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Hybrid compounds from thiosemicarbazone and triazole as antidiabetic agents and their antioxidant potentials.

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Abstract

Diabetes mellitus is a metabolic disease that threatens and reduces the quality of life. Eight hybrids (1a-h) of thiosemicarbozone and triazole were screened for their effects on genes related to type 2 diabetes as well as their antioxidant activity. The influence of the hybrids on glucose transport genes (*Glut-4*, *Mef2a* and *Nrf-1*) was carried out using quantitative real time polymerase chain reaction (PCR). Antioxidant assays were carried out using established techniques. Hybrids 1b, 1d, 1e and 1g exhibited high expression of *Glut-4* gene relative to insulin and control. All the hybrids tested except 1h and 1f expressed the *Nrf-1* while only 1h did not express *Mef-2a* relative to control. Among all the compounds, 1b showed the highest 1-diphenyl-2-picryl- hydrazyl (DPPH) radical scavenging ability and Trolox Equivalent Antioxidant Capacity (TEAC) values. In terms of Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC), 1c and 1d had the highest values, respectively. In all the antioxidant assays carried out, 1a was shown to have the lowest antioxidant activities. Hybrids 1d and 1g showed consistent pattern of glucose transport pathway gene transcription with all the hybrids showing antioxidant potentials though at varying extents. These hybrids could be potential candidates eliciting antidiabetic and antioxidant effects.

Keywords: Thiosemicarbazone, Triazole, Free radical, Oxidative stress, Antioxidant, Diabetes.

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Introduction

Diabetes mellitus is a metabolic disorder in which a combination of hereditary and environmental factors results in abnormally high blood sugar levels [1] and it is associated with absolute or relative deficiency in insulin secretion or insulin action [2,3]. Diabetes is characterized by hyperglycemia, lipoprotein abnormalities, raised basal metabolic rate, defect in enzymes, and high oxidative stress which induces damage to pancreatic beta cells [1]. It is a common disorder that affects people at all ages which include children, adolescents and young adults, and also sometimes in older people [4,5]. The increasing occurrence of diabetes around the world constitutes a global public health problem and it is predicted that by 2025, the world will have an increase of 72% of diabetic patients [4]. Diabetes is divided into two types; i) type 1 diabetes, which accounts for less than 10% of all diabetic cases and results from the autoimmune destruction of beta cells with patients depending on exogenous insulin and, ii) type 2 diabetes, which affects about 90% of all people with diabetes and is characterized by defects in insulin secretion and action [6].

Free radicals belong to a group of reactive oxygen species (ROS) [7] which are unstable, short-lived and have one or more unpaired electrons [8]. They attack important cellular macromolecules such as protein, lipid and DNA and cause cell damage [9]. Antioxidants help to scavenge free radicals in the body [10] and act through inhibiting initiation and generation steps that leads to termination of the chain reaction and delay the oxidation process [11]. They exhibit their antioxidant activities by donating hydrogen atoms or the single-electron transfer to a radical [12,13]. Antioxidant systems, both enzymatic and non-enzymatic help to balance ROS in order to keep their level constant in living organisms and an imbalance

by over production of ROS and/or reduction of antioxidants leads to oxidative stress [14]. Oxidative stress, an imbalance between the production of free radicals and the ability the body's antioxidant system to fight back, has been implicated in the pathogenesis of many chronic diseases. Antioxidants alleviate oxidative stress, the adverse effects of free radical [15] and reportedly help in slowing down aging process and fight diseases such as diabetes mellitus, hypertension and cancer [16,17].

Semicarbazone is synthesized by the condensation of semicarbazide and aldehyde/ketone [18]. Thiosemicarbazone is an analog of a semicarbazone which contains a sulphur atom in place of the oxygen atom. A considerable number of thiosemicarbazones derivatives have been shown to have a wide spectrum of biological activities which include antidiabetic [19], antiviral [20], anticancer [21], antibacterial [22,23], antifungal [22], and antimalarial [24] activities. Triazoles are heterocyclic compounds featuring five member ring of two carbon atoms and three nitrogen atoms as part of the aromatic five-member ring with molecular formula $C_2H_3N_3$ [25]. Triazole derivatives have also been reported to have antidiabetic [19], antifungal [26,27], antibacterial [26], anticancer [28,29], antimalaria [30], and anti-inflammatory [31] benefits.

