Synthesis, antidiabetic, antioxidant and anti-inflammatory activities of novel hydroxytriazenes based on sulpha drugs

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\textbf{A B S T R A C T}

The present study is aimed to investigate the anti-inflammatory, antioxidant and antidiabetic activities of three series of hydroxytriazenes based on sulfa drugs viz; Sulphathiazole (ST), Sulfoxazole (SF) and Sulphamethoxazole (SM). Antidiabetic activities of the synthesized hydroxytriazenes were investigated by \( \alpha \)-glucosidase and \( \alpha \)-amylase inhibition method and IC\textsubscript{50} values were recorded. The compounds presented significant \( \alpha \)-glucosidase and \( \alpha \)-amylase inhibition effect with IC\textsubscript{50} values ranging from 122 to 341 \( \mu \)g/mL. Anti-inflammatory activity was also investigated by carrageenan-induced paw edema (CPE) method, where \% inhibition was up to 89\% after 4 h of treatment and antioxidant properties of the similar compounds were assessed by DPPH and ABTS radical scavenging assays. Antioxidant capacity of all the hydroxytriazenes detected by ABTS assay, was significantly higher as compared to DPPH assay. The hydroxytriazenes having highest antioxidant capacity presented IC\textsubscript{50} values for compound ST-1 and ST-6 are 488 \( \mu \)g/mL for DPPH, 54.12 \( \mu \)g/mL for ABTS and 858.5 \( \mu \)g/mL for DPPH, 48.0 \( \mu \)g/mL for ABTS, respectively. These results suggested that ABTS assay may be more useful than DPPH assay for synthetic antioxidants. The findings from the molecular docking experiments may also expand the formation of new potent sulpha drugs based hydroxytriazenes targeting towards the subunit of C-terminal of human maltase-glucoamylase for the treatment of diabetes metabolic disorder. Overall, highlight the multifunctional role of hydroxytriazenes as antidiabetic, antioxidant and anti-inflammatory agents.

\textbf{1. Introduction}

Diabetes mellitus is a chronic metabolic disorder (Golbidi et al., 2012) characterized by means of abnormally high plasma glucose, give rise to diabetic complications which include diabetic neuropathy (Ziegler et al., 1995), retinopathy (Hammes et al., 2003), nephropathy (Lim, 2014) and cardiovascular diseases (Levine et al., 2006). Diabetes is a fast increasing life style related disease (Taslimi et al., 2020; Bouguerra et al., 2007) that requires the significant persisted research, therefore, there is an urgent need for novel approaches to prevent and deal with this pandemic.

Although, obesity and physical inertness are regarded to be primary causes associated with type-2 diabetes, however, few latest researches suggest that oxidative stress may make contributions to the pathogenesis of diabetes through enhancing insulin resistance or by impairing secretion of insulin (Montonen et al., 2004).

Diabetes can be effectively managed by decreasing postprandial hyperglycaemia (DeFronzo, 2000) by delaying the glucose absorption through inhibiting the enzymes particularly \( \alpha \)-glucosidase and \( \alpha \)-amylase which hydrolyze carbohydrate (Chiasson et al., 2002). \( \alpha \)-Glucosidase enzyme catalyse the digestion of carbohydrates and \( \alpha \)-amylase splits the \( \alpha \)-1,4-glucosidic linkage to produce maltose and glucose. Thus, these inhibitors inhibit the release of D-glucose from dietary carbohydrates and delay the absorption of glucose from intestine, leading to diminished postprandial hyperglycaemia (Hanefeld et al., 2004; Bösenberg and Van Zyl, 2008).

Although, treatment of diabetes is mostly centred on the management of hyperglycaemia, the approaches targeting reduction of oxidative stress perceived to be an effective way of treatment of diabetes and related complications. Oxidative stress is the accretion of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that cannot be coerced by the...
endogenous move of free radical neutralizing agents and antioxidants (Türkän et al., 2020). ROS and RNS are produced through regular cellular metabolism and have harmful effect to living systems (Valko et al., 2006). The antioxidant therapy would provide a therapeutic strategy to defend the lCeils against oxidative stress and prevent related diabetic vascular complication (Golbidi et al., 2011).

Antioxidants are enzymes or synthetic or natural substances, that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans (Huang et al., 2005). An antioxidant diminishes free radicals, enhance scavenging of free radicals and antioxidant defence mechanism. The measurement of total antioxidant capacity of a substance using one method seems to be somewhat impractical and not easy. However, there are several methods reported claiming to measure total antioxidant capacity in vitro. Due to the unavailability of standard assays, it is very difficult to select reliable method to measure antioxidant capacity of synthetic, natural and biological samples. Therefore, it is planned to examine antioxidant capacity of hydroxytriazenes by two methods DPHI and ABTS radical scavenging assays for the possible benefits in management of diabetes (Baragob et al., 2014; Rajendiran et al., 2018).

