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Specificities of Calreticulin Transacetylase to acetoxy derivatives of 3-alkyl-4-methylcoumarins: Effect on the activation of nitric oxide synthase

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ABSTRACT

Calreticulin Transacetylase (CRTAase) catalyzes the transfer of acetyl groups from polyphenolic acetates (PAs) to the receptor proteins and modulates their biological activities. CRTAase was conveniently assayed by the irreversible inhibition of cytosolic glutathione *S*-transferase (GST) by the model acetoxycoumarin, 7,8-diacetoxy-4-methylcoumarin (DAMC). We have studied earlier, the influence of acetoxy groups on the benzenoid ring, the effect of reduction of double bond at C-3 and C-4 position, the effect of methyl/phenyl group at C-4, and the influence of position of carbonyl group with respect to oxygen heteroatom in the benzopyran nucleus, for the catalytic activity of CRTAase. In this communication, we have extended our previous work; wherein we studied the influence of an alkyl group (ethyl, hexyl and decyl) at the C-3 position of the acetoxy coumarins on the CRTAase activity. The substitution at C-3 position of coumarin nucleus resulted in the reduction of CRTAase activity and related effects. Accordingly the formation of NO in platelets by C-3 alkyl substituted acetoxy coumarins was found to be much less compared to the unsubstituted analogs. In addition the alkyl substitution at C-3 position exhibited the tendency to form radicals other than NO.

1. Introduction

The enzymatic acetylation of proteins was so far known to involve the participation of specific acetyl transferases acting upon acetyl CoA in the transfer of acetyl group to the proteins; example being the histone acetyl transferase.[1] The enzymatic acetylation of proteins independent of Acetyl CoA was unknown till we identified the membrane bound novel enzyme Acetoxy Drug: protein Transacetylase (TAase) catalyzing the transfer of acetyl group from polyphenolic acetates to certain functional proteins such as Glutathione *S*-transferase (GST), NADPH Cytochrome *c* reductase and nitric oxide synthase.[2–9] The acetylation of these enzymes resulted in the modification of their catalytic activities. Our later studies confirmed TAase as the Calreticulin (CR), a calcium binding protein. We then designated TAase as Calreticulin Transacetylase (CRTAase).[10]

Accordingly, PAs were found effective in the inhibition of Cytochrome *c* mediated activation of mutagens because of which, the acetoxy derivatives of several classes of polyphenols were found to be the substrates of CRTAase.[11,12]

CRTAase was found to catalyze the transfer of acetyl group from 7,8-diacetoxy-4-methylcoumarin (DAMC), a model acetoxy drug, to glutathione *S*-transferase (GST) resulting in the acetylation of several lysine residues in its active site and subsequently to inhibition of catalytic activity of GST.[3,4] Acetoxy coumarins were also found to modulate the activities of liver microsomal cytochrome P-450 catalyzed mixed function oxidases (MFO) and NADPH Cytochrome *c* reductase catalyzed by CRTAase, possibly by way of acetylation of these enzyme proteins.[5,6] Later, a number of acetoxy derivatives of coumarins and flavones were examined for their specificity to liver microsomal CRTAase. These investigations highlighted the structural features of polyphenolic acetates such as the proximity of acetoxy groups to the oxygen heteroatom, cardinal role of carbonyl group, and the influence of phenyl group substitution on the pyran ring, in controlling their specificities for the CRTAase.[7,8]

In the present investigation, efforts have been made to compare the specificities of diacetoxy and monoacetoxy coumarins on TAase activity and also to delineate the structure activity relationship (SAR) with special reference to the effect of alkyl group at the C-3 position of the pyran moiety of the polyphenolic acetates. The results clearly demonstrated that the specificity of polyphenolic acetates for TAase of 3-alkyl diacetoxy coumarins are almost twice as that of 3-alkyl monoacetoxy coumarins. However, the TAase activ-

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ities of both the mono/diacetoxy 3-alkyl coumarins are lower than the corresponding non alkylated analogs, that is, 7-acetoxy-4-methyl coumarin (MAMC) and 7,8-diacetoxy-4-methyl coumarin (DAMC). It can also be concluded that the the increase in chain length of the alkyl substituent at the pyran nucleus lead to decrease in CRTAase activity.

The acetylation of NOS by acetoxy coumarins is an effective approach to activate NOS thereby enhancing NO levels in human platelets. We have in this report demonstrated the effect of substitution at C-3 position of acetoxy coumarins on the enhancement of NO levels in platelets. From the present investigations it can be inferred that alkyl substitution at C-3 position of the coumarin nucleus led to inhibition of NOS as compared to the corresponding unsubstituted coumarins, that is, MAMC/DAMC.

2. Results

In our earlier work we elucidated the role of acetoxy groups on the benzenoid ring of coumarin moiety in facilitating the acetylation of the receptor protein catalyzed by Calreticulin Transacetylase. 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.[7–9]

However, the effect of substituent at the C-3 position of coumarin has not been thoroughly studied earlier. In this report, we have meticulously compared the specificities of acetoxy derivatives of a number of 3-alkyl-4-methylcoumarins as substrate to Calreticulin Transacetylase by the replacement of C-3 hydrogen with different alkyl groups (ethyl, hexyl and decyl). As alkyl groups are hydrophobic in nature, these may enhance hydrophobic interactions between the substrate (acetoxycoumarins) and the target protein thus influencing the CRTAase catalytic activity. Such a study would allow us to study the effect thereof on the rate of catalytic activity of CRTAase and the efficacy of these acetoxy coumarins to activate platelet NOS. The C-3 alkyl coumarins (**2a**–**c** and **3a**–**c**) were synthesized via Pechmann condensation of 2-alkyl ethyl acetoacetates (**1a**–**c**) with resorcinol and pyragallol. The 2-alkyl ethyl acetoacetates in turn were prepared from ethyl acetoacetate by its alkylation using alkyl bromide and sodium hydride. The mono/dihydroxy coumarins thus obtained were acetylated using DMAP (*N*,*N*-dimethyl aminopyridine) and acetic anhydride to give the corresponding monoacetoxy (**4a–c**) and diacetoxy coumarins (**5a–c**) bearing different alkyl substituents at the C-3 position and a methyl group at C-4 position (Schemes 1 and 2). All the compounds were fully characterized on the basis of their physical and spectral data, and of these coumarins, **2c**, **3c**, **4c**, **5b** and **5c** are novel.