The semicarbazone is an electron withdrawing group and exhibits antioxidant activity and its favourable substitution may increase its free radical scavenging effect [32,33]. The synthesis of high nitrogen containing heterocyclic systems has been attracting increasing interest over the past decade because of their utility in various applications [25]. Novel therapeutic strategies support the use of the ROS scavengers, chelators of transition metal ions or modulators of a cell signaling for the treatment of diseases [34-36]. In our previous studies, the anti-malaria [37] and anti-obesity [38] activities of these newly synthesized hybrid compounds have been investigated. Currently, drugs in use to treat or manage type 2 diabetes which include metformin and the glitazones i.e. rosiglitazone have several negative side-effects [39,40]. Metformin, for instance, is associated with gastric disturbances while rosiglitazone is known to increase the risk of heart attack and stroke by as much as 43% [41]. There is therefore an urgent need for better and new classes of drugs that can possibly treat or better manage the disease than those currently in use. The present study was carried out to assess the influence of the hybrids from thiosemicarbazone and triazole on genes related to type 2 diabetes as well as investigating their antioxidant potentials.

Materials and Methods

Synthesis of hybrid compounds

The hybrids (**1a-h**) were synthesized according to a published protocol as outlined in Scheme 1 [37,38]. Briefly, the synthesis started with alkylation of commercially available 4-hydroxybenzaldehyde with propargyl bromide in the presence of K_2CO_3 to give 4 which incorporates an alkynyl group which is required for click chemistry. Compound **4** was subjected to click chemistry with freshly prepared benzyl azide to give 1,4-disubstituted triazole **5**. Methylhydrazinecarbodithioate **7**, that was prepared in a one pot synthesis from the condensation of hydrazine monohydrate and methyl iodide reacted with triazole **5** under Schiff's base condensation reaction conditions to produce compound **8** as presented in Scheme 1 and this went through nucleophilic substitution reactions with a series of primary amines to produce the hybrid compounds (**1a-h**) as shown in Table 1. The reagents and conditions which are involved in the synthesis include (i) K_2CO_3, propargyl bromide, acetone, reflux, 2.5 h, 92%; (ii) BnN_3, $CuSO_4.5H_2O$, sodium ascorbate, DMF: H_2O (4:1), 60°C, 3 h, 80%; (iii) CS_2, KOH, CH_3I, H_2O: isopropanol (1:1), RT, 4 h, 90%; (iv) 5, 7, MeOH, reflux, overnight, 89%; (v) RNH_2, MeOH, reflux, 24 h, 62-85% (Figure 1).

Table 1. *Series of primary amines that provided the new hybrid compounds. The hybrid compounds were labelled 1a-1h as shown in the table below.*

Hybrid Compounds	Amines (R-NH_2)
1a	$PhCH_2NH_2$
1b	$CH_3CH_2CH_2CH_2CH_2NH_2$
1c	$PhCH_2CH_2NH_2$
1d	$(CH_3)_2NCH_2CH_2NH_2$
1e	$HOCH_2CH_2CH_2CH_2CH_2CH_2NH_2$
1f	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2NH_2$
1g	$CH_3CH(CH_3)CH_2NH_2$
1h	$HOCH_2CH_2NH_2$

Cell culture protocol

In the study, 3T3-L1 adipocytes cell lines (mouse) were cultured using Dulbecco's modified Eagles medium (DMEM) (GIBCO, USA) supplemented with 10% Foetal calf serum (BioWest, France) and 1% penicillin/streptomycin/fungizone (GIBCO, USA) at 37°C with 5% CO_2 and 95% humidity. Cells were maintained in continuous passage by trypsinization of subconfluent cultures with Trypsin/Versene (Highveld, RSA). Differentiation was induced by introduction of medium containing 2% Foetal calf serum and 2% penicillin/streptomycin/fungizone when pre-adipocytes were 80% confluent. Cells were kept in this medium for 5 days until adipocytes were well formed. The resultant adipocytes were then treated with 5 µl of the hybrid compounds (10 mg/ml in DMSO) for 4 days. After 4 days treatment, the medium was changed with non-supplemented DMEM medium, incubated for 4 hours.