Oxidative stress and inflammation are largely related, many studies indicated that vascular inflammation facilitating arterial diseases. Under the condition of oxidative stress by generation of ROS and RNS species also play crucial role in activation of signalling pathway which affect intra and extra cellular pathway. At the site of inflammation, accumulation of ROS occurs because mast cells and leucocytes are produced, which leads to a ‘respiratory burst’ due to an increased uptake of oxygen (Pollack et al., 2016; Donath, 2013).

Hydroxytriazenes is a bidentate ligand which has alpha hydroxyl group relative to azo group and diazo group. This class of compounds were also investigated by CPE and radical scavenging methods, acetone powder was procured from Sigma Aldrich and 2,2-Diphenyl-1-sidase, p-nitrophenyl-

were recorded by using DMSO

in vitro capacity

for the possible bene

EXO,G2QTOF. MS ES+ spectrometry (Waters Instrument, USA) at MRC MNIT, Jaipur. The antioxidant activity was studied using Synergy H4 Hybrid Multimode Reader (Bio-TEK Instrument, Inc Winooski, VT, USA) at department of environmental sciences, MGCCV, Chitrakoot, Satna.

2.2. Synthesis of hydroxytriazenes

Hydroxytriazenes based on sulpha drugs have been synthesized as per reported method (Elkins and Hunter, 1938; Sogani and Bhattacharya, 1956). This method involves reduction of nitro compounds using Zn dust in neutral medium to get hydroxyamines and its coupling with diazonium salt resulted from diazotization of sulpha drugs [Sul-phathiazone (ST), Sulfoisoxazole (SF) and Sulphamethoxazole (SMI) at 0–5 °C, in pH 5–6 (Figure 1). Synthesized compounds were recrystallized many times to ascertain purity by means of methanol/aceton/DMF as a solvent.

2.3. Structural characterisation

Synthesized compounds were characterized using standard spectroscopic techniques. Detailed data and structures of all the compounds are given in supporting information (S6).

3. hydroxy_3. methyl4.1.1.(N. (thiazol_2. yl)sulfonyl)phenyl)tria

azene (ST-1): Reddish brown shining powder (methanol); mp 153 °C; FTIR (KBr): \( \nu_{max} \) 3472, 3208, 1529, 1286, 1138 cm\(^{-1}\). \( ^1 \)H NMR (DMSO\(_d6\), 400 MHz): \( \delta \) 3.95 (3H, s, H1), 11.62 (1H, s, H2), 7.70 (2H, d, J = 8.8 Hz, H5), 7.37 (2H, d, J = 8.8 Hz, H6), 6.81 (1H, d, J = 7.6 Hz, H4); \( ^13 \)C NMR (DMSO\(_d6\), 100 MHz): \( \delta \) 30.6 (1C, C1), 144.4 (1C, C2), 113.7 (2C, C4a), 134.4 (2C, C5b), 128.8 (1C, C6), 168.9 (1C, C8), 141.0 (1C, C10), 107.9 (1C, C12); HRMS (m/z) calc. for C\(_{12}H_{15}N_5O_3S_2\), [M+H]+ 314.303, found 314.3635.

3. hydroxy_3. ethyl4.1.1.(N. (thiazol_2. yl)sulfonyl)phenyl)tri

azene (ST-2): Light brown powder (methanol); mp 184 °C; FTIR (KBr): \( \nu_{max} \) 3453, 3222, 1526, 1299, 1137 cm\(^{-1}\). \( ^1 \)H NMR (DMSO\(_d6\), 400 MHz): \( \delta \) 4.52 (2H, q, J = 7.32 Hz, H2), 11.55 (1H, s, H3), 7.70 (2H, d, J = 8.8 Hz, H5), 7.37 (2H, d, J = 8.8 Hz, H6), 6.81 (1H, d, J = 4.4 Hz, H4); \( ^13 \)C NMR (DMSO\(_d6\), 100 MHz): \( \delta \) 58.32 (1C, C1), 12.37 (1C, C2), 143.9 (1C, C3), 113.5 (2C, C4a), 127.6 (2C, C5a), 124.3 (1C, C6), 168.5 (1C, C8), 134.3 (1C, C10), 107.9 (1C, C12); HRMS (m/z) calc. for C\(_{11}H_{13}N_5O_3S_2\) [M+H]+ 312.075, found 312.3878.