The CRTAase activities with these compounds as the substrate were compared with DAMC and MAMC. The results clearly indicated that among all the acetoxy coumarins DAMC caused highest inhibition of GST (Fig. 1), and as well the activation of NADPH Cytochrome *c* reductase (Fig. 2). However, among the alkylated coumarins it is the 3-ethyl-4-methyl-7,8-diacetoxycoumarin (**5a**) is the better substrate for the CRTAase in comparison to other C-3 alkyl diacetoxycoumarins. Further, it is clearly evident from Figures 1 and 2 that activities with diacetoxy 3-alkyl-4-methylcourmarins are twice as high as those with the corresponding monoacetoxy coumarins.

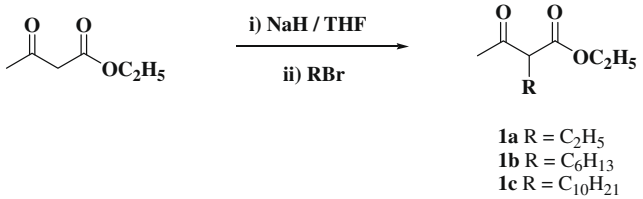
CRTAase mediated inhibition of glutathione *S*-transferase (GST) by alkylated diacetoxy coumarins **5a–c** is almost twice as compared to the corresponding monoacetoxy analogs, that is, 7-acetoxy alkylcoumarins **4a–c**. Acetoxycoumarins **5a**, **5b** and **5c** when compared with DAMC showed a gradual decline with the increase in chain length of the alkyl substituent at the C-3 position in the coumarin moiety. Similar trend was observed with 7-acetoxy-4-methyl-3-alkylcoumarins **4a**, **4b** and **4c**.

Similarly, in case of CRTAase catalyzed activation of NADPH Cytochrome *c* reductase (Fig. 2), the results revealed that 3-alkyl-4-methyl-7,8-diacetoxycoumarin **5a–c** upon incubation with liver microsomes resulted in the activation of the reductase to the maximum extent. The monoacetoxy 3-alkyl-4-methylcoumarins **4a**, **4b** and **4c** caused significantly lesser activation of the reductase as compared to their diacetoxy coumarin analogs. Also, smaller the alkyl group present at the C-3 position, the higher was the CRTAase catalyzed activation of NADPH Cytochrome *c* reductase. However, among the mono and di acetoxycoumarins, it is the C-3 unsubstituted coumarins, viz. MAMC and DAMC that have the maximum substrate specificity for CRTAase.

Studies were extended with the view to examine the influence of acetoxy coumarins (**4a–c**, **5a–c**) on activation of NOS. The platelets upon incubation with L-arginine and ethyl/hexyl substituted mono and diacetoxy coumarins exhibited enhanced DCF fluorescence. It is worth noting that the inclusion of L-NAME failed to reduce the fluorescence to the control level indicating the possible formation of oxygen radicals apart from NO. The decyl substituted acetoxy coumarins virtually produced no enhancement of NO levels in platelets.

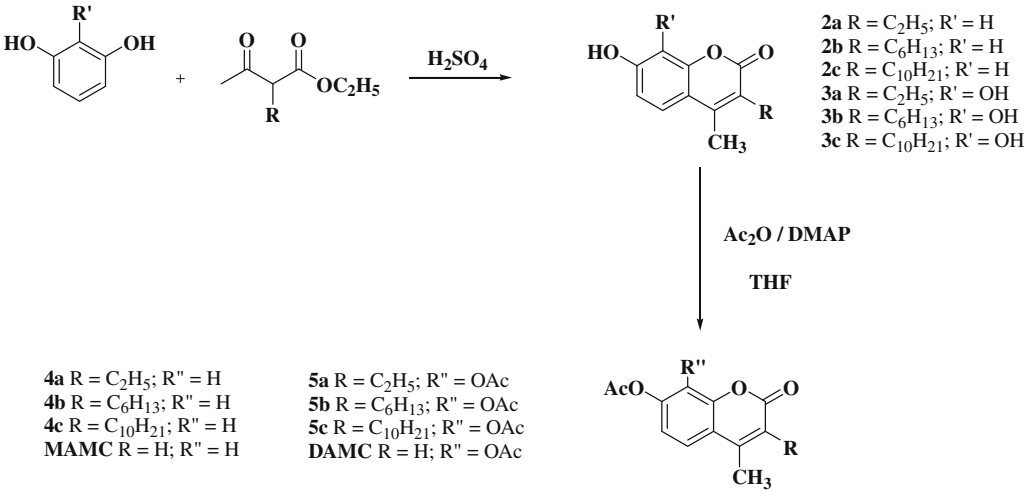
3. Discussion

The existence of the unique enzyme acetoxy drug: protein Transacetylase (TAase) now identified as Calreticulin Transacetylase (CRTAase) in rat liver microsomes catalyzing transfer of the acetyl group from acetoxy polyphenols to specific proteins was evidenced from the studies published earlier.[6–8] As a part of the quantitative structure–activity relationship (QSAR) studies on CRTAase, we have demonstrated earlier (a) positional specificity of acetoxy groups on the benzenoid ring of certain polyphenols and (b) absolute requirement of carbonyl group on pyran ring.[6,7] We have in this report highlighted the outcome of the replacement of the C-3 hydrogen by an alkyl group on the catalytic activity of CRTAase. For this task, a number of acetoxy coumarins and diacetoxy coumarins were synthesized and their specificities to CRTAase were determined. The compounds **4a**–**c** and **5a**–**c** apart from bearing mono acetoxy and diacetoxy groups also differ in substitution at the C-3 position of pyran ring. The compounds with ethyl group at the C-3 position yielded higher CRTAase activities (Figs. 1 and 2), in comparison to their hexyl or decyl analogs. However, the highest catalytic activity was found with C-3 unsubstituted acetoxy coumarins MAMC and DAMC. These results convincingly suggested that substitution at C-3 by (larger) alkyl groups accounted for significant decrease in CRTAase activity. The presence of bulky alkyl groups at C-3 position in the pyran ring of coumarins reduces their abilities to transfer acetyl group to the functional proteins, suggesting that bulkier alkyl groups might be causing steric hindrance for the acetoxy substrates from accessing the active site of CRTAase. This results in the decreased rate of transfer of the acetyl group to the receptor protein in the case of hexyl or decyl

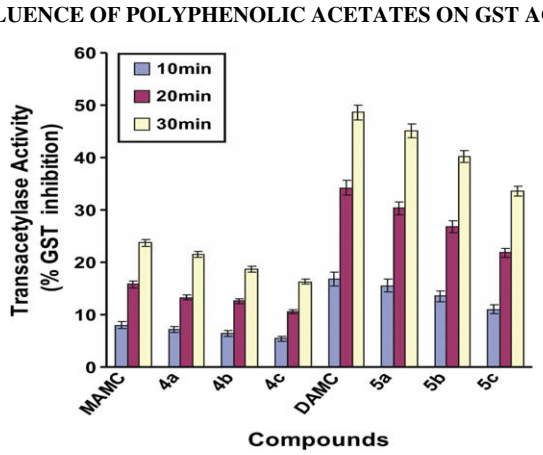


1a R = C_2H_5
1b R = C_6H_{13}
1c R = $C_{10}H_{21}$

Scheme 1. Alkylation of ethyl acetoacetate.