Quantitative real-time PCR

Total RNA was extracted from the cells using QIAzol lysis reagent (QIAGEN Sciences, USA) and RNA clean and Concentrator-25 (Inqaba Biotech, SA). Double stranded cDNA

was synthesized from 3 µg of total RNA using Superscript Reverse Transcriptase III (Invitrogen, USA). Real time PCR performed in triplicate using Rotor gene-3000 Quantitative real-time PCR machine using Sensi Mix SYBR No-ROX One-Step Kit (Bioline, UK). Primers used are mouse *Glut-4* gene (Forward primer- 5' GCA GCG AGT GAC TGG AAC A 3'; Reverse primer- 5'CCA GCC ACG TTG CAT TGT AG 3'), *Nrf-1* gene (Forward primer- 5' AAA CAC AAA CTC AGG CCA CC 3'; Reverse primer-5' CCA TCA GCC ACA GCA GAG CA 3') and *Mef2a* gene (Forward primer-5' GTG TAC TCA GCA ATG CCG AC 3'; Reverse primer-5' AAC CCT GAG ATA ACT GCC CTC 3'). The amplification occurred in a three-step cycle: denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec and extension at 72°C for 15 sec. Relative mRNA expression normalized to mouse Actin reference gene (Forward primer- 5' GAG ACC TTC AAC ACC CCA GCC 3'; Reverse primer- 5' GGA GAG CAT AGC CCT CGT AG 3') and calculated according to relative standard method.

In vitro glucose uptake in 3T3-L1 adipocytes

To assess glucose uptake, the fully differentiated adipocytes were exposed to the respective representative compounds (**1c, 1f, 1g, 1h**) at lower concentrations of 0.05, 0.5, 5 and 50 µg/ml. Insulin (1 µM) was included as a positive control and metformin (1 µM) as a reference drug control. The vehicle control containing 0.01% DMSO was also included and plates were incubated for 3 hours. Since **1g** was the compound that exhibited the highest gene expression in our previous publication [38], we assessed glucose transport using fewer selected compounds including **1g** where highest gene expression was observed with glucose transport genes. After incubation with these compounds, glucose uptake in 3T3-L1 adipocytes was determined using pulse-labeling with [3H]-2-deoxy-D-glucose (3H-2-DOG) in glucose-free DMEM containing the extracts for 15 min.

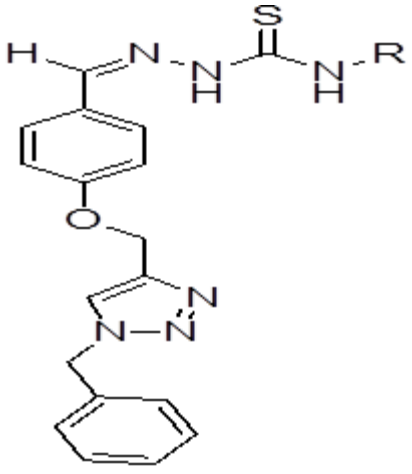


Figure 1. *Chemical structure of the thiosemicarbazone-triazole hybrid compounds. R``-represents the various alkyl and aryl groups as listed in Table 1.*

Liquid Scintillation counting for glucose uptake

Liquid scintillation counting (2200 CA, ParkardTricarb, USA) was used to measure intracellular 3H-2-DOG. Activity of the compounds were determined as fmol (3H-2-DOG CPM/mg protein) and expressed below as 100% relative to the DMSO control. Insulin and metformin were expressed as % relative to the media control.

ABTS radical scavenging activity

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was done according to a method described by Re *et al.* [42]. ABTS+ solution was prepared a day before use by mixing ABTS salt (8 mM) with potassium persulfate (3 mM) and kept in the dark. The ABTS+ solution was further diluted with distilled water before use. Twenty five microlitres (25 µl) of the sample was mixed with 300 µl ABTS+ solution in a 96-well clear microplate. The plate was read after 30 min incubation at room temperature in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) at 734 nm in triplicates. Trolox was used as the standard and result expressed as µmol TE/g sample.