3. hydroxy_3. propyl4.1.1.(N. (thiazol_2. yl)sulfonyl)phenyl)tri

azene (ST-3): Buff color powder (methanol); mp 160161 °C; FTIR (KBr): \( \nu_{max} \) 3455, 3248, 1531, 1297, 1137 cm\(^{-1}\). \( ^1 \)H NMR (DMSO\(_d6\), 400 MHz): \( \delta \) 3.89 (3H, s, H1), 1.86 (2H, m, H5), 4.08 (2H, t, J = 6.76 Hz, H6), 11.57 (1H, s, H3); 7.70 (2H, d, J = 8.76 Hz, H5), 7.37 (2H, d, J = 8.76 Hz, H6), 12.64 (1H, s, H1), 7.22 (1H, d, J = 4.32 Hz, H4), 6.80 (1H, d, J = 4.56 Hz, H14); \( ^13 \)C NMR (DMSO\(_d6\), 100 MHz): \( \delta \) 64.5 (1C, C1), 20.2 (1C, C2), 10.6 (1C, C3), 143.9 (1C, C4), 113.5 (2C, C4a), 124.3 (2C, C5), 127.4 (1C, C6), 168.5 (1C, C8), 134.3 (1C, C10), 107.9 (1C, C12); HRMS (m/z) calc. for C\(_{11}H_{13}N_5O_3S_2\) [M+H]+ 342.07, found 341.5714.

3. hydroxy_3. phenyl4.1.1.(N. (thiazol_2. yl)sulfonyl)phenyl)tri

azene (ST-4): Light skin color powder (methanol); mp 179 °C; FTIR (KBr): \( \nu_{max} \) 3446, 3210, 1526, 1137 cm\(^{-1}\). \( ^1 \)H NMR (DMSO\(_d6\), 400 MHz): \( \delta \) 7.86 (2H, d, J = 8.18 Hz, H2), 7.57 (2H, d, J = 7.92 Hz, H3), 2.98 (1H, t, J = 7.64 Hz, H9), 12.21 (1H, s, H1), 7.71 (2H, d, J = 8.8 Hz, H10), 12.64 (1H, s, H5), 7.18 (1H, d, J = 4.8 Hz, H9), 6.67 (1H, d, J = 4.32 Hz, H3); \( ^13 \)C NMR (DMSO\(_d6\), 100 MHz): \( \delta \) 143.4 (1C, C1), 130.1 (2C, C3a), 130.8 (2C, C2a), 142.3 (1C, C4), 142.9 (1C, C6), 114.5 (2C, C9,13), 135.4 (2C, C10,12), 127.4 (1C, C11), 168.6 (1C,
C13), 139.0 (1C, C16); HRMS ESI (m/z) calc. for C19H17N5O4S, [M+H]+ 376.05, found 376.4989.

3. Hydroxy-3,3-dimethyloxazol-5-yl)sulfamoyl)phenyl)triazene (SF-3): Dark yellow color powder (acetone); mp 162 °C. FTIR (KBr): \( \nu_{\mathrm{max}} 3484, 3379, 1594, 1336, 1162 \) cm\(^{-1}\); \(^1\)H NMR (DMSO-d6, 400 MHz): \( \delta 1.62 (3H, s, H13), 2.07 (2H, m, H2), 4.0 (2H, t, J = 7.2 Hz, H7), 10.49 (1H, s, H12), 6.62 (2H, d, J = 8.7 Hz, H9), 7.36 (2H, d, J = 7.2 Hz, H10), 11.20 (1H, s, H11), 2.50 (3H, s, H14), 2.07 (3H, s, H15); \(^13\)C NMR (DMSO-d6, 100 MHz): \( \delta 26.6, 29.5, 106.8, 111.7, 106.7, 132.4 (2C, C7,9), 148.8 (1C, C12), 151.3 (1C, C13), 161.2 (1C, C8), 162.5 (1C, C14), 123.9 (1C, C6)\); HRMS ESI (m/z) calc. for C19H17N5O4S, [M+H]+ 334.06, found 334.0303.