Scheme 2. Pechmann condensation of phenols with alkylated ethyl acetoacetate.



- Polyphenolic acetates were separately preincubated with rat liver microsomes (25 μg) and rat liver cytosol (12.5 μg) followed by assay of GST, the activity was expressed in terms of inhibition of GST.
- Each histogram indicates % inhibition of GST at 10 min intervals (10-30 min) in succession.
- Values are mean ± SD of three observations.

Figure 1. CRTAase catalyzed inhibition of GST by acetoxycoumarins.

substituted acetoxy coumarins as compared to ethyl substituted or unsubstituted counterparts.

The irreversible activation of NADPH Cytochrome *c* reductase was established as the basis for some of the CRTAase related biological effects.[5,6] As observed earlier with other polyphenolic acetates, the acetoxy derivatives of 3-alkyl-4-methylcoumarins modulate reductase and GST activities in a manner proportional to their specificity to CRTAase. Accordingly, CRTAase catalyzed activation of reductase and inhibition of GST by 3-decylcoumarins and 3-hexylcoumarins were found to be significantly lower than those by the 3-ethylcoumarin counterparts.

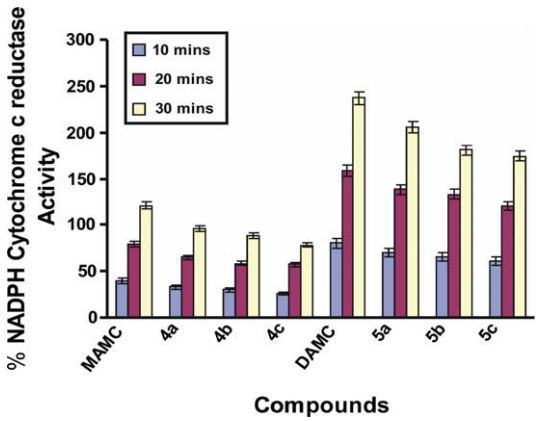
The role of CRTAase in transferring the acetyl group of acetoxy coumarins to amino acids of the active site of NADPH Cytochrome *c* reductase causing irreversible enzymatic activation is postulated.[13] Since NADPH Cytochrome *c* reductase forms a domain of NOS, the activation by way of acetylation of reductase caused the enhancement of the activity of the platelet NOS. It was thought interesting to probe whether variation in alkyl chain length at C-3 position in acetoxy coumarins could activate NOS. We examined this proposition using human platelets as the experimental system. The platelets preincubated with 3-substituted acetoxy coumarins and L-arginine exhibited enhancement in the level of NO, however the inclusion of L-NAME, a well known inhibitor of NOS failed to reduce DCF fluorescence (a measure of NO level) to the control level. These observations are indicative of the possible production of radicals other than NO by C-3 alkyl acetoxy coumarins (Fig. 3). Especially, 3-decyl acetoxycoumarins appeared to produce more free radicals as compared to the other test compounds. Further studies are warranted to decipher the mechanism of the action of 3-alkyl acetoxy coumarins.

4. Experimental

4.1. Materials and methods

4.1.1. Chemicals

The organic solvents (acetone, acetic anhydride, chloroform, tetrahydrofuran, petroleum ether and ethyl acetate) were dried and distilled prior to their use. Reactions were monitored by precoated TLC plates (Merck Silica Gel $60F_{254}$); the spots were visual-



- Polyphenolic acetates were separately preincubated with rat liver microsomes followed by assay of NADPH Cytochrome c reductase.
- The activity was expressed in terms of activation of NADPH Cytochrome c reductase.
- Values are mean ± SD of three observations.

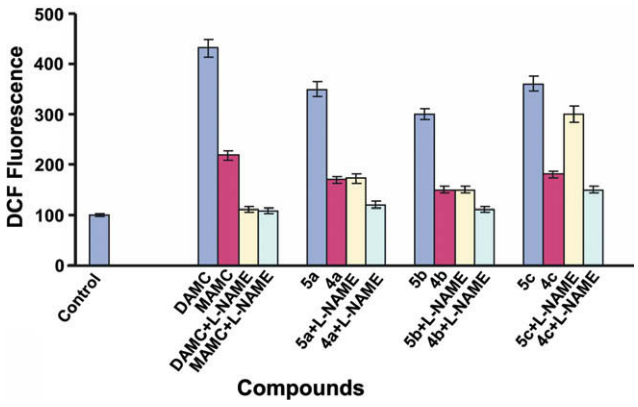
Figure 2. CRTAase catalyzed activation of NADPH Cytochrome *c* reductase by acetoxycoumarins.

ized 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, and washed each time with petroleum ether. Melting points were recorded in capillaries in sulfuric acid bath and are uncorrected. Infrared spectra were recorded on Perkin–Elmer FT-IR model 9 spectrophotometer. 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 (**1a**), NADPH, Cytochrome *c*, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), dichloro fluorescein-diacetate (DCFH-DA), *N*-nitro L-arginine methyl ester (L-NAME) and L-arginine were purchased from Sigma Chemical Co., St. Louis, MO (USA). Sodium nitrite was purchased from Thomas Baker Chemicals Ltd, Mumbai, India.

4.1.2. Synthesis of alkylated ethyl acetoacetate

4.1.2.1. Preparation of ethyl 2-acetyloctanoate (1b) and ethyl 2-acetyldodecanoate (1c). To a cold solution (0 °C) of ethyl acetoacetate (10 g, 76.84 mmol) in THF (30 mL) was added sodium hydride (3.13 g, 130.62 mmol). Once the addition was complete, the reaction mixture was heated at 60 °C for 2 h. The contents of the flask were then allowed to attain room temperature, further, addition of 1-bromohexane (13 g, 80 mmol) or 1-bromodecane (17.4 g, 80 mmol) dissolved in THF was carried out under ice cold conditions. The resultant mixture thus obtained was heated at 100 °C for 10–12 h. The reaction was monitored by TLC (10% ethyl



- Polyphenolic acetates were incubated with platelets, L-arginine and DCF at 37 ºC for 30 mins.
- L-NAME was also added in a set polyphenolic acetates to confirm the appearance of NO.
- Values are mean ± SD of three observations.