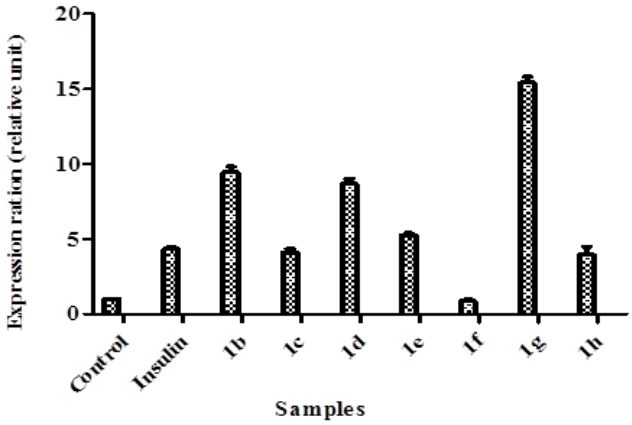


Figure 2. *Glut-4 gene expression in response to treatment with the hybrids.*

Ferric reducing antioxidant power assay

The FRAP assay was carried out using the method described by Benzie and Strain [43]. Briefly, 10 µl of the sample was mixed with 300 µl FRAP reagent in a 96-well clear plate. The FRAP reagent was a mixture (10:1:1, v/v/v) of acetate buffer (300 mM, pH 3.6), tripyridyl triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3.6H_2O$ (20 mM). After incubation at room temperature for 30 min, the plate was read at a wavelength of 593 nm in a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) in triplicates. Ascorbic acid (AA) was used as the standard and the result expressed as µmol AAE/g sample.

DPPH radical scavenging activity assay

DPPH free radical scavenging activity was conducted using a method described by Zheleva-Dimitrova [44] with slight modifications. Briefly, 10 µl of the sample was reacted with 190 µl of DPPH solution in a 96-well clear plate. The sample

was left for 30 min at room temperature and the plate was read using a Multiskan Spectrum plate reader (Thermo Fisher Scientific, USA) at 517 nm in triplicates. Free radical scavenging activity of the samples was expressed according to the equation below.

$$Percent(\%)\ inhibition\ of\ DPPH\ activity = \frac{A^0 - A}{A^0} \times 100$$

where A^0 is the absorbance of DPPH• in solution without an antioxidant, and A is the absorbance of DPPH.

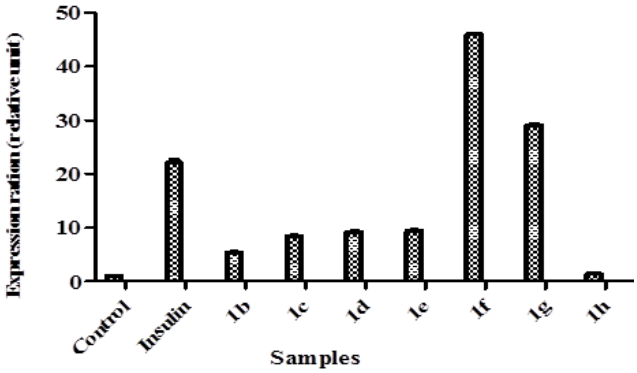


Figure 3. *Mef2a gene expression in response to treatment with hybrids.*

Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out according to the method of Ou et al. [45] using a fluorescence plate reader (Thermo Fisher Scientific, Waltham, Mass., USA). The reaction consisted of 12 µl of the samle and 138 µl of fluorescein (14 µM), which was used as a target for free radical attack. The reaction was initiated by the addition of 50 µl AAPH (768 µM) and the fluorescence (emission 538 nm, excitation 485 nm) recorded every 1 min for 2 hours in triplicates. Trolox was used as the standard and the result expressed as µmol TE/g sample.

Results and Discussion

Glut-4 is an insulin-responsive glucose transporter that regulates glucose uptake [46,47]. *Glut-4* transports glucose from blood to the cells thereby maintaining glucose homeostasis (euglycemia). Some reports have indicated that *Glut-4* levels are low in diabetes mellitus [48-52] hence, up-regulation of *Glut-4* transcription is seen as a means to alleviate type 2 diabetes [53-56]. *Glut-4* gene is regulated by myocyte enhancing (*Mef2*) transcription factor which binds to its cis-elements as a hetero-dimer (*Mef2a*/D) resulting in *Glut-4* expression [55,56]. Recent studies showed that *Glut-4* expression is also regulated by nuclear respiratory factor (*Nrf-1*), a mitochondrial transcription factor, which controls *Mef2a* gene to regulate *Glut-4* [57,58]. Although exercise and diet can potentially protect individuals from type 2 diabetes, the prevalence of type 2 diabetes continues to rise worldwide driving a need for new more effective therapeutics in the management or treatment of the disease [59]. These could include therapeutics that mimic the induction of genes comparable to those responses brought about by regular exercise. Thus, owing to their essential roles in blood sugar metabolism, these genes have become targets for pharmacological therapeutics in the management and treatment of type 2 diabetes.