3. Hydroxy-3,3-dimethyloxazol-5-yl)sulfamoyl)phenyl)triazene (SF-4): Light yellow color powder (methanol); mp 109110 − C. FTIR (KBr): \( \nu_{\mathrm{max}} 3474, 3380, 1595, 1339, 1159 \) cm\(^{-1}\); \(^1\)H NMR (DMSO-d6, 400 MHz): \( \delta 1.67 (3H, s, H13), 2.05 (2H, m, H2), 4.0 (2H, t, J = 7.2 Hz, H7), 10.57 (1H, s, H12), 6.57 (2H, d, J = 8.7 Hz, H9), 7.56 (2H, d, J = 7.2 Hz, H10), 11.20 (1H, s, H11), 2.50 (3H, s, H14), 2.07 (3H, s, H15); \(^13\)C NMR (DMSO-d6, 100 MHz): \( \delta 26.6, 29.5, 106.8, 111.7, 106.7, 132.4 (2C, C7,9), 148.8 (1C, C12), 151.3 (1C, C13), 161.2 (1C, C8), 162.5 (1C, C14), 123.9 (1C, C6)\); HRMS ESI (m/z) calc. for C19H17N5O4S, [M+H]+ 388.09, found 388.1493.

3. Hydroxy-3,3-dimethyloxazol-5-yl)sulfamoyl)phenyl)triazene (SF-5): Orange brown color powder (methanol); mp 8082 °C. FTIR (KBr): \( \nu_{\mathrm{max}} 3483, 3379, 1597, 1336, 1159 \) cm\(^{-1}\); \(^1\)H NMR (DMSO-d6, 400 MHz): \( \delta 1.67 (3H, s, H13), 2.05 (2H, m, H2), 4.0 (2H, t, J = 7.2 Hz, H7), 10.57 (1H, s, H12), 6.57 (2H, d, J = 8.7 Hz, H9), 7.56 (2H, d, J = 7.2 Hz, H10), 11.20 (1H, s, H11), 2.50 (3H, s, H14), 2.07 (3H, s, H15); \(^13\)C NMR (DMSO-d6, 100 MHz): \( \delta 26.6, 29.5, 106.8, 111.7, 106.7, 132.4 (2C, C7,9), 148.8 (1C, C12), 151.3 (1C, C13), 161.2 (1C, C8), 162.5 (1C, C14), 123.9 (1C, C6)\); HRMS ESI (m/z) calc. for C19H17N5O4S, [M+H]+ 388.09, found 388.1493.
sH2O, 2.07 (3H, s, H2O); 13C NMR (DMSO-d6, 100 MHz): δ 153.2 (1C, C1), 114.8 (2C, C2,3), 124.5 (1C, C4), 121.8 (1C, C5), 130.3 (1C, C6), 156.2 (1C, C7), 120.2 (2C, C8,9), 125.4 (2C, C10,11,12), 151.0 (1C, C13), 104.3 (1C, C14), 144.5 (1C, C15), 10.2 (1C, C16), 5.8 (1C, C17), 20.8 (1C, C18); HRMS (m/z) calc. for C23H36N3O3S, [M+H] + 402.40, found 402.46.

3. hydroxy-4.3-methylphenyl)

4.1.1.1. Biological activity

2.4.1. Anti-diabetic

To evaluate anti-diabetic activity of synthesized hydroxytriazinyl α-glucosidase and α-amylase inhibition methods (Tripathi et al., 2014) were used. Several dilutions of primary solution (5 μg/mL, DMSO) were made and assayed accordingly to obtain concentration of the test sample required to inhibit 50% activity of the enzyme (IC50). Quantification was performed with respect to the standard curve of acarbose (Y = 0.2262x + 47.244, R2 = 0.9937) for α-amylase and results were expressed as standard acarbose in 10, 30, 60, 90, 120, 150 μg/mL.

2.4.1.1. Assay for α-glucosidase inhibition method. In this method different concentrations viz 300, 240, 180, 120, and 20 μg/mL of each test compounds were prepared. Rat-intestinal powder was dissolved in 100 mL of saline water and sonicated properly at 4 °C. After sonication, the suspension was centrifuged (1008 g, 4 °C) and the resulting supernatant was used for assay. A reaction mixture containing 50 μL of phosphate buffer (50 mM, pH 6.8), 75 μL of rat α-glucosidase and 50 μL sample of varying concentrations (20–300 μg/mL) was pre-incubated for 5 min at 37 °C, then 5 μL of 3 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) was added to the mixture as a substrate. After incubation at 37 °C for 30 min, enzymatic activity was quantified by measuring the absorbance at 405 nm in Multi-Mode Reader. Acarbose was used as a positive control, which is the standard drug and water as a negative control. Experiments were done in triplicate, detailed results are shown in supporting information (S1) and IC50 values are given in Table 1. The inhibition of α-glucosidase activity was calculated using the following formula:

\[
\% \text{ inhibition} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100
\]

