In vitro and computational studies on the antiglycation activity of compounds isolated from antidiabetic Tetracera alnifolia stem bark

Kelly Oriakhi, Collins U. Ibeji, Emmanuel E. Essien, Nkeiruka Eluehike, Kissinger Orumwensodia, Patrick Uadia and Iqbal M. Choudhary

CONTACT
Kelly Oriakhi, kelly.oriakhi@uniben.edu
Collins U. Ibeji, ugochukwu.ibeji@unn.edu.ng

ABSTRACT
The continuous search for new compounds in natural-based plants is a promising strategy for the prevention of diseases. This work examined antiglycation activity compounds isolated from the antidiabetic extract of T. alnifolia stem bark via in vitro and computational (molecular dynamics [MD]) approaches. Phytochemical investigation of ethyl acetate fraction and the application of spectroscopic methods led to the isolation and elucidation of 3 compounds: quercetin, kaempferol, and gallic acid. Compounds 1, 2 and 3 were then screened for antioxidant and antiglycation activities. Results show that the ethanol extract of T. alnifolia demonstrated good antioxidant activity compared to the standard gallic acid. There was a significant reduction in fasting blood glucose level progressively in diabetic rats, for 21 days compared to diabetic control. Consequently, the antiglycation activity of ethyl acetate fraction had the highest antiglycation activities, followed by dichloromethane (DCM) fraction. Compounds isolated from ethyl acetate fraction, exhibited the highest antiglycation effect for kaempferol followed by quercetin, while gallic acid had the least antiglycation effect. The root mean square of deviation (RMSD) and MM/GBSA energies obtained from molecular dynamics agree with the in vitro antiglycation activity with the sequence of structural stability in the order: kaempferol > quercetin > gallic acid. Therefore, findings from these results suggest that compounds isolated from T. alnifolia possess antiglycation activity.

Introduction
Diabetes mellitus is a metabolic disorder of multiple aetiologies characterized by high blood sugar (Brealey & Singer, 2009). The sugar in the blood (mainly glucose) reacts non enzymatically with protein to form advanced glycation end product (AGEs) in poorly controlled diabetes mellitus (Negre-Salvoyre et al., 2009). Glycation of proteins in the extracellular matrix alters their properties and after a series of biochemical processes, the plasma proteins will accumulate. In particular, the accumulation of LDL in the walls of blood vessels can contribute to atherogenesis or damage the basement membrane of the glomeruli (Ohtsubo & Marth, 2006). Endothelial cells and macrophages have AGE receptor on their surfaces, uptake of glycated proteins by these receptors can activate the transcription factor nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1) to increase the expression of vascular cell adhesion molecule-1 and cytokines, such as tumor necrosis factor and interleukin-6 and other proinflammatory molecules, leading to endothelial dysfunction (Yonekura et al., 2005). Diabetes mellitus (type 2) occurs as a result of impaired sensitivity of peripheral tissues (adipose tissues and muscle) to insulin action, via the GLUT-4 transporter. Protein kinase B (Akt) plays an important role in metabolism and insulin signaling by promoting GLUT-4 translocation, to the cell surface of target tissues and ultimately increases blood glucose uptake. Advanced glycation endproducts (AGEs) may interfere with insulin intracellular signaling and glucose transporters (GLUT 4) in tissues, as implicated in insulin resistance cells.