Figure 3. The effect of C-3 substituted coumarins on activation of platelet NOS.

acetate–petroleum ether). Unreacted sodium hydride was deactivated by addition of ethyl acetate, the solution was then filtered to remove sodium bromide formed during the reaction. The solution was then washed with water, the organic layer dried over anhydrous sodium sulfate and the solvent evaporated. The crude product so obtained was subjected to column chromatography using silica gel (100–200 mesh) and the desired products were eluted with 2% ethyl acetate–petroleum ether as viscous oils.

4.1.2.2. Ethyl 2-acetyloctanoate (1b). Obtained from the reaction of ethyl acetoacetate with 1-bromohexane as a pale yellow coloured liquid (8.72 g, 53%); bp: 147–148 °C (literature bp: 148–149 °C);[14] ^{1}H NMR (chloroform-*d*, 300 MHz): δ 0.86 (t, 3H, J = 7.3 Hz, –$CH_2(CH_2)_4CH_3$), 1.25–1.29 (m, 10H, –$(CH_2)_5CH_3$), 1.82 (t, 3H, J = 7.4 Hz, –OCH_2CH_3), 2.22 (s, 3H, –$COCH_3$), 3.39 (t, 1H, J = 7.3 Hz, –$CH(CH_2)_5CH_3$), 4.18 (q, 2H, J = 7.1 Hz, –OCH_2CH_3); ^{13}C NMR (chloroform-*d*, 75.5 MHz): δ 13.76 (–$(CH_2)_5CH_3$), 14.04 (–OCH_2CH_3), 22.12 (–$COCH_3$), 23.45, 27.23, 28.42, 29.86, 32.54 (–$(CH_2)_5CH_3$), 60.32 (–$CH(CH_2)_5CH_3$), 61.50 (–OCH_2CH_3), 172.13 (–$C(O)OCH_2CH_3$), 207.87 (–$COCH_3$).

4.1.2.3. Ethyl 2-acetyldodecanoate (1c). Obtained from the reaction of ethyl acetoacetate with 1-bromodecane as a pale yellow coloured liquid (10.38 g, 50%); bp: 170–172 °C (literature bp: 171–171.5 °C);[15] ^{1}H NMR (chloroform-*d*, 300 MHz): δ 0.88 (t, 3H, J = 6.9 Hz, –$(CH_2)_9CH_3$), 1.20–1.26 (m, 18H, –$(CH_2)_9CH_3$), 1.83 (t, 3H, J = 6.9 Hz, –OCH_2CH_3), 2.23 (s, 3H, –$COCH_3$), 3.41 (t, 1H, J = 6.7 Hz, –$(CH(CH_2)_9CH_3$), 4.19 (q, 2H, J = 7.0 Hz, –OCH_2CH_3); ^{13}C NMR (chloroform-*d*, 75.5 MHz): δ 14.00 (–$(CH_2)_9CH_3$), 14.44 (–OCH_2CH_3), 23.01 (–$COCH_3$), 27.73, 28.08, 28.56, 29.03, 29.66, 29.86, 29.89, 32.23 (–$(CH_2)_9CH_3$), 60.30 (–$CH(CH_2)_9CH_3$), 61.58 (–OCH_2CH_3), 170.30 (–$C(O)OCH_2CH_3$), 204.12 (–$COCH_3$).

4.1.3. General procedure for the synthesis of 3-alkyl-7-hydroxy-4-methylcoumarins (2a–c)

To a mixture of resorcinol (1.0 g, 9 mmol) in alkylated ethyl acetoacetate [**1a** (1.63 mL, 15.43 mmol) or **1b** (3.22 mL, 15.43 mmol) or **1c** (4.06 mL, 15.43 mmol)] was slowly added concentrated sulfuric acid (5 mL, dropwise) at 0 °C. The mixture was stirred at room temperature for 3–4 h. The progress of reaction was monitored on TLC (5% methanol–chloroform). On completion of the reaction 100 mL ice/water was added. The crude solid so obtained was then filtered, washed with water, dried and crystallized from ethanol to give the coumarins **2a–c** [**2a** (1.44 g, 78%), **2b** (1.88 g, 80%) or **2c** (2.2 g, 79%)] as pale yellow coloured crystals.

4.1.3.1. 3-Ethyl-7-hydroxy-4-methylcoumarin (2a). The coumarin **2a** was obtained as pale yellow coloured crystals (1.44 g, 78%); mp: 196–198 °C (literature mp: 196–198 °C);[16,17] ^{1}H NMR (acetone-d_6, 400 MHz): δ 1.05 (t, 3H, J = 7.6 Hz, –CH_2CH_3), 2.36 (s, 3H, C-4 CH_3), 2.59 (q, 2H, J = 7.6 Hz, –CH_2CH_3), 6.67 (d, 1H, J = 2.4 Hz, C–8H), 6.80 (dd, 1H, J = 2.4 and 8.8 Hz, C–6H), 7.56 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (acetone-d_6, 100 MHz): δ 12.55 (–CH_2CH_3), 13.75 (C-4 CH_3), 20.42 (–CH_2CH_3), 102.11 (C-8), 112.46, 113.49, 123.89 and 126.24 (C-3, C-5, C-6 and C-10), 146.00 (C-4), 153.83 and 159.82 (C-7 and C-9), 160.18 (C-2 carbonyl); IR (Nujol) ν_{max}: 3425.9 (OH), 1720.9 (CO), 1672.5, 1614.4, 1581.0, 1508.2, 1459.6, 1378.1, 1356.6, 1356.6, 1309.4, 1244.6, 1216.7, 1124.2, 1115.8, 1093.2, 1051.6, 954.1, 921.2, 798.5, 776.1, 722.1, 673.4 cm^{-1}; UV (acetonitrile) λ_{max}: 317 nm; EI MS: $C_{12}H_{12}O_3$ [M]$^+$: 204.00.