2.4.1.2. Assay for α-amylase inhibitory method. Six different concentrations 300, 240, 180, 120, 60 and 20 μg/mL of each of the test compounds were prepared in test tubes and six more solutions (10, 30, 60, 90, 120, 150 μg/mL) of standard drug acarbose were also prepared for comparison. A control test tube was prepared which did not contain any sample or drug. To each of the test compounds, 50 μL of the α-amylase solution (0.5 μg/mL in 0.02 M sodium phosphate buffer solution, pH 6.5) was added and allowed to incubate for 10 min at room temperature. After this, 50 μL starch solution (1% starch in 0.02 M phosphate buffer solution) was added to all the test tubes and kept for incubation at 25 °C for 10 min. Then, 100 μL 3.5-dinitrosalicylic acid (DNSA) was added to each of the test tube and incubated again in boiling water for 5 min to stop the

s. ODcontrol = ODsample x 100

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reaction. The test tubes were diluted to 350 μL by adding distilled water. Their absorbance was recorded using the Multi-Mode Reader at 540 nm. The % inhibition of α-glucosidase activity was calculated using same formula used in α-glucosidase inhibition method. The results, % inhibition as mean ± SD and IC50 values are shown in supporting information (S2) and Table 1 respectively.

2.4.2. Antioxidant activity

Antioxidant activities of all the hydroxytriazenes were screened by DPPH and ABTS radical scavenging method (Tripathi et al., 2013). The quantification of radicals was done using Multimode Reader with 2.5 mL DPPH solution. After shaking vigorously, the mixture was incubated at room temperature in the dark place for 30 min, the absorbance was measured at 517 nm. The % DPPH scavenging effects of the drugs were estimated in terms of percent inhibition and procedure was repeated at 2, 3 and 4 h after carrageenan injection.

2.4.3. Anti-inflammatory activity

Anti-inflammatory activity of the compounds was determined by carrageenan induced paw edema inhibition method in rats as reported in literature (Winter et al., 1962). In this method, Male or female Wistar albino rats with a body weight between 150 to 200 g were used. The animals have been starved overnight with free access of water. Thirty minutes after the oral administration of the compounds, the thickness of right hind paw was measured by a plethysmograph. Carrageenan (0.1 mL of 1%) was injected subcutaneously into the plantar surface of the right hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. One hour later the volume of the edema was measured again and the antiedematous effects of the drugs were estimated in percent inhibition and procedure was repeated at 2, 3 and 4 h after carrageenan injection.

Wister albino rats (150–200 g) were obtained from the animal house B.N. College of Pharmacy, Udaipur (Registration No. 870/ac/08/ CPCSEA) and Protocol No. is 52/ACR/BNCP-10/IAEC. They were housed at temperature of 25 ± 2 °C for 12 h, light-dark cycle at 40–60 % humidity, in polypropylene cages and feed a standard rodent diet with water and libitum. Animal were deprived of food but not water 4 h before experiment.

The rats were divided into 21 groups (n = 6) total 126 animals were used, each receiving distilled water (control), diclofenac 12.5 mg/kg p.o. (reference standard) and test compounds groups (ST-1 to 6, SF-1 to 6 and SM-1 to 6) at a dose of 100 mg/kg, bw, p.o. A 1% solution of carrageenan (0.1 mL/kg, p.o.) was injected into subplantar tissue of the right hind paw of each rat. The paw volume at different time intervals was recorded using plethysmometer and compared with control and the percentage inhibition was calculated by this formula.

\[
\text{Anti-inflammatory activity} \% = \left[ \frac{(V_t - V_c) - (V_t - V_o)}{(V_t - V_o)_{\text{carrageenan}}} \right] \times 100
\]
V₀ and Vᵣ indicate volume of hind paw edema of at 0 and 1, 2, 3, 4 h after injection of carrageenan respectively. Comprehensive data of study (supporting information (SS)) and results as % inhibition are described in Table 1 and Figures 3, 4, 5.

2.5. Statistical analysis

The data were recorded as mean ± standard error mean (S.E.M). The significance of variance of the groups was calculated using one way and multiple way analyses of variance (ANOVA). The test followed by Dunnett’s test and P values less than 0.01 were noted as significance (Mahajan 1989).