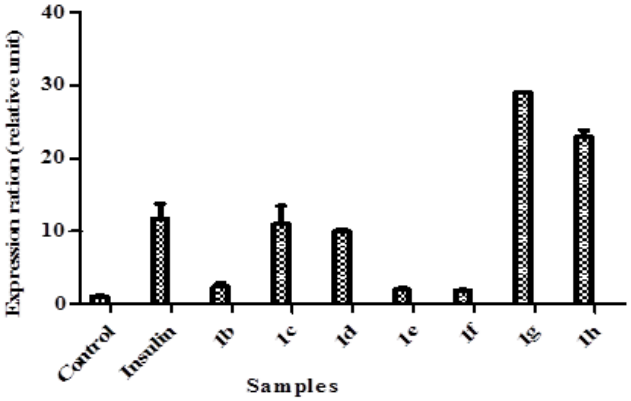


Figure 4. *Nrf-1 gene expression in response to treatment with the hybrids.*

After successful synthesis of hybrids **1a-h**, we investigated their effect on *Glut-4* gene expression in 3T3-L1 adipocytes using quantitative real time PCR (qPCR). Expression of *Glut-4* gene in relation to test compounds was compared with that of insulin as a standard drug that induces *Glut-4* gene expression. Upon treatment with 1a, the cells lifted from the plate hence, they could not be used for RNA extraction. As shown in Figure 2, it is evident that **1b**, **1d**, **1e** and **1g** exhibited high expression of *Glut-4* gene relative to insulin and control. **1g** showed a more pronounced increase (4 fold of the insulin and 15 folds of the control) followed by **1b** and **1d** with 2 folds of the insulin under the experimental conditions employed. On the contrary, **1f** did not exhibit any influence on *Glut-4* gene expression. The *Glut-4* gene is now seen as one of the potential targets for therapeutic modalities aimed at alleviating type 2 diabetes because it is the rate-limiting step in glucose transport and maintenance of normal blood glucose levels (euglycemia). Increase in *Glut-4* gene in this study by these hybrid compounds more than insulin that is usually used to treat diabetes has huge potential in the treatment and management of diabetes.

Hybrids **1b-h** were further investigated if they can express *Mef2a* gene which is the transcription regulator of *Glut-4*. Interestingly, all except **1h** expressed the *Mef2a* (Figure 3) relative to control. The effect of **1f** was almost 50 folds higher than the control while **1g** displayed 31 folds more expression. The increase in *Mef2a* followed the same pattern observed with that seen with the *Glut-4* gene. These results further reinforce systematic increase in the transcriptional cascade involved in glucose transport mechanisms. Deletion of the *Mef2a* binding sites in the *Glut-4* gene has shown to abolish *Glut-4* transcription indicating the importance of *Mef2a* in glucose transport. The consistent increase of both *Glut-4* and *Mef2a* by these compounds shows they can be potential agents in mechanisms involved in improving glucose transport. To

ascertain further the increases observed above with *Glut-4* and *Mef2a* genes, we assayed the effects of the hybrids on *Nrf-1* and the results showed that **1c**, **1d**, **1g** and **1h** greatly stimulated *Nrf-1* expression compared to control (Figure 4) with **1g** and **1h** even stimulating more than insulin. As it is evident from the results, the consistent higher stimulating effect of **1g** followed by **1d** on the three genes suggests that the presence of a non-polar short-branched chain of the amine moiety might be important in the up-regulation of *Glut-4*, *Mef2a* and *Nrf-1* genes. The increase in *Nrf-1* also strengthens the increases in *Glu-4* and *Mef2a*. Reports have shown that *Nrf-1* binds and controls *Mef2a* which in turn regulate *Glut-4*. This means *Nrf-1* regulate *Glut-4* indirectly by controlling *Mef2a*.