4.1.3.2. 3-Hexyl-7-hydroxy-4-methylcoumarin (2b). The coumarin **2b** was obtained as pale yellow coloured crystals (1.88 g, 80%); mp: 110 °C (literature mp: 110–112 °C);[18] ^{1}H NMR (methanol-d_4, 400 MHz): δ 0.86 (t, 3H, J = 7.2 Hz, –$CH_2(CH_2)_4CH_3$), 1.19–1.44 (m, 8H, –$CH_2(CH_2)_4CH_3$), 2.34 (s, 3H, C-4 CH_3), 2.53 (t, 2H, J = 7.8 Hz, –$CH_2(CH_2)_4CH_3$), 6.61 (d, 1H, J = 2.4 Hz, C–8H), 6.74 (dd, 1H, J = 2.4 and 8.8 Hz, C–6H), 7.50 (d, 1H, J = 8.8 Hz, C–6H); ^{13}C NMR (methanol-d_4, 100 MHz): 13.12 (–$(CH_2)_5CH_3$), 13.66 (C-4, CH_3), 23.55, 25.57, 26.86, 29.40, 31.28 (–$(CH_2)_5CH_3$), 101.77 (C-8),112.79, 113.14, 121.94 and 125.86 (C-3, C-5, C-6 and C-10), 147.76 (C-4), 153.54 and 160.40 (C-7 and C-9), 162.96 (C-2 carbonyl); IR (Nujol) ν_{max}: 3413.2 (OH), 1714.3 (CO), 1361.9, 1222.1, 1091.4, 530.0 cm^{-1}; UV (acetonitrile) λ_{max}: 318 nm; EI MS: $C_{16}H_{20}O_3$ [M]$^+$: 260.30.

4.1.3.3. 3-Decyl-7-hydroxy-4-methylcoumarin (2c). The coumarin **2c** was obtained as pale yellow coloured crystals (2.2 g, 79%); mp: 58–60 °C; ^{1}H NMR (chloroform-*d*, 400 MHz): δ 0.83 (t, 3H, –$CH_2(CH_2)_8CH_3$), 1.21–1.47 (m, 16H, –$CH_2(CH_2)_8CH_3$), 2.35 (s, 3H, C-4 CH_3), 2.58 (t, 2H, J = 7.6 Hz, –$CH_2(CH_2)_8CH_3$), 6.84 (d, 1H, J = 8.8 Hz, C–6H), 7.02 (s, 1H, C–8H), 7.44 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (chloroform-*d*, 100 MHz): δ 14.22 (–$(CH_2)_9CH_3$), 15.01 (C-4 CH_3), 22.78, 23.98, 27.53, 29.43, 29.71, 29.73, 31.99, 43.98 (–$(CH_2)_9CH_3$), 103.04 (C-8), 113.45, 113.99, 123.02 and 125.81 (C-3, C-5, C-6 and C-10), 147.56 (C-4), 153.43 and 159.37 (C-7 and C-9), 163.44 (C-2 carbonyl); IR (Nujol) $\nu_{max:}$ 3425.9 (OH), 1720.9 (CO), 1672.5, 1614.4, 1581.0, 1508.2, 1459.6, 1378.1, 1356.6, 1309.4, 1244.6, 1216.7, 1144.8, 1124.2, 1093.2, 1051.6, 954.1, 798.5, 776.1, 722.1, 673.4 cm^{-1}; UV (acetonitrile) $\lambda_{max:}$ 320 nm; EI MS: calculated for $C_{20}H_{28}O$ [M+H]$^+$ 317.2120, found 317.2121.

4.1.4. General procedure for synthesis of 7,8-dihydroxy-3-alkyl-4-methylcoumarins (3a–c)

To a mixture of pyrogallol (1.0 g, 8 mmol) in alkylated ethyl acetoacetate [**1a** (1.53 mL, 9.5 mmol) or **1b** (2.81 mL, 13.48 mmol) or **1c** (3.55 mL, 13.48 mmol)] was slowly added concentrated sulfuric acid (5 mL, dropwise) at 0 °C. The mixture was stirred at room temperature for 3–4 h. The progress of reaction was monitored on TLC (5% methanol–chloroform). On completion of the reaction 100 mL ice/water was added. The solid so obtained was then filtered, washed with water, dried and crystallized from ethanol to give corresponding dihydroxycoumarins **3a–c** [**3a** (1.33 g, 77%), **3b** (1.63 g, 75%) or **3c** (1.95 g, 75%)] as colourless crystals.

4.1.4.1. 7,8-Dihydroxy-3-ethyl-4-methylcoumarin (3a). The coumarin **3a** was obtained as colourless crystals (1.33 g, 77%); mp: 218–220 °C (literature mp: 218 °C);[16] ^{1}H NMR (acetone-d_6, 400 MHz): δ 1.04 (t, 3H, J = 7.6 Hz, –CH_2CH_3), 2.35 (s, 3H, C-4 CH_3), 2.58 (q, 2H, J = 7.6 Hz, –CH_2CH_3), 6.81 (d, 1H, J = 8.8 Hz, C–6H), 7.09 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (acetone-d_6, 100 MHz): δ 12.53 (–CH_2CH_3), 13.86 (C-4 CH_3), 20.45 (–CH_2CH_3), 111.81, 115.55, 114.02 and 123.65 (C-3,C-5, C-6 and C-10), 131.66 (C-8), 141.85 and 146.85 (C-7 and C-9), 151.96 (C-4), 160.57 (C-2 carbonyl); IR (Nujol) $\nu_{max:}$ 3333.8 (OH), 1694.7 (CO), 1607.7, 1507.6, 1464.3, 1378.8, 1335.4, 1249.8, 1268.5, 1146.8, 1146.8, 1177.0, 1101.0, 1123.9, 974.6, 861.0, 802.3, 779.1, 724.8 cm^{-1}; UV (acetonitrile) λ_{max}: 259 and 314 nm; EI MS: $C_{12}H_{12}O_4$ [M]$^+$: 220.03.