3. Results and discussion

3.1. Anti-diabetic activity

One therapeutic approach for treating diabetes is to decrease post-prandial hyperglycemia. This is done by hindering the absorption of glucose through inhibition of the carbohydrate hydrolysing enzymes particularly α-amylase and α-glucosidase in the digestive tract. Results of the activity were expressed as IC₅₀ values. Acarbose has been used as reference drug, which have IC₅₀ values 12.21 and 69.74 μg/mL for α-glucosidase and α-amylase enzymes, respectively.

In α-glucosidase activity the most active hydroxytriazene was SM-1 (IC₅₀ = 160.7 μg/mL) followed by SM-2 (IC₅₀ = 201.99 μg/mL) and ST-2 (IC₅₀ = 219 μg/mL), SM-3 (IC₅₀ = 222.91 μg/mL). Compounds ST-6 (IC₅₀ = 314 μg/mL), SM-5 (IC₅₀ = 314.43 μg/mL) and SF-6 (IC₅₀ = 329 μg/mL), ST-5 (IC₅₀ = 341 μg/mL) were showed the lowest activity. Other samples displayed medium range of α-glucosidase activity. In α-amylase activity the most active hydroxytriazene was SM-2 (IC₅₀ = 122.17 μg/mL) followed by SF-3 (IC₅₀ = 148.08 μg/mL) and SM-6 (IC₅₀ = 164.62 μg/mL), SF-1 (IC₅₀ = 172.65 μg/mL), SF-5 (IC₅₀ = 219 μg/mL), ST-2 (IC₅₀ = 231.35 μg/mL) and SF-6 (IC₅₀ = 245.55 μg/mL), ST-6 (IC₅₀ = 326.89 μg/mL) showed the lowest activity. Other samples displayed medium range of α-amylase activity.

3.1.1. SAR studies

To look at the structure-activity relationship (SAR) of the novel hydroxytriazenes incorporated sulfonamide drugs moiety, the variations were determined on one end of hydroxytriazene moiety with alkyl/aryl alkyl groups. On the other hand sulfonamide group with five membered heterocyclic of methyl substituted thiazole and isoxazole rings were substituted. Among the three series, compound SM-1 seems to be more favourable inhibitor towards the α-glucosidase enzyme due to the presence of small methyl substituents at the nitrogen atom of the hydroxytriazene and isoxazole ring. The increment of alkyl carbons from methyl to ethyl & propyl (compounds ST-3,4, SF-3,4 & SM-3,4) on the hydroxytriazene led to decrease the α-glucosidase enzyme inhibition activity by moderate level. The potency of α-glucosidase enzyme inhibition drastically reduced while increasing the bulky groups from methyl to phenyl ring at the hydroxytriazene moiety (compounds ST-5,6, SF-5,6 & SM-5,6). This indicated that only smaller alkyl substituents are tolerable at this region. Concerning the enzyme inhibition towards the α-amylase, compounds with mono and dimethyl substituents of isoxazole moiety at sulfonamide group showed better enhancement of activity (compounds SF-3,4, SM-2,4 & 6) than the thiazole nucleus placed at the same position. The hydroxytriazenes integrated sulfonamide series exhibited moderate inhibition against the both α-glucosidase and α-amylase enzymes when compared with standard acarbose drug. This preliminary SAR investigation gave a structural requirement guidance to further lead optimisation of the series against these enzymes.

Recent advances have developed the newer strategies in management of diabetes by hindering the activity of intestinal enzymes particularly α-amylase and α-glucosidase, both play important role in carbohydrate digestion by degrading starch and oligosaccharides to monosaccharides before they can be absorbed and glucose absorption. Suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation (Kim et al., 2005). Alpha-glucosidase and α-amylase inhibitors inhibit the digestion of carbohydrates and slows down the absorption. Acarbose, voglibose, miglitol etc. are well known drug used in the treatment of diabetes mellitus by inhibiting the digestive enzymes in intestines (Derosa and Maffoili, 2012). However, this drug has some unwanted effects such as abdominal or stomach pain, diarrhea and flatulence (Agu et al., 2019). On the other hand, synthetic compounds possessing antioxidant proprieties have much less side effects and are thus being preferred. Thus, there is need to explore compounds having antioxidant properties for the control and treatment of diabetes.

In our present study, we have examined the in-vitro antidiabetic activities of the 18 hydroxytriazenes by α-amylase and α-glucosidase enzyme inhabitation methods and comparing them with the standard drug acarbose. From Table 1, we can see that all the compounds have shown significant inhibition of α-amylase and α-glucosidase enzymes. Comparison of results of both the activities revealed that hydroxytriazenes are stronger inhibitor for α-amylase than α-glucosidase enzyme. The mechanisms involved in inhibition of the enzymes by hydroxytriazenes might be due to interaction with proteins of enzymes.

