H str), 2855.18, 2367.37, 1765.40, 1656.22, 1564.92, 1511.10, 1459.84, 1370.74, 1223.68, 1014.71, 924.77 cm^{-1}; UV (methanol) λ_{max}: 323 and 368 nm; HRMS: $C_{18}H_{23}O_3N$ [M]$^+$: 301.9141.

4.1.4.4. 4-Methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate[25] (22). It was obtained as white solid (85%); mp: 270 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.30 (s, 3H, C-4 CH_3), 2.40 (s, 3H, OCOCH_3), 6.46 (s, 1H, H-3), 7.29–7.35 (m, 2H, H-5 and H-7), 7.47 (dd, 1H, J = 1.5 and 7.5 Hz, H-8), 11.71 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 18.39 (C-4 CH_3), 20.76 (OCOCH_3), 116.27, 117.06 (C-5 and C-7), 120.01 (C-10), 121.47 (C-8), 124.63 (C-3), 136.38 (C-9), 144.72 (C-4), 147.43 (C-6), 161.45 (C-2), 169.53 (O$COCH_3$); IR (KBr) ν_{max}: 3433.37, 2835.40, 1751.37, 1656.34, 1559.57, 1502.72, 1425.27, 1373.09, 1212.40, 1166.23, 1135.37, 1012.90, 905.94, 871.27, 842.91, 683.29 cm^{-1}; UV (methanol) λ_{max}: 264 and 334 nm; HRMS: $C_{12}H_{11}O_3N$ [M+H]$^+$: 218.4582.

4.1.4.5. 3-Ethyl-4-methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate (23). It was obtained as white crystals (80%); mp: 258–260 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 0.99 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.26 (s, 3H, C-4 CH_3), 2.35 (s, 3H, OCOCH_3), 2.62 (q, 2H, J = 7.2 Hz, CH_2CH_3), 7.19–7.29 (m, 2H, H-5 and H-7), 7.44 (s, 1H, H-8), 11.67 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.10 (CH_2CH_3), 14.45 (C-4 CH_3), 19.68 (CH_2CH_3), 20.78 (OCOCH_3), 115.76, 116.95 (C-5 and C-7), 120.50 (C-10), 123.36 (C-8), 133.32 (C-9), 134.89 (C-3), 141.59 (C-4), 144.60 (C-6), 161.15 (C-2), 170.04 (O$COCH_3$); IR (KBr) ν_{max}: 2934.63, 1752.29, 1644.31, 1500.24, 1458.60, 1420.02, 1386.26, 1371.75, 1258.49, 1221.17, 1182.79, 1046.76, 1020.62, 953.84, 897.95, 705.93, 681.39 cm^{-1}; UV (methanol) λ_{max}: 269 and 332 nm; HRMS: $C_{14}H_{15}O_3N$ [M+H]$^+$: 246.5055.

4.1.4.6. 3-Hexyl-4-methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate (24). It was obtained as white crystals (80%); mp: 158–160 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.90 (t, 3H, J = 6.8 Hz, CH_2CH_3),1.34–1.57 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.34 (s, 3H, C-4 CH_3), 2.45 (s, 3H, OCOCH_3), 2.80 (t, 2H, J = 7.5 Hz, CH_2CH_2), 7.18 (dd, 1H, J = 2.4 and 8.7 Hz, H-7), 7.39–7.43 (m, 2H, H-5 and H-8) 12.46 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 14.15 (CH_2CH_3), 15.15 (C-4 CH_3), 21.11 (OCOCH_3), 22.65, 27.05, 28.95, 29.51, 31.76 (($CH_2)_5CH_3$), 116.53, 117.04 (C-5 and C-7), 121.63 (C-10), 123.06 (C-8), 132.62 (C-3), 134.70 (C-9), 142.45 (C-4), 145.38 (C-6), 163.83 (C-2), 169.90 (O$COCH_3$); IR (KBr) ν_{max}: 3432.83, 2926.24, 1761.26, 1656.07, 1500.98, 1369.83, 1219.35, 1176.07, 1014.16, 940.47, 628.69 cm^{-1}; UV (methanol) λ_{max}: 269 and 333 nm; HRMS: $C_{18}H_{23}O_3N$ [M]$^+$: 301.6057.