4.1.4.2. 7,8-Dihydroxy-3-hexyl-4-methylcoumarin (3b). The coumarin **3b** was obtained as colourless crystals (1.63 g, 75%); mp: 122–124 °C; ^{1}H NMR (methanol-d_4, 400 MHz): δ 0.87 (t, 3H, –$CH_2(CH_2)_4CH_3$), 1.25–1.31 (m, 8H, –$CH_2(CH_2)_4CH_3$), 2.34 (s, 3H, C-4 CH_3), 2.56 (t, 2H, –$CH_2(CH_2)_4CH_3$), 6.76 (d, 1H, J = 8.4 Hz, C–6H), 7.05 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (methanol-d_4, 100 MHz): δ 13.11 (–$(CH_2)_5CH_3$), 13.78 (C-4, CH_3), 23.54, 25.56, 26.91, 29.09, 31.53 (–$(CH_2)_5CH_3$), 111.89,115.10, 113.86 and 121.76 (C-3, C-5, C-6 and C-10), 131.81 (C-8), 141.76 and 148.14 (C-7 and C-9), 148.23 (C-4), 162.62 (C-2 carbonyl); IR (Nujol) ν_{max}:

3396.1 (OH), 1711.6 (CO), 1608.1, 1091.1 cm^{-1}; UV (acetonitrile) $\lambda_{max:}$ 260 and 314 nm; EI MS: $C_{16}H_{20}O_4$ $[M]^+$: 276.30.

4.1.4.3. 7,8-Dihydroxy-3-decyl-4-methylcoumarin (3c). The coumarin **3c** was obtained as colourless crystals (1.95 g, 75%); mp: 118–120 °C; ^{1}H NMR (methanol-d_4, 400 MHz): δ 0.86 (t, 3H, $-CH_2(CH_2)_8CH_3$), 1.20–1.25 (m, 16H, $-CH_2(CH_2)_8CH_3$), 2.35 (s, 3H, C-4 CH_3), 2.57 (t, 2H, J = 7.8 Hz, $-CH_2(CH_2)_8CH_3$), 6.76 (d, 1H, J = 8.8 Hz, C–6H), 7.06 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (methanol-d_4, 100 MHz): δ 13.12 ($-(CH_2)_9CH_3$), 13.95 (C-4 CH_3), 23.91, 26.92, 28.51, 28.93, 29.23, 29.30, 29.44, 29.69, 31.75 ($-(CH_2)_9CH_3$), 111.91, 115.11, 113.88, and 121.79 (C-3, C-5, C-6 and C-10), 131.83 (C-8), 137.45 and 141.79 (C-7 and C-9), 148.23 (C-4), 162.63 (C-2 carbonyl); IR (Nujol) ν_{max}: 3333.8 cm^{-1} (OH), 1694.7 (CO), 1607.7, 1507.6, 1464.3, 1378.8, 1335.4, 1268.5, 1249.8, 1177.0, 1146.8, 1123.9, 1101.8, 974.6, 880.4, 861.0, 798.1, 724.8 cm^{-1}; UV (acetonitrile) λ_{max}: 259 and 312 nm; HRMS: calculated for $C_{20}H_{28}O_4$ $[M+H]^+$ 333.2066, found 333.2067.

4.1.5. General procedure for synthesis of 3-alkyl-7-acetoxy-4-methylcoumarin (4a–c)

To a mixture of 3-alkyl-7-hydroxy-4-methyl-coumarin (**2a–c**) (500 mg) and dimethylaminopyridine (10 mg) in THF was added acetic anhydride (1.4 mL, 15 mmol) and the reaction mixture was stirred at room temperature for 24 h. On completion of the reaction, 50 mL ice/water was added. The crude solid was then filtered, washed with water, dried and crystallized from ethanol to give colourless crystals of 7-acetoxycoumarins **4a–c** [**4a** (0.43 g, 72%), **4b** (0.41 g, 70%) or **4c** (0.40 g, 71%)].

4.1.5.1. 3-Ethyl-7-acetoxy-4-methylcoumarin (4a). The coumarin **4a** was obtained as colourless crystals (0.43 g, 72%); mp: 108–110 °C (literature mp: 110 °C);[17] ^{1}H NMR (chloroform-d, 300 MHz): 1.15 (t, 3H, J = 7.5 Hz, $-CH_2CH_3$), 2.33 (s, 3H, C-4 CH_3), 2.41 (s, 3H, $-OCOCH_3$), 2.71 (q, 2H, J = 7.5 Hz, $-CH_2CH_3$), 7.03 (d,1H, J = 2.2 Hz, C–8H), 7.07 (dd, 1H, J = 2.2 and 8.5 Hz, C–6H), 7.60 (d, 1H, J = 8.5 Hz, C–5H); ^{13}C NMR (chloroform-d, 75.5 MHz): 12.95 ($-CH_2CH_3$), 14.58 (C-4 CH_3), 20.93 and 21.06 ($-OCOCH_3$ and $-CH_2CH_3$), 109.96 (C-8), 117.79, 118.56, 125.16 and 127.58 (C-3, C-5, C-6 and C-10), 145.02 (C-4), 151.90 and 152.56 (C-7 and C-9), 162.01 (C-2 carbonyl), 168.01 ($-OCOCH_3$); IR (Nujol) ν_{max}: 1758.5 (OCO), 1708.8 (CO), 1613.0, 1573.6, 1502.7, 1462.3, 1423.0, 1376.3, 1277.2, 1250.1, 1201.9, 1154.1, 1134.9, 1092.7, 1066.3, 1016.5, 970.3, 933.6, 911.8, 874.6, 853.7, 830.6, 778.5, 722.4 cm^{-1}; UV (acetonitrile) λ_{max}: 272 and 309 nm; EI MS: calculated for $C_{14}H_{14}O_4$ $[M]^+$: 246.10.

4.1.5.2. 7-Acetoxy-3-hexyl-4-methylcoumarin (4b). The coumarin **4b** was obtained as colourless crystals with (0.41 g, 70%); mp: 58–60 °C; ^{1}H NMR (chloroform-d, 300 MHz): δ 0.88 (t, 3H, J = 7.6 Hz, $-CH_2(CH_2)_4CH_3$), 1.25–1.38 (m, 8H, $-CH_2(CH_2)_4CH_3$), 2.33 (s, 3H, C-4 CH_3), 2.40 (s, 3H, $-OCOCH_3$), 2.65 (t, 2H, J = 7.6 Hz, $-CH_2(CH_2)_4CH_3$), 7.06–7.08 (m, 2H, C–6H and C–8H), 7.60 (d, 1H, J = 8.5 Hz, C–5H); ^{13}C NMR (chloroform-d, 75.5 MHz): δ 14.05 ($-(CH_2)_5CH_3$), 14.90 (C-4 CH_3), 21.08 ($-OCOCH_3$), 22.58, 27.66, 28.66, 29.32, 31.65 ($-(CH_2)_5CH_3$), 109.98 (C-8), 117.80, 118.60, 125.19 and 126.60 (C-3, C-5, C-6 and C-10), 145.25 (C-4), 151.87 and 152.56 (C-7 and C-9), 161.43 (C-2 carbonyl), 168.91 ($-OCOCH_3$); IR (Nujol) ν_{max}: 1747.2 (OCO), 1709.8 (CO), 1617.3, 1463.1, 1377.1, 1239.5, 1158.5, 1140.0, 1092.6, 1050.5, 1020.4, 915.1, 874.9, 823.9, 755.1, 722.3, 646.4 cm^{-1}; UV (CH_3OH) λ_{max}: 274 and 310 nm; EI MS: $C_{18}H_{22}O_4$ $[M]^+$: 302.60.