Medicinal plants endowed with antiglycation and antioxidant properties are potential therapeutic recipes in the treatment of long-term hyperglycemia. Some hypoglycemic antidiabetic therapeutic agents are known to have antiglycation activities with the ability of increasing glucose disposal within the body. GLUT4 is the major insulin-responsive glucose transporter primarily expressed in adipose tissues, skeletal muscle, and cardiac muscle (Fukumoto et al., 1989; Jewell et al., 2010). It is found within the intracellular vesicles where it is fully expressed (Richter & Hargreaves, 2013). Upon increase in blood glucose levels, insulin acts on the cell surface membrane thereby producing a cascade of events that lead to the translocation of GLUT4 from the...
interior of the cell to the plasma membrane (Jewell et al., 2010), thereby enhancing the uptake of glucose into the cells. The cascade of signal transduction events leading to the translocation and trafficking of GLUT4 molecules in response to insulin is mediated by a host of proteins collectively known as the mitogen-activated protein kinases (MAPKs) which is relevant in signal transduction pathways. In the folkloric medicine of Nigeria, *T. alnifolia* Wild is used in the treatment of various diseases and infections, including diabetes mellitus (Arowosegbe et al., 2015). *Tetracera alnifolia* belongs to the family Dillenaceae. This family is rare in Africa, where it is represented only by members of the pantropical genus *Tetracera* (Horn, 2009). Flavonoids, coumarin derivatives, and terpenoids are the main chemical constituents isolated from these plants genera (Lima et al., 2014). Isolation of phytochemicals such as kaempferol, kaempferol-3-sulphate, quercetin, quercetin-3-O-galactopyranoside, quercetin-3-glucuronide, rhamnocitrin, rhamnocitrin-3-sulphate, and procyanidin in the leaves of *T. alnifolia* has been reported (Gurni & Kubitzki, 1981). The leaves have been reported to possess anti-inflammatory, analgesic, and anti-tuberculosis activities (Lawal et al., 2011; Nsonde Ntandou et al., 2017). In addition, *T. scandens* and *T. indica* leaves have been shown to exhibit hypoglycaemic effects (Ahmed et al., 2012). In this work, we report for the first time the isolation of antiglycation compounds from the antidiabetic and antioxidant extract of *T. alnifolia* stem bark. To further support the *in vitro* antiglycation activity, molecular dynamics simulation was performed to understand the structural stability of ligands and their interaction with Glucose Transporter 4 (GLUT4) protein.

**Materials and methods**

**General procedures**

Proton spectra were run on an Avance AV-400 NMR spectrometer. The samples were dissolved in Deuterated methanol (CD3OD). Tetramethylsilane (TMS) was used as reference (δH = 0) and coupling constants in Hz. Mass spectrometry was carried out on an electron Impact Mass Spectrometer (EI-MS) (JEOL MS 600H-1) using positive mode. Analytical precoated TLC silica gel 60 from Sigma–Aldrich was used for thin-layer chromatography and visualized under short (254 nm) and long (366 nm) wavelength UV light. Column chromatography was conducted using silica gel Kieselgel 60 (60-200 mesh, Merck) or Sephadex LH-20 from Sigma–Aldrich. All solvents used were analytical grade (Merck).

**Plant material and extraction**

The stem bark of *T. alnifolia* was collected from tropical forest in Ibadan. The plant was identified accordingly by a taxonomist in the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Nigeria where a voucher specimen with voucher number (UBHt0145) was deposited in the herbarium. The plant stem bark was air-dried and pulverized. 2 kg of the pulverized sample was extracted with absolute ethanol (2 L) at room temperature for 72 h. The samples were filtered with Whatman No. 50 filter paper and the filtrate evaporated to dryness using a rotary evaporator (RE 300, Bibby Scientific, UK) to give 220 g (percentage yield of 11%). The ethanol extract was subjected to solvent-solvent partitioning with hexane (500 ml x 3) for defatting, dichloromethane (500 ml x 4), and ethyl acetate (500 ml x 4) using a separating funnel to obtain the respective fractions. Fractions collected were evaporated to dryness and concentrated for use.