4.1.4.7. 4-Methyl-2-oxo-1,2-dihydroquinolin-8-yl acetate (25). It was obtained as white solid (85%); mp: 242–244 °C; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.19 (s, 3H, C-4 CH_3), 2.25 (s, 3H, OCOCH_3), 6.27 (s, 1H, H-3), 7.01 (t, 1H, J = 7.9 Hz, H-6), 7.12 (d, 1H, J = 7.7 Hz, H-5), 7.43 (d, 1H, J = 7.9 Hz, H-7), 11.27 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 18.68 (C-4 CH_3), 21.35 (OCOCH_3), 121.19 (C-9), 121.23, 121.48 (C-7 and C-3), 122.36, 123.84 (C-5 and C-6), 131.54 (C-10), 136.74 (C-4), 148.00 (C-8), 161.63 (C-2), 169.64 (O$COCH_3$); IR (KBr) ν_{max}: 3431.86, 2996.30, 1763.12, 1671.14, 1647.83, 1605.69, 1475.41, 1422.01, 1366.81, 1195.13, 1168.09,

1138.04, 1016.39, 932.32, 869.41, 744.18, 689.19 cm^{-1}; UV (methanol) λ_{max}: 268 and 328 nm; HRMS: $C_{12}H_{11}O_3N$ $[M+H]^+$: 218.1072.

4.1.4.8. 3-Ethyl-4-methyl-2-oxo-1,2-dihydroquinolin-8-yl acetate (26). It was obtained as white solid (80%); mp: 210–212 °C; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.04 (t, 3H, J = 7.5 Hz, CH_2CH_3), 2.37 (s, 3H, C-4 CH_3), 2.44 (s, 3H, $OCOCH_3$), 2.66 (q, 2H, J = 7.5 Hz, CH_2CH_3), 7.15–7.25 (m, 2H, H-5 and H-6), 7.64 (dd, 1H, J = 1.5 and 7.8 Hz, H-7), 11.42 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 13.11 (CH_2CH_3), 14.72 (C-4 CH_3), 19.67 (CH_2CH_3), 21.35 ($OCOCH_3$), 120.99 (C-7), 121.60 (C-3), 122.11, 122.58 (C-5 and C-6), 129.99 (C-9), 133.23 (C-10), 136.46 (C-4), 141.50 (C-8), 161.34 (C-2), 169.61 ($OCOCH_3$); IR (KBr) ν_{max}: 3162.22, 1770.93, 1632.36, 1421.68, 1361.50, 1188.50, 1021.12, 955.93, 867.96, 735.74, 675.53 cm^{-1}; UV (methanol) λ_{max}: 272 and 328 nm; HRMS: $C_{14}H_{15}O_3N$ $[M]^+$: 245.4751.

4.1.4.9. 3-Hexyl-4-methyl-2-oxo-1,2-dihydroquinolin-8-yl acetate (27). It was obtained as white solid (80%); mp: 162–164 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 0.89 (t, 3H, J = 6.6 Hz, CH_2CH_3), 1.31–1.54 (m, 8H, $CH_2(CH_2)_4CH_3$), 2.47 (s, 3H, C-4 CH_3), 2.48 (s, 3H, $OCOCH_3$), 2.75 (t, 2H, J = 6.9 Hz, CH_2CH_2), 7.16–7.31 (m, 2H, H-5 and H-6), 7.56 (d, 1H, J = 8.4 Hz, H-7), 9.65–9.74 (br m, 1H, NH); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.11 (CH_2CH_3), 15.37 (C-4 CH_3), 21.20 ($OCOCH_3$), 22.68, 27.20, 29.00, 29.57, 31.82 ($(CH_2)_5CH_3$), 121.34, 121.74 (C-5 and C-7), 121.80 (C-6), 122.53 (C-3), 128.95 (C-9), 132.84 (C-10), 136.37 (C-4), 142.65 (C-8), 162.16 (C-2), 168.80 ($OCOCH_3$); IR (KBr) ν_{max}: 3428.77, 2930.79, 2372.19, 1754.72, 1650.93, 1565.36, 1461.77, 1367.69, 1209.86, 1017.50, 907.94, 781.27, 738.35, 698.94 cm^{-1}; UV (methanol) λ_{max}: 273 and 327 nm; HRMS: $C_{18}H_{23}O_3N$ $[M]^+$: 301.4821.

4.1.5. General procedure for the synthesis of N- and O-substituted alkyl esters of quinolin-2-ones (28–31)

To a mixture of acetoxy-4-methylquinolin-2-ones (7.3 mmol), K_2CO_3 (1 g, 7.3 mmol) in anhydrous DMF (10 mL), was added ethyl bromoacetate (1.2 g, 7.3 mmol) and the reaction mixture was refluxed for 12 h. The reaction was then cooled to room temperature and the mixture was poured in ice cold water and extracted with ethyl acetate. The crude product was purified through column chromatography to give two isomeric products.