4.1.5.3. 7-Acetoxy-3-decyl-4-methylcoumarin (4c). The coumarin **4c** was obtained as colourless crystals (0.40 g, 71%); mp: 91–92 °C; ^{1}H NMR (chloroform-d, 300 MHz): 0.88 (t, 3H, $-CH_2(CH_2)_8CH_3$), 1.26–1.51 (m, 16H, $-CH_2(CH_2)_8CH_3$), 2.33 (s, 3H, C-4 CH_3), 2.40 (t, 3H, $-OCOCH_3$), 2.54 (t, 2H, $-CH_2(CH_2)_8CH_3$), 7.06 (d, 1H, J = 8.4 Hz, C–6H), 7.14 (s, 1H, C–8H), 7.58 (d, 1H, J = 8.4 Hz, C–5H); ^{13}C NMR (chloroform-d, 75.5 Hz): δ 14.11 ($-(CH_2)_9CH_3$), 14.93 (C-4 CH_3), 21.11 ($-OCOCH_3$) 22.78, 23.85, 27.69, 28.73, 29.30, 29.58, 31.87, 31.45, 43.98 ($-(CH_2)_9CH_3$), 110.00 (C-8), 117.80, 118.62, 125.18 and 126.54 (C-3, C-5, C-6 and C-10), 145.21 (C-4), 151.87 and 152.58 (C-7 and C-9), 161.41(C-2 carbonyl), 168.89 ($-OCOCH_3$); IR (Nujol) ν_{max}: 1746.2 (OCO), 1710.5 (CO), 1617.7, 1464.5, 1376.7, 1243.8, 1197.2, 1159.2, 1140.4, 1093.8, 1050.84 cm^{-1}; UV (acetonitrile) λ_{max}: 273 and 308 nm; HRMS: Calculated for $C_{22}H_{30}O_4$ $[M+H]^+$ 359.2230, found 359.2231.

4.1.6. General procedure for synthesis of 7,8-diacetoxy-3-alkyl-4-methylcoumarin (5a–c)

To a mixture of 7,8-dihydroxy-3-alkyl-4-methylcoumarin (**3a–c**) (500 mg), dimethylaminopyridine (10 mg) in THF was added acetic anhydride (1.28 mL, 13.66 mmol) and the reaction mixture was stirred at room temperature for 24 h. On completion of the reaction 50 mL ice/water was added. The crude solid was then filtered, washed with water, dried and crystallized from ethanol to give colourless crystals of 7,8-diacetoxycoumarins **5a–c** [**5a** (0.53 g, 78%) or **5b** (0.48 g, 75%) or **5c** (0.44 g, 72%)].

4.1.6.1. 7,8-Diacetoxy-3-ethyl-4-methylcoumarin (5a). The coumarin **5a** obtained as colourless crystals (0.53 g, 78%); mp: 130 °C (literature mp: 130–131 °C);[16] ^{1}H NMR (chloroform-d, 300 MHz): δ 1.14 (t, 3H, J = 7.3 Hz, $-CH_2CH_3$), 2.33 (s, 3H, C-4 CH_3), 2.41 (s, 6H, $-2 \times (-OCOCH_3)$), 2.67 (q, 2H, J = 7.4 Hz, $-CH_2CH_3$), 7.14 (d, 1H, J = 8.8 Hz, C–6H), 7.49 (d, 1H, J = 8.8 Hz, C–5H); ^{13}C NMR (chloroform-d, 75.5 Hz): δ 12.87 ($-CH_2CH_3$), 14.70 (C-4, CH_3), 20.29 and 20.61 ($2 \times -OCOCH_3$), 21.03 ($-CH_2CH_3$), 118.19, 121.50, 118.53 and 119.64 (C-3, C-5, C-6 and C-10), 128.05 (C-8), 144.01 and 144.97 (C-7 and C-9), 145.19 (C-4), 160.05 (C-2 carbonyl), 167.51 and 167.89 ($2 \times -OCOCH_3$); IR (Nujol) ν_{max}: 1758.2 (OCO), 1708.4 (CO), 1613.2, 1573.8, 1462.7, 1377.1, 1200.4, 1154.1, 1134.9, 1092.3, 1016.6, 912.2, 830.4, 778.5, 722.0 cm^{-1}; UV (acetonitrile) λ_{max}: 276 and 308 nm; EI MS: $C_{16}H_{16}O_6$ $[M]^+$: 303.80.

4.1.6.2. 7,8-Diacetoxy-3-hexyl-4-methylcoumarin (5b). The coumarin **5b** obtained as colourless crystals (0.48 g, 75%); mp: 89–90 °C; ^{1}H NMR (chloroform-d, 300 MHz): δ 0.81 (t, 3H, $-CH_2(CH_2)_4CH_3$), 1.20–1.23 (m, 8H, $-CH_2(CH_2)_4CH_3$), 2.25 (s, 3H, C-4 CH_3), 2.33 (s, 6H, $2 \times (-OCOCH_3)$), 2.57 (t, 2H, J = 7.3 Hz, $-CH_2(CH_2)_4CH_3$), 7.06 (d, 1H, J = 8.3 Hz, C–6H), 7.41 (d,1H, J = 8.4 Hz, C–5H); ^{13}C NMR (chloroform-d, 75.5 MHz): δ 14.06 ($-(CH_2)_5CH_3$), 15.03 (C-4 CH_3), 20.33 and 20.64 ($2 \times -OCOCH_3$), 22.57, 23.69, 27.74, 30.29, 31.64 ($-(CH_2)_5CH_3$), 118.18, 121.52, 119.69 and 125.50 (C-3, C-5, C-6 and C-10), 127.00 (C-8), 141.05 and 144.00 (C-7 and C-9), 145.16 (C-4), 160.20 (C-2 carbonyl), 167.55 and 167.93 ($2 \times -OCOCH_3$); IR (Nujol) $\nu_{max:}$ 1773.0 (OCO), 1709.0 (CO), 1612.5, 1461.2, 1377.3, 1274.7, 1221.6, 1114.7, 1164.4, 1078.8, 1016.0, 952.3, 880.1, 823.5, 773.9, 723.1 cm^{-1}; UV (acetonitrile) λ_{max}: 279 nm; HRMS: calculated for $C_{20}H_{24}O_6$ $[M]^+$ 360.1572, found 360.1569.