**Isolation of compounds from ethyl acetate fraction of *T. alnifolia* stem bark**

6 g of ethyl acetate fraction was subjected to silica gel column chromatography (60x3 cm, internal diameter, 90 g). The gradient elution using various solvent combinations started with n-hexane, followed by dichloromethane, ethyl acetate and methanol. The 68 fractions obtained were bulked into four groups based on similarity in TLC characteristics. Fraction B (2.7 g) was subjected to further purification using silica gel column chromatography; 34 bulked fractions were bulked into three groups. Fraction Bii (2.03 g) was loaded on sephadex LH-20 (100 x 5 cm i.d., 700 g); and was eluted repeatedly with methanol (2 L) to give compound 1 (1.10 g) and 2 (800 mg), which was code TA 1 and TA 2 respectively (Figure 1). Fraction C (1.00 g) was also subjected to further purification with silica gel column chromatography to obtain 30 fractions. The fractions monitored by TLC afforded three bulked fractions. Fraction Cii gave a spot with a faint tail on TLC and showed positive for phenolic test using 5% FeCl3 spray reagent, while fractions Ci and Cii gave a negative reaction. Cii was subjected to preg. TLC (solvent system: ethyl acetate 100%) to afford a pure compound 3 coded TA 3 (700 mg) (Figure 1).

**Animals**

Ten weeks old male albino rats (Wistar strain) (141–200 g), bred in the Department of Biochemistry, Faculty of Life Science, University of Benin, Benin City, Nigeria, were used for the study. The animals were housed in galvanized rat cages and acclimatized for two weeks before the experiment. They were fed with guinea growers’ mash and had access to water ad libitum. The guide and care of the animals were according to the principles of laboratory animal care (NIH, 1985). The institutional Ethical Review Committee of the Faculty of Life Sciences, University of Benin, Nigeria approved the use of the experimental rats.

**Induction of diabetes mellitus in rats**

Diabetes mellitus was induced in rats following an overnight fast, by a single intraperitoneal injection of streptozotocin (0.09 g of STZ in 2.93 ml of cold citrate buffer at pH 4.5) at a dose of 50 mg/kg body weight, then 10% glucose solution was administered via oral gavage after 6 h. Hyperglycaemia was monitored for seventy-two hours after injection by measuring the blood glucose level using a glucometer (Accu-check, Roche diabetes care Inc. USA). Under this
condition, only the streptozotocin rats with fasting glucose levels of ≥200 mg/dL were selected for the study.

**Evaluation of antidiabetic activity**

The 25 male rats were randomly divided into five groups of five rats each and treated as follows:

**Group I**: rats were fed with standard pellet diet and allowed access to clean water daily.

**Group II**: rats were given a single dose of streptozotocin (50 mg/kg body weight, i.p) and fed the standard pellet diet and clean water daily.

**Group III**: rats were given a single dose of streptozotocin (50 mg/kg body weight, i.p), thereafter, was given 5 mg/kg body weight of standard drug aminoguanidine (positive control) for 21 days.

**Group IV**: rats were given a single dose of streptozotocin (50 mg/kg body weight, i.p) thereafter, was given 250 mg/kg body weight ethanol extract of *T. alnifolia* stem bark orally for 21 days.

**Group V**: rats were administered a single dose of streptozotocin (50 mg/kg body weight, i.p) thereafter, was given 500 mg/kg body weight ethanol extract of *T. alnifolia* stem bark orally for 21 days.

Fasting blood glucose levels obtained from the tail vein of the animals was checked before the start of the experiment (day 0 or the day streptozotocin injection was administered) and after administration of streptozotocin injection (day 3). Thereafter the blood glucose was monitored on days 7 and 14 with a glucometer (Accu-check). The animals were weighed at the beginning and end of the feeding period. On the 21st day, the animals after an overnight fast were anaesthetized and blood was collected via cardiac puncture into fluoride oxalate tube and plain tube, respectively. Blood collected in the fluoride oxalate tube was centrifuged immediately and plasma glucose assayed using the glucose oxidase method.

**Determination of antioxidant activity**

Diphenyl-2-picryl-hydrazyl (DPPH) scavenging effects of ethanol extract of *T. alnifolia* stem bark was determined by the method described previously (Jain et al., 2008).