4.1.5.1. Ethyl 2-(7-acetoxy-4-methyl-2-oxoquinolin-1(2*H*)-yl)acetate (28). It was obtained as white crystals (60%); mp: 110 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.25 (t, 3H, J = 7.1 Hz, CH_2CH_3), 2.34 (s, 3H, C-4 CH_3), 2.48 (s, 3H, $OCOCH_3$), 4.23 (q, 2H, J = 7.1 Hz, CH_2CH_3), 5.04 (s, 2H, NCH_2), 6.59 (s,1H, H-3), 6.87 (s, 1H, H-8), 7.03 (d, 1H, J = 8.7 Hz, H-6), 7.73 (d, 1H, J = 8.7 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.09 (CH_2CH_3), 19.23 (C-4 CH_3), 21.20 ($OCOCH_3$), 43.76 (NCH_2), 61.82 (CH_2CH_3), 107.08, 116.10 (C-6 and C-8), 119.32 (C-3), 120.18 (C-10), 126.70 (C-5), 140.14 (C-9), 147.10 (C-4), 152.43 (C-7), 161.73 (C-2), 168.04 ($OCOCH_3$), 168.97 (COO); IR (KBr) ν_{max}: 2999.98, 2925.16, 1766.13, 1745.40, 1658.88, 1594.89, 1437.53, 1386.80, 1371.56, 1324.86, 1243.22, 1204.97, 1177.62, 1111.11, 1019.91, 958.23, 909.97, 864.10, 816.25, 745.32, 714.35, 649.75, 604.10, 561.14 cm^{-1}; UV (methanol) λ_{max}: 324 nm; HRMS: $C_{16}H_{17}O_5N$ $[M]^+$: 304.0316.

4.1.5.2. Ethyl 2-(7-acetoxy-4-methylquinolin-2-yloxy)acetate (30). It was obtained as white crystals (30%); mp: 98 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.28 (br s, 3H, CH_2CH_3), 2.35 (s, 3H, C-4 CH_3), 2.62 (s, 3H, $OCOCH_3$), 4.26 (br s, 2H, CH_2CH_3), 4.99 (s, 2H, OCH_2CO), 6.87 (s, 1H, H-3), 7.17 (d, 1H, J = 7.8 Hz, H-6), 7.51 (s, 1H, H-8), 7.88 (d, 1H, J = 6.3 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.16 (CH_2CH_3), 18.77 (C-4 CH_3), 21.15 ($OCOCH_3$), 61.01 (CH_2CH_3), 62.41 (OCH_2CO), 112.33, 118.89 (C-6 and C-8), 119.20 (C-3), 123.72 (C-10), 124.79 (C-5), 146.89 (C-9), 147.29 (C-4), 151.31 (C-7), 161.07 (C-2), 169.24 (–$OCOCH_3$), 169.35 ($COOCH_2CH_3$); IR (KBr) ν_{max}: 2989.04, 2928.22, 1761.41, 1641.32, 1580.26, 1516.42, 1463.00, 1426.20, 1371.03, 1210.92, 1175.98, 1136.43, 1081.82, 1014.69, 943.65, 912.83, 862.57, 797.55, 692.03, 599.54 cm^{-1}; UV (methanol) λ_{max}: 324 nm; HRMS: $C_{16}H_{17}O_5N$ $[M]^+$: 303.5980.

4.1.5.3. Ethyl 2-(6-acetoxy-4-methyl-2-oxoquinolin-1(2*H*)-yl)acetate (29). It was obtained as white crystals (60%); mp: 166 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.26 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.34 (s, 3H, C-4 CH_3), 2.45 (s, 3H, $OCOCH_3$), 4.23 (q, 2H, J = 6.9 Hz, CH_2CH_3), 5.08 (s, 2H, NCH_2), 6.65 (s,1H, H-3), 7.10 (d, 1H, J = 9.0 Hz, H-8), 7.28 (dd, 1H, J = 2.4 and 8.7 Hz, H-7), 7.44 (d, 1H, J = 2.1 Hz, H-5); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.07 (CH_2CH_3), 19.07 (C-4 CH_3), 21.03 ($OCOCH_3$), 43.68 (NCH_2), 61.74 (CH_2CH_3), 114.77, 117.86 (C-5 and C-7), 121.37 (C-8), 122.17 (C-10), 124.24 (C-3), 136.81 (C-9), 145.36 (C-4), 146.74 (C-6), 161.35 (C-2), 168.04 ($OCOCH_3$), 169.54 (COO); IR (KBr) ν_{max}: 3474.88, 2988.51, 1763.26, 1745.76, 1664.42, 1600.02, 1570.50, 1441.61, 1424.06, 1373.46, 1313.63, 1207.62, 1170.43, 1122.79, 1024.86, 953.86, 899.96, 813.98, 681.48, 615.15 cm^{-1}; UV (methanol) λ_{max}: 276 and 336 nm; HRMS: $C_{16}H_{17}O_5N$ $[M]^+$: 303.8917.