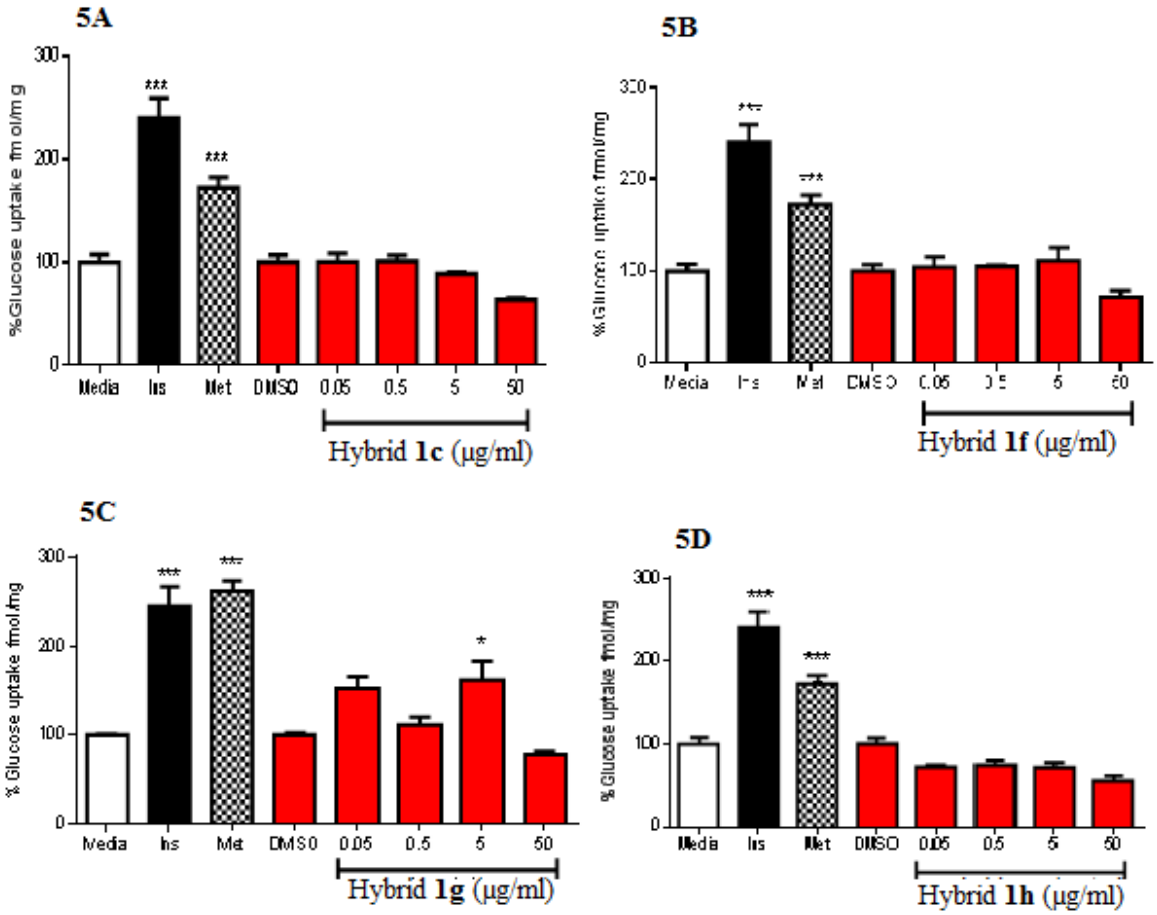


Figure 5. *Glucose uptake in 3T3-L1 adipocytes using pulse-labeling with [3H]-2-deoxy-D-glucose (3H-2-DOG) in glucose-free DMEM containing the compounds for fifteen minutes. The graph shows glucose uptake activity in 3T3-L1 adipocytes exposed to compounds 1c (A), 1f (B), 1g (C), 1h (D), at different concentrations over three (3) hours. The mean percentage ± SEM is expressed relative to the control at 100%. Statistical analysis ANOVA with Dunnet's post hoc test; (*p ≤ 0.05, and ***p<0.01, respectively).*

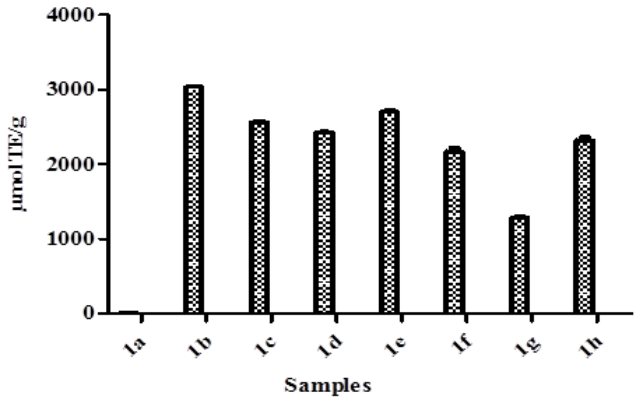


Figure 6. *ABTS radical scavenging activity of the hybrids.*

From the observed increased transcription of the genes involved in glucose transport metabolism, we decided to investigate if this would have any influence on the functional assays to determine if the observed increased in gene expression corresponds to improved glucose transport. This is because gene transcription does not always translate to enhanced functionality of a particular gene. Results from the assay indicated that only **1g** showed increased glucose transport (Figure 5). Using lower concentrations of the hybrids, the increased glucose transport induced by **1g** corresponded with the observed gene transcription with *Glu-4*, the major glucose transporter in skeletal and adipose tissues. The positive controls, metformin and insulin also increased glucose transport as expected. These are very promising results warranting further investigation on the mechanisms through which this compound (**1g**) enhances gene transcription of