**Determination of antiglycation activity**

The fructose mediated production of fluorescent AGES on Human Serum Albumin (HSA) assay was employed with slight modifications for the determination of antiglycation activity (Rahbar & Figarola, 2003). The hexane, dichloromethane, and ethyl acetate fractions of *T. alnifolia* stem bark was dissolved in absolute DMSO concentrations of 0.25-2.0 mg/mL. Isolated compounds (quercetin, kaempferol, and gallic acid) and standard rutin were also prepared using DMSO concentrations of 0.5-2.0 mM. Human Serum Albumin was employed as the model protein to be glycated at 10 mg/mL with 0.5 M fructose as a glycating agent. Samples were incubated in triplicates on 96-well plate at various concentrations with 10 mg/mL HSA, 0.5 M fructose, 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sodium azide as a bactericidal agent and incubated for 4 weeks at 37°C. For positive control, HSA, fructose, and phosphate buffer were incubated with the same concentration and conditions with absolute DMSO. After 4 weeks of incubation, the 96-well
plate was observed for a fluorescence at a wavelength of 330 – 440 nm on microtiter plate spectrophotometer (SpectraMax M2, Molecular Devices, USA). The sample concentration required for 50% of inhibition was calculated using Ez-fit software (Perrella Scientific, USA). Percentage of inhibition was calculated using the formula:

\[
\% \text{ Inhibition} = \left(1 - \frac{\text{fluorescence of test sample}}{\text{fluorescence of control group}}\right) \times 100
\]

**Computational details**

**Preparation of enzyme-inhibitor complex**

Docking was performed on the three-dimensional X-ray crystallographic structure of Glucose Transporter 4 (GLUT4) using Autodock tools 1.5.4 (Sanner, 1999). The protein data bank (PDB) access code, 3PCU (Zhang et al., 2012) was retrieved from PDB database for the docking. Protein was prepared by adding polar hydrogen atoms and Gasteiger charges were added using the AutoDock Tools graphical user interface by MGL Tools (Morris et al., 2009). The ligand preparation which involved geometric optimization to obtain minimal structures was carried out using Gaussian 09, (Frisch et al., 2009). The optimum binding site for the ligand was obtained using the Lamarckian genetic algorithm method.

**Molecular dynamic simulation (MD)**

Compounds 1, 2 and 3 were subjected to MD simulation using AMBER 18 (Case et al., 2005). The systems were optimized using ANTECHAMBER (Wang et al., 2001) and LEAP module of Amber 18 was applied to ensure all the appropriate parameters were available for MD simulations. The protein parameters were assigned using FF14SB (Perez et al., 2015) version of the Amber force field. Missing hydrogen atoms were added using The X-leaf (Case et al., 2008) software. MD simulations were performed with (GAFF) (Wang et al., 2004) for inhibitors and ff99SB (Hornak et al., 2006) force field for protein. The complexes were submerged in TIP3P explicit water in a cubic box of distance 10 Å. 5 Na + Counterions were added to neutralize the complexes each. The SHAKE algorithm (Ryckaert et al., 1977) was used to contained all bonds involving hydrogen atoms. The same procedure as previously reported (Ibeji, 2020) was applied in this study for MD. Bad clashes were eliminated by two-phase minimizations, 2500 steps of steepest minimization tracked by 2500 of conjugated gradient. A restrained minimization with a force of 500 kcal mol\(^{-1}\) on the solute molecule was performed firstly and water molecules, with ions, were relaxed. Full minimization of the complexes was done unrestrained. Heating from 0 to 300 K under the NVT ensemble for 500ps was performed slowly applying the Langevin dynamics with a collision frequency of 1.0 ps\(^{-1}\). Force constraint of 10 kcal (mol Å\(^{-1}\))\(^{-1}\) was applied during a 1 ns of retrained MD equilibration. 100 ns MD production with a time step of 2 fs with no restraints was run. The periodic boundary condition under the NPT ensemble at 300 K and a pressure of 1 atm was used (Ibeji, 2020) and Long ranged electrostatic forces with a cut-off of 12 Å was carried out using partial Mesh Ewald (PME) (Harvey & De Fabritiis, 2009; Ibeji, 2020).