3.2. Antioxidant activity

Antioxidant tests using free radical traps are relatively straightforward to perform. Among free radical scavenging methods available, the DPPH and ABTS methods are rapid, simple, highly reproducible and inexpensive in comparison to other models (Gulcin, 2020). Both methods are based on the reduction of DPPH and ABTS radical solution in the presence of hydrogen donating antioxidants, due to the formation of the non-radical form DPPH-H (Figure 2) and ABTS-H.

The synthesized compounds were able to reduce the stable radical DPPH (purple to yellow) and ABTS (blue-green to colourless), in a concentration-dependent manner. Results were expressed as IC₅₀ (Table 1) indicating all the synthesized compounds showed a fair DPPH and ABTS scavenging activity, as compare to ascorbic acid which was used as standard.

In this study two methods DPPH and ABTS assay are used, both methods are technically simple. It has been observed that IC₅₀ values are lower in DPPH assay in comparison to ABTS assay. This may be attributed to difference in redox potential and slow reaction of DPPH radicals because it is a long lived nitrogen radical, which is not reactive like peroxyl radicals involved in lipid peroxidation. This would result in result in low readings for antioxidant capacity of samples (Bondet et al., 1997).

The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with a standard. The methods is rapid and can be used over a wide range of pH values, in both aqueous and organic solvent systems. It also has good repeatability and is simple to perform; hence, it is widely reported.

In DPPH scavenging activity the most active hydroxytriazene was SM-2 (IC₅₀ = 367.17 μg/mL) followed by SF-5 (IC₅₀ = 423 μg/mL) and SM-1 (IC₅₀ = 426 μg/mL), ST-3 (IC₅₀ = 662 μg/mL), SM-5 (IC₅₀ = 660 μg/mL) and ST-6 (IC₅₀ = 858 μg/mL) showed the lowest activity. Other samples displayed medium range of DPPH scavenging activity. In ABTS scavenging activity the most active hydroxytriazenes were ST-6 (IC₅₀ = 48.09 μg/mL), ST-4 (IC₅₀ = 54.217 μg/mL) and ST-1 (IC₅₀ = 54.13 μg/mL) followed by SF-6 (IC₅₀ = 70 μg/mL), SM-5 (IC₅₀ = 81.50 μg/mL), SF-4 (IC₅₀ = 88.14 μg/mL) and SF-1 (IC₅₀ = 89.032 μg/mL), SM-2 (IC₅₀ = 401.72 μg/mL), SM-3 (IC₅₀ = 282.32 μg/mL), SM-6 (IC₅₀ = 250.26 μg/mL), SM-1 (IC₅₀ = 252.96 μg/mL) and ST-2 (IC₅₀ = 232.39 μg/mL) showed the lowest activity. Other samples displayed medium range of ABTS scavenging activity.
3.2.1. SAR studies

In particular, DPPH scavenging activity, only SM-2 demonstrated the moderate activity among the series. The activity fully based upon the presence of ethyl and methyl isoxazole moieties at the both end of the hydroxytriazenes integrated sulfonamide scaffold. The other substituents like phenyl, tolyl, propyl and methyl made the compounds less active. Therefore, analogue SM-2 could be considered as a lead compound for further development for the DPPH scavenging activity. But in the ABTS scavenging activity, thiazole containing three compounds ST-1, ST-4 and ST-6 exhibited the best activity than the isoxazole containing compounds, when compared with ascorbic acid drug. In the thiazole installed series, compounds with more hydrophobic aryl/alkyl groups at triazenyl (compound ST-6) displayed high ABTS scavenging activity. In the dimethyl isoxazole substituted derivatives, three compounds such as SF-1, SF-4 & SF-7 showed equipotent activity with the standard ascorbic acid drug. Except compound SM-5, the remaining compounds in the monomethyl isoxazole substituted derivatives showed lesser activity possibly due to reducing the nonpolar methyl at the isoxazole ring. Therefore, the thiazole fragment in the ST series important for enhancement of ABTS scavenging activity as well as for further structural optimization.

3.3. Anti-inflammatory activity

Synthesized hydroxytriazenes and marketed formulation of diclofenac sodium produced dose-dependent inhibition of carrageenan-induced paw edema as compared to the control and the values are reported in terms of % inhibition in Table 1 and Figures 3, 4 and 5. These results indicated that all hydroxytriazenes with a dose of 100 mg/kg, bw, p.o. shows a significant anti-inflammatory activity as compared to the reference drug diclofenac sodium.