4.1.5.4. Ethyl 2-(6-acetoxy-4-methylquinolin-2-yloxy)acetate (31). It was obtained as white crystals (30%); mp: 62 °C; ^1H NMR ($CDCl_3$, 300 MHz): δ 1.25–1.34 (m, 3H, CH_2CH_3), 2.36 (s, 3H, C-4 CH_3), 2.60 (s, 3H, $OCOCH_3$), 4.21–4.30 (m, 2H, CH_2CH_3), 5.00 (s, 2H, OCH_2CO), 6.91 (s, 1H, H-3), 7.34 (dd, 1H, J = 2.7 and 9.0 Hz, H-7), 7.57 (d, 1H, J = 2.4 Hz, H-5), 7.79 (d, 1H, J = 9.0 Hz, H-8); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.21 (CH_2CH_3), 18.73 (C-4 CH_3), 21.16 ($OCOCH_3$), 61.04 (CH_2CH_3), 62.49 (OCH_2CO), 113.13, 115.17 (C-5 and C-7), 124.04 (C-8), 126.00 (C-10), 129.15 (C-3), 144.02 (C-9), 146.85 (C-4), 147.10 (C-6), 160.47 (C-2), 169.34 ($OCOCH_3$), 169.78 (COO); IR (KBr) ν_{max}: 3478.18, 2990.84, 1762.23, 1750.29, 1609.84, 1580.73, 1522.33, 1467.00, 1439.28, 1421.11, 1384.17, 1364.61, 1341.67, 1219.46, 1168.78, 1088.15, 1036.15, 1017.43, 942.95, 908.61, 887.67, 847.01, 824.95, 736.28, 620.01 cm^{-1}; UV (methanol) λ_{max}: 324 nm; HRMS: $C_{16}H_{17}O_5N$ $[M]^+$: 304.0379.

4.1.5.5. General procedure for the synthesis of 2-oxo-1,2-dihydroquinolin-4-yl acetate (33). Aniline (5 g, 54 mmol) was added to a mixture of fused zinc chloride (21.5 g, 161 mmol), malonic acid (5.6 g, 54 mmol), and phosphorus oxychloride (108 mmol, 16.5 g). The reaction mixture was heated at 65 °C for 36 h. It was then cooled and poured on ice.[26] The precipitate was filtered and purified through column chromatography using silica gel (100–200 mesh) in methanol/chloroform (1:100). A solution of acetic anhydride and acetic acid (1:4) was added to 4-hydroxyquinolin-2-one (**32**) and the reaction mixture was refluxed for 6 h and then poured on crushed ice (Scheme 3). The resulting precipitate was filtered and washed with water to yield 2-acetoxyquinolin-2-one (**33**).

4.1.5.6. 4-Hydroxyquinolin-2(1*H*)-one (32). It was obtained as yellow solid (70%); mp: 320 °C, (Literature value = 318–320 °C);[27] ^1H NMR (DMSO-d_6, 300 MHz): δ 5.76 (s, 1H, H-3), 7.11–7.78 (m, 4H, H-5, H-6, H-7 and H-8), 11.18 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 98.18 (C-3), 114.95, 115.10 (C-6 and C-8), 121.04 (C-5), 122.62 (C-10), 130.82 (C-7), 139.13 (C-9), 162.43 (C-4), 163.57 (C-2); IR (KBr) ν_{max}: 3430.12, 3094.44, 2953.09, 2861.12, 2639.30, 2364.36, 1669.52, 1633.94, 1594.78, 1560.44, 1506.18, 1471.81, 1420.44, 1378.93, 1332.01, 1259.95, 1235.09, 1160.89, 1145.18, 1102.39, 1035.00, 909.53, 867.70, 773.96, 762.96, 755.55, 671.97, 630.01 cm^{-1}; UV (methanol) λ_{max}: 269 and 314 nm; HRMS: $C_9H_7O_2N$ $[M]^+$: 161.7715.