**Thermodynamic calculations**

MM/GBSA a crucial tool applied for understanding protein-ligand binding affinity and macromolecular stability (Genheden & Ryde, 2015; Zhou et al., 2009; Zhou & Madura, 2004) was calculated. The binding free energy was averaged over 1000 snapshots which were extracted from the 100 ns trajectory as described by the set of equation.

\[
\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{protein}} - G_{\text{ligand}}
\]

The free energy term, \(\Delta G_{\text{bind}}\) is computed using the following equations:

\[
\Delta G_{\text{bind}} = \Delta E_{\text{gas}} + \Delta G_{\text{solvation}} - T\Delta S_{\text{normalmodeanalysis}}
\]

Where,

\[
\Delta E_{\text{gas}} = E_{\text{int}} + E_{\text{vdw}} + E_{\text{elec}}
\]

\[
E_{\text{int}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{torsion}}
\]

\[
G_{\text{solvation}, GB} = G_{\text{GB}} + G_{\text{nonpolar,solvation}}
\]

\[
\Delta G_{\text{nonpolar}} = \gamma SASA + \beta
\]

The gas-phase energy (\(\Delta E_{\text{gas}}\)) defines the sum of the internal (\(E_{\text{int}}\)), van der Waals (\(E_{\text{vdw}}\)) and Coulombic (\(E_{\text{elec}}\)) energies, (Equation 4). The solvation free energy refers to the polar (\(G_{\text{GB}}\)) and nonpolar (\(G_{\text{nonpolar,solvation}}\)) contributions (Equation 5). \(G_{\text{GB}}\) polar solvation was calculated by Generalized Born (GB) solvation model with the dielectric constant 1 for solute and 80.0 for the solvent (Onufriev et al., 2000). The nonpolar free energy contribution was calculated using Equation 6. The surface tension proportionality constant, \(\gamma\), and the free energy of nonpolar solvation of a point solute, \(\beta\), were set to 0.00542 kcal mol\(^{-1}\) Å\(^{-2}\) and 0 kcal mol\(^{-1}\), respectively (Gohlke et al., 2003).

**Results and discussion**

**Antioxidant activity of ethanol extract of T. alnifolia stem bark**

The DPPH radical scavenging activities of T. alnifolia extract is presented in Table 1. The extract demonstrated effective antiradical activity (88.72%, 13.39 ± 0.18 IC\(_{50}\)) compared to

| Table 1. DPPH radical scavenging activity of ethanol extract of T. alnifolia. |
|-----------------------------------------------|
| Samples | % Inhibition | IC\(_{50}\) (µg/ml) |
|---------|-------------|------------------|
| Extract of T. alnifolia | 88.72 ± 0.25 | 13.39 ± 0.18* |
| Gallic acid | 93.93 ± 0.31 | 0.14 ± 0.05a |
| N-acetyl cysteine | 93.95 ± 0.22 | 0.07 ± 0.01b |

Values are presented as mean ± SEM (n = 3). Values with different alphabet letters are significantly different (p < 0.05) from one another.
the standards gallic acid and N-acetyl cysteine used in the assay. The observed result may be attributed to the polyphenol content of the extract. Quercetin, kaempferol, and gallic acid isolated from the ethyl acetate fraction of *T. alnifolia* stem bark are polyphenol compounds and known antioxidant agents. A report has also suggested a relationship between the free radical scavenging activity of polyphenolic substances and their chemical structures (Samak et al., 2009), hence the ability of constituents inherent in the extract to act as hydrogen atoms or electrons donor in the conversion of the stable purple coloured DPPH to the reduced yellow coloured DPPH-H.

**Antidiabetic activity of ethanol extract of *T. alnifolia* stem bark**

The antidiabetic activity of ethanol extract of *T. alnifolia* stem bark in streptozotocin induced diabetic rats is shown in Table 2. The rats induced with diabetes showed significant elevation (p<0.05) of the blood glucose level when compared to the normal control group on day three (Table 2). Also, there was a dose-dependent decrease in blood glucose levels in the diabetic treated (250 and 500 mg/mg body weight) rats on day 7, 14 and 21 when compared to diabetic control. The observed biochemical effect of *T. alnifolia* extract on these diabetic rats may be due to its phytochemical profile: flavonoids, coumarin derivatives, and terpenoids are the main chemical constituents isolated from *Tetracera* genera (Lima et al., 2014).