genes involved in glucose transport concomitantly with corresponding glucose transport in these cells. However, other tested compounds did not show increase in glucose transport in relation to the gene transcription (Figures 2 and 5).

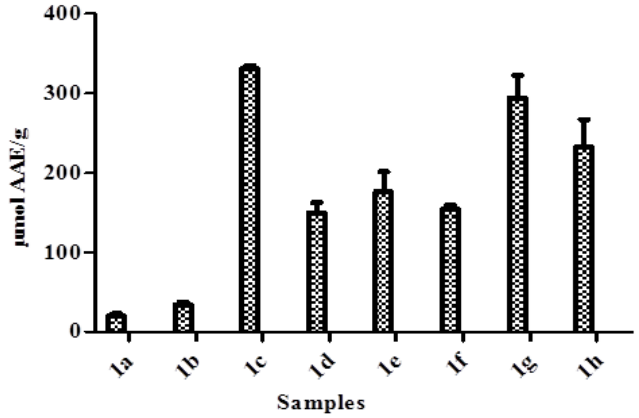


Figure 7. Ferric reducing antioxidant power of the hybrids.

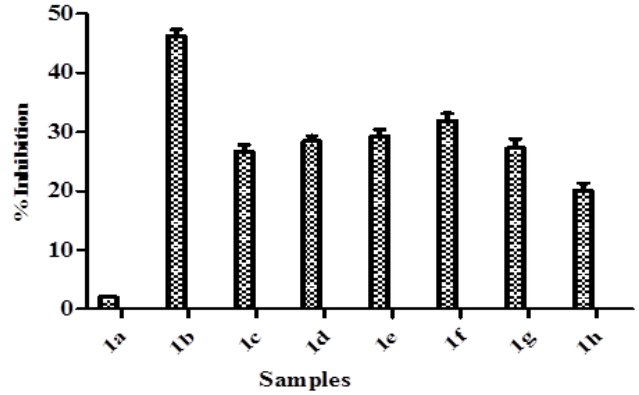


Figure 8. DPPH radical scavenging activity of the hybrids.

ROS are produced during both normal and altered cellular metabolism and they include singlet oxygen (oxygen molecule in the electronically excited state), superoxide anion radical, peroxy radical, alkoxy radical, hydrogen peroxide [60-62]. ROS can both be beneficial and harmful to the physiology of cells [62]. As previously mentioned, excessive production of ROS over the body antioxidant system results in the destruction of the cell components and causes oxidative stress. Oxidative stress has been implicated in a wide array of diseases which include inflammation, neurodegenerative disorders (Alzheimer's disease, Parkinson's disease), atherosclerosis, diabetes and cancer [62,63]. Scavenging of free radicals is one of the well-recognized mechanisms by which antioxidants inhibit lipids and other biomolecules oxidation [13].

ABTS method involves a technique that produces a blue/green ABTS+ chromophore through the reaction of ABTS and potassium persulfate [7,64]. It is dependent on the inhibition of the absorbance of radical cation ABTS [65]. The decolorization of ABTS radical in a sample shows the capacity of an antioxidant to donate electron or hydrogen atoms to inactivate the radical cation [66,67]. In this study, the results indicated that all the hybrid compounds had ABTS radical scavenging activities. In this study, the hybrid compound **1e** was the highest while **1a** was the lowest (Figure 6). These results confirmed the potency of the hybrid compounds to protect against oxidative damage. FRAP assay is a novel method for the assessment of antioxidant power where the ferric reducing ability of samples are investigated [68]. The reduction of ferric to ferrous ion at low pH generated coloured ferrous-tripyridyltriazine complex. This method is based on the reaction between antioxidant potentials and Fe^{3+}-TPTZ complex to produce blue color Fe^{2+}-TPTZ form [69]. In this study, all the hybrids compound showed FRAP potentials through their reducing abilities (Figure 7) as evident in the conversion of ions Fe3+ to Fe2+ by the formation of blue color. Among the FRAP values for the hybrid compounds, **1c** was the highest while **1a** was the lowest. The higher the FRAP value, the greater is the antioxidant activity. The change in absorbance is directly related to the total reducing power of the electron-donating antioxidants present in the samples.