4.1.5.7. 2-Oxo-1,2-dihydroquinolin-4-yl acetate (33). It was obtained as yellow solid (85%); mp: 220 °C, (Literature value = 217–219 °C);[28] ^{1}H NMR (DMSO-d_6, 300 MHz): δ 2.45 (s, 3H, –OCOCH_3), 6.65 (s, 1H, H-3), 7.22–7.66 (m, 4H, H-5, H-6, H-7 and H-8), 11.89 (br s, 1H, NH); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 20.75 (–OCOCH_3), 98.19 (C-3), 112.28, 115.48 (C-6 and C-8), 122.03 (C-5), 122.44 (C-10), 131.47 (C-7), 138.87 (C-9), 156.04 (C-4), 162.39 (C-2), 172.04 (–OCOCH_3); IR (KBr) ν_{max}: 3396.31, 3009.90, 2965.78, 2858.41, 1768.58, 1668.77, 1615.91, 1561.20, 1506.34, 1475.03, 1434.55, 1398.53, 1370.26, 1268.81, 1189.23, 1160.47, 1143.99, 1078.60, 984.19, 953.88, 887.47, 770.84, 758.32, 660.04 cm^{-1}; UV (methanol) λ_{max}: 264 and 326 nm; HRMS: $C_{11}H_9O_3N$ $[M+H]^+$: 204.1366.

4.2. Isolation of platelet rich plasma (PRP)

The citrated blood was used for the preparation of PRP by the method of Vickers and Thompson.[29] Venous blood (9 mL) was collected from healthy human volunteer and mixed with 1.0 mL of 3.8% trisodium citrate and centrifuged at 180*g* for 10 min. The upper two-third fraction of plasma (PRP) was transferred to another centrifuge tube leaving behind lower one-third layer to avoid contamination with WBC's and RBC's. Platelet poor plasma (PPP) was obtained by centrifugation of the remaining sample at 2500*g* for 10 min. Platelet count was determined in PRP using electronic counter, SYSMEX Model No. FA 20 and were adjusted to 250×10^6/mL with PPP.

4.3. Aggregometry

The test compounds (in appropriate concentrations) were separately preincubated with PRP in a final volume of 0.5 mL at 37 °C. Platelet aggregation was induced by the addition of ADP (15 μM) and assessed by using a platelet aggregation profiler (BIODATA CORPORATION, Model No. PAP-4) and the results were expressed as the maximum percentage of light transmittance change (% max) from the baseline at the end of the recording time, using PPP as a reference. Platelet aggregation curves were recorded for 6 min and analyzed according to internationally established standards.

4.4. Platelet aggregation in vivo

Male sprague dawely rats (weight 200–250 g) housed in mesh cages maintained at 25 °C and illuminated at 12:12 h light dark cycles. The known amount of test compound was suspended in appropriate volume of saline, sonicated for 30 s and the preparation was administered to the rats orally at a dose of 133 μmoles/kg. The animals were sacrificed after 24 h, and blood samples were taken by cardiac puncture. The blood was centrifuged according to above mentioned procedure and then analyzed for the assessment of platelet aggregation.[30]

4.5. Cox-1 activity assay

Platelets lysate were prepared from the above mentioned sample in 100 μL of lysis buffer (50 mmol/L tris, 150 mmol/L NaCl, 10 mmol/L EGTA, 1% triton X-100, 1% sodium deoxycholate, 1 mmol/L sodium vanadate, 50 mmol/L NaF, 2 mmol/L EDTA (pH 8.0), 1 mmol/L phenylmethylsulfonyl fluoride, and 5 g/mL of leupeptin/pepstatin A/aprotinin for 15 min at 4 °C and assessed for Cox-1 activity.[31] The assay was carried out using Cox-1 assay ELISA kit (*Cayman Chemical*), according to the manufacturer's protocol. Briefly, 50 μL of each lysed samples were added to the wells, the enzymatic reaction was initiated by adding 100 μM *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD) and 100 μM arachidonic acid (saturating condition) in assay buffer. Inhibitors were added to the incubation reaction at different time intervals before the addition of TMPD and arachidonic acid. The enzyme activity was measured calorimetrically by monitoring the appearance of oxidized TMPD at 590 nm.

4.6. Assay of platelet CRTAase

Platelet CRTAase was assayed as mentioned in the earlier publications.[7] The unit of CRTAase activity was expressed in terms of % inhibition of GST activity under the conditions of the assay.

4.7. Platelet measurement of NO level by flow cytometry

The method outlined by Imrich and Kobzik as described earlier was followed for the assay of NOS in platelets by flow cytometry.[32]

4.8. Calculation and statistics

Calculations and statistics were performed using the graph pad prism 3.02 software. The one-way analysis of variance (ANOVA) tests followed by the Turkey multiple comparisons test were used. Data were expressed as mean ± standard error. Statistical significance was calculated using the Student's *t*-test. p values less than 0.05 (p <0.05) were considered to be statistically significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.04.011.

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