The inflammation in animals is a protective response to a cell injury. Edema, erythema, hyperalgesia, pain and loss of function at microscopic level are the clinical signal of injury. The non-steroidal anti-inflammatory drug (NSAID) normally works at the periphery and not at CNS. NSAID block the production of cytokines and cyclooxygenase pathway (COX) by hindering synthesis of eicosanoids at injured tissue and thereby reducing the production of prostaglandins (Vane and Botting, 1998) and inhibiting release of histamine (Han et al., 2012). Thus the tentative mechanism of action demonstrated by hydroxytriazenes is proposed to be NSAID like.

3.3.1. SAR studies

Hydroxytriazenes SM-1, SM-3, SM-4 and SM-6 particularly from SM series showed excellent activity comparable to standard diclofenac (52%) after 2 h of treatment. Compounds having alkyl chain substituted derivatives particularly ST-3(82.28%), SF-1(77.42%), SM-2(88.85%) and SM-6(88.57%) showed excellent anti-inflammatory activity after 4 h of treatment compared with their phenyl or substituted phenyl analogues. It has been observed that presence of alkyl substituent linked to triazene moiety may enhance the anti-inflammatory activity. Compounds having monomethyl isoxazole unit at sulfonamide nucleus showed superior potency among the screened compounds. In the case of SM series, the percentage of inhibition of inflammation values were ranged from 80.28 % to 88.85 % except the compound SM-5. The other series of (ST-1 to 6 & SF-1 to 6) having di-methyl isoxazole and thiazole substituents at the sulfonamide nucleus resulted in moderate anti-inflammatory activity. The percentage inflammation reducing activity ranging from 60.57 to 77.57 %. Hence molecules having monomethyl isoxazole unit represent promising leads for the development of novel class of potent anti-inflammatory agents. The significant high anti-inflammatory activity of hydroxytriazenes may be due to the inhibition of the mediators of inflammation such as histamine, serotonin and prostaglandin.

3.4. Molecular docking

To investigate the binding mode of the sulphur drug based hydroxytriazenes derivatives further, *In silico* molecular docking was performed for

![Figure 2. Conversion of DPPH* (purple) to its corresponding hydrazine form (yellow) by the addition of synthesized compounds to DPPH* due to proton transfer.](image)

![Figure 3. Anti-inflammatory activity of ST-series of hydroxytriazenes with % Inhibition of paw edema.](image)
active compounds SF-1 and SM-1 to determine their binding affinity in to the C-terminal subunit (PDB: 3TOP) of human maltase-glucamylase of the α-glucosidase enzymes (Ren et al., 2011). The enzymes X-ray crystal structure coordinates and the docking method were estimated before docking the SF-1 and SM-1 by replicating the bound conformation of acarbose in to the corresponding enzymes active site. Docking simulations of these molecules was accomplished by using the Molecular Operating Environment (MOE 2015.1001) software package (Chemical Computing Group Inc. 2016). The triangle matcher placement technique followed by rigid receptor protocol was selected to search and generate the good fit conformers in the active site of the enzymes. We used GBVI/WSA dG scoring function to determine the binding energy values.

Figure 4. Anti-inflammatory activity of SF-series of hydroxytriazenes with % Inhibition of paw edema.

Figure 5. Anti-inflammatory activity of SM-series of hydroxytriazenes with % Inhibition of paw edema.

Figure 6. 3D (Panel A) and 2D (Panel B) structural representations of binding mode of compounds SF-1 (pink colour) and SM-1 (brown colour). Both molecules are shown as a ball and stick model in the active site of C-terminal subunit of human maltase-glucamylase of α-glucosidase.
of the best protein-ligand complex (pose). Molecular modelling showed that, SF-1 and SM-1 molecules have privileged binding affinity scores of 6.61 and 6.68 kcal/mol towards the active site of C-terminal of maltase-glucoamylase, respectively. As shown in Figure 6, both docked compounds showed similar binding manner inside the pocket of the enzyme. Theoretically, the hydroxyl group which is attached at the triazene moiety have entirely projected towards the solvent interface of the enzyme. It revealed that restriction for lengthening at this triazene position is essential. The sulphonamide oxygen make a hydrogen bonding interaction with the Arg1510 residue by the amino group of guanidine. The phenyl ring in SF-1 and SM-1 molecules primarily formed an arene-H interaction with the Arg1510 residue by the amino group of guanidine. The synthesized compounds showed similar binding manner inside the pocket of the enzyme.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

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