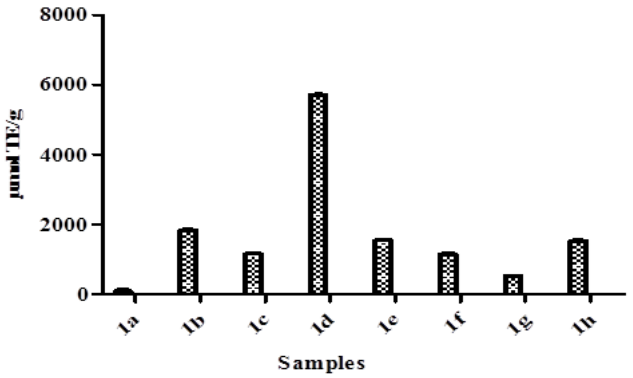
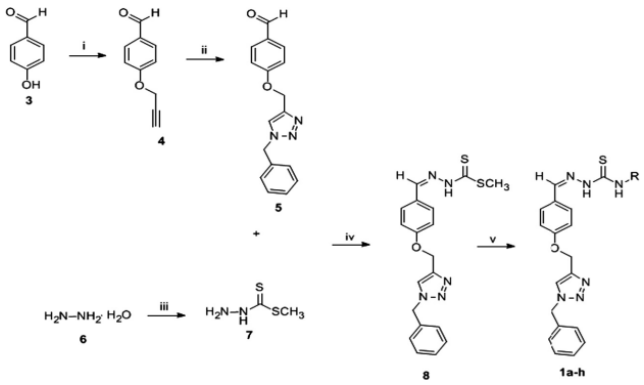


Figure 9. Oxygen radical absorbance capacity of the hybrids.



Scheme 1. The scheme for the synthesis of the novel hybrid compounds.

DPPH radical scavenging assay is a common method of assessing intrinsic free radical scavenging activity in a cell free system with 2,2-diphenyl-1-picryhydrazyl (DPPH), a free-radical generating compound [15]. It has many advantages than other methods which include as good stability, credible sensitivity, simplicity and feasibility [70-72]. This method is based on the formation of the DPPH-H non radical form in the presence of hydrogen donating antioxidants at 517 nm [69]. DPPH radical is a stable free radical and accepts an electron or

hydrogen radical to become a stable diamagnetic molecule [15,73]. In this study, DPPH scavenging activities were found in all the hybrid compounds (Figure 8). The degree of decrease in absorbance values is indicative of the antioxidant power of the compounds. From the results, the highest DPPH inhibition value was **1b** while the **1a** was the lowest. The exhibited antioxidant activities of these hybrid compounds on DPPH radical scavenging may be attributed to their hydrogen donating abilities. ORAC assay entails the use of fluorescent probe known as fluorescein and the loss of fluorescein over time due to peroxyl radical formation by the breakdown of AAPH is an indication of oxidative damage [74]. In this study, all the hybrid compounds showed ORAC values (Figure 9). The highest ORAC value was **1d** while the lowest was **1a**. The ORAC results confirmed the ability of the hybrid compounds to donate hydrogen atoms which resulted into the quenching of generated peroxyl radicals.

Conclusion

A series of thiosemicarbazone-triazole hybrids were evaluated for their effect on *Glut-4*, *Mef2a* and *Nrf-1* genes. There was consistency in the pattern of glucose transport pathway gene transcription by hybrids **1d** and **1g** with **1g** showing the most pronounced increase. Other compounds studied did not show a consistent up-regulation of all the relevant glucose transport genes investigated. This present study suggests that **1g** could be potential therapeutic agent to treat type 2 diabetes. The antioxidant activities of hybrids were examined for the first time in this study. The results showed that all the hybrid compounds have antioxidant potentials at varying degrees. As a result, they could play important protective roles to counteract the onset and progression of several diseases of the free radical aetiology such as diabetes mellitus. It can be speculated that the interactions of these hybrid compounds with free radicals and their abilities to donate electrons depend on their structures and hence, further research studies in the understanding of the mechanisms through which they exert their antioxidants effects are needed.

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