4.1.6.3. 3-Decyl-7,8-diacetoxy-4-methylcoumarin (5c). The coumarin **5c** obtained as colourless crystals (0.44 g, 72%); mp: 94–96 °C; ^{1}H NMR (chloroform-d, 300 MHz): δ 0.86 (t, 3H, $-CH_2(CH_2)_8CH_3$), 1.20–1.26 (m, 16H, $-CH_2(CH_2)_8CH_3$), 2.32 (s, 3H, C-4 CH_3), 2.40 (s, 6H, $2 \times (-OCOCH_3)$), 2.62 (t, 2H, J = 7.6 Hz, $-CH_2(CH_2)_8CH_3$), 7.12 (d, 1H, J = 8.6 Hz, C–6H), 7.48 (d, 1H, J = 8.8 Hz, C–6H); ^{13}C NMR (chloroform-d, 75.5 MHz): δ 13.65 ($-(CH_2)_9CH_3$), 14.63 (C-4 CH_3), 19.85 and 20.17 ($2 \times -OCOCH_3$),

22.14, 27.23, 28.12, 29.05, 31.34, 39.08, 39.36, 39.64, 39.91 ($-(CH_2)_9CH_3$), 117.84, 121.23, 119.15 and 126.33 (C-3, C-5, C-6 and C-10), 129.46 (C-8), 143.54 and 144.60 (C-7 and C-9), 144.99 (C-4), 159.69 (C-2 carbonyl), 166.95 and 167.40 ($2 \times -OCOCH_3$); IR (Nujol) ν_{max}: 1775.4 (OCO), 1709.5 (CO), 1613.0, 1550.2, 1462.7, 1377.4, 1271.9, 1222.1, 1165.1, 1080.0 cm^{-1}; UV (acetonitrile) λ_{max}: 275 nm; EI MS: $C_{24}H_{32}O_6$ $[M]^+$: 415.70.

4.1.7. Preparation of microsomes and cytosol

Rats were sacrificed by decapitation. The liver from rats was pooled and washed in chilled saline and the tissue was minced and homogenized in 30% 10 mM phosphate buffer containing 0.25 M sucrose and 1.4 mM β-mercaptoethanol, pH = 7.0 using Potter Eljvehjem homogenizer. The homogenate was centrifuged using Sorvall Centrifuge at 10,000*g* for 30 min at 4 °C. The pellet was discarded and supernatant was again centrifuged in Beckmann Ultracentrifuge Model L7 at 1,00,000*g* for 1 h at 4 °C. The cytosolic fraction was set-aside at −20 °C. The pellet obtained was washed with 0.15 M KCl and then resuspended in storage buffer (20% glycerol in 10 mM K_3PO_4, 2 mM EDTA (ethylenediamine tetraacetic acid), 2 mM DTT (dithiothreitol), 2 mM PMSF (phenylmethanesulfonylfluoride), pH = 7.2) and stored at −20 °C in aliquots.

4.1.8. CRTAase assay

The method of Habig et al.[19] was followed for GST assay using GSH (Glutathione reduced form) and CDNB (1-chloro-2,4-dinitrobenzene) as substrates. The assay was carried out in 1 mL spectrophotometric cuvette. The reaction mixture consisted of 0.25 M phosphate buffer (pH = 6.5), cytosol (12–13 μg protein) and 1 mM CDNB and 1 mM GSH in a total volume of 1.0 mL. The contents were mixed and the progress of reaction was followed at 340 nm using Cary Spectrophotometer Model Cary 100 with kinetic software. It was ensured that reaction should be linear with respect to time of incubation and enzyme concentration.

For the assay of CRTAase rat liver microsomes (25 μg) was preincubated for different time intervals with test compounds and cytosol (12–13 μg) followed by GST assay. The percentage inhibition in GST activity, as compared to control, is taken as units of CRTAase activity.

4.1.9. Modulation of rat liver microsomal NADPH Cytochrome *c* reductase

The assay mixture consisted of 0.05 M phosphate buffer (pH = 7.7), 0.1 mM EDTA liver microsomes (25 μg protein), 36 μM Cytochrome *c* and 1 mM NADPH. Accordingly, polyphenolic acetates were separately incubated along with liver microsomes at 37 °C in shaking water bath followed by addition of EDTA, Cytochrome *c* and NADPH in a total volume of 1 mL. The progress of the reaction NADPH cytochrome P450 reductase assay was determined by monitoring absorption at 515 nm. In the control samples DMSO replaced PA. The increment in reductase activity due to compounds over the control was expressed as % activation.

4.1.10. Isolation of platelet rich plasma (PRP)

The citrated blood was used for the preparation of PRP. Venous blood (9 mL) was collected from healthy human volunteer and was mixed with 1.0 mL of 3.8% trisodium citrate (anticoagulant). The citrated blood was centrifuged at 1200 rpm for 10 min at room temperature. 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. PRP was then further centrifuged at 4000 rpm for 5 min to produce a platelet button. The platelet button was then suspended in PBS.

4.1.11. Assay of NOS by flow cytometry

The method outlined by Imrich and Kobzik was followed for the assay of NOS by flow cytometry.[20] Measurements were made with a 488 nm laser based flow cytometer (FACS calibur, Becton and Dickenson, USA) and data (light scatter and green fluorescence) was acquired using the Cell Quest software (Becton and Dickinson, USA). Analysis was performed by applying appropriate gates with reference to the auto fluorescence measured under similar conditions. Platelets isolated as described earlier were suspended in phosphate buffer saline and platelets count was adjusted to 10^6/mL using electronic particle counter (SYSMEX, Model no. FA 20). DCFH-DA (Dichloro fluoresein diacetate) (20 μM) Polyphenolic acetates (100 μM) were then added to the cell suspension and separately preincubated at 37 °C for 30 min In order to confirm the enhancement of NO level in platelets, L-NAME (50 μM), a specific nitric oxide inhibitor was also added. To quench the assay, samples were placed on ice for 10 min in dark. Relative green DCF fluorescence was measured.

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