**Antiglycation activities of fractions of *T. alnifolia* stem bark**

The ethanol extract of *T. alnifolia* stem bark was subjected to solvent-solvent partitioning on a separating funnel to obtain four fractions which were screened against human serum albumin (antiglycation assay); ethyl acetate fraction had the highest antiglycation activity, with dichloromethane and hexane fractions; quercetin, kaemferol, and gallic acid have been reported as antiglycation compounds in different models (Kim et al., 2010; Li et al., 2014), and these compounds also exhibited profound antiglycation activity, however, the most significant was quercetin (91.9% inhibition, IC₅₀, 0.28 ± 0.03 mM). Quercetin, kaempferol, and gallic acid have been reported as antiglycation compounds in different models (Kim et al., 2010; Li et al., 2014), and these compounds may be responsible for the antiglycation efficacy of *T. alnifo- lius* stem bark. Studies have shown that flavonoids are the group of polyphenols with the highest potential for the inhibition of glycoxidation; also, the inhibitory effects of polyphenols on glycation have been thought to be mainly due to their antioxidant rather than metal-chelating properties (Sadowska-Bartosz et al., 2014).

**Isolation and characterization of polyphenolic compounds**

Isolation and structural elucidation of polyphenolic compounds is shown in Fig. S1 & S2. The active ethyl acetate fraction of *T. alnifolia* stem bark afforded compound 1, 2, and 3 when subjected to chromatographic separations and compound 1 was obtained as a pale-yellow amorphous powder. El-MS result revealed a molecular ion (M + H) of 302 which corresponded to a molecular formula of C₁₅H₁₀O₆ and base peak at m/z 286 with the recognizable fragment at 69, 121 and 258. The ¹H-NMR data of the compound produced four proton doublet peaks at the aromatic region. The aromatic proton doublets at δ 6.21 and δ 6.40 were due to the meta coupled protons of a 5,7 substituted ring A. This accounted for protons H-6 & H-8 respectively. The two proton doublets at δ 6.90 & δ 8.00 accounted for the protons H-2’, H-6’ and H-3’, H-5’ of a 4’-substituted ring B respectively. The spectral data were in agreement with kaempferol reported in the literature (Markham et al., 1978). Thus, compound 2 is identified as kaempferol (Supplementary Figure S3 & S4).

**Antiglycation activities of compounds isolated from ethyl acetate fraction**

The antiglycation potential of quercetin, kaempferol, gallic acid, and fractions of *T. alnifolia* stem bark, at various concentrations is depicted in Table 3 and 4 respectively. This plant fractions and isolated compounds inhibited the formation of cross-linked AGEs in a dose-dependent manner. The ethyl acetate fraction demonstrated significant antiglycation activity (90% inhibition; IC₅₀, 0.011 mg/mL) compared to the hexane and dichloromethane fractions; quercetin, kaemferol, and gallic acid isolated from the ethyl acetate fraction also exhibited profound antiglycation activity, however, the most significant was quercetin (91.9% inhibition, IC₅₀, 0.28 ± 0.03 mM). Quercetin, kaempferol, and gallic acid have been reported as antiglycation compounds in different models (Kim et al., 2010; Li et al., 2014), and these compounds may be responsible for the antiglycation efficacy of *T. alnifo- lius* stem bark. Studies have shown that flavonoids are the group of polyphenols with the highest potential for the inhibition of glycoxidation; also, the inhibitory effects of polyphenols on glycation have been thought to be mainly due to their antioxidant rather than metal-chelating properties (Sadowska-Bartosz et al., 2014).

**Computational analysis**

The binding energies and 2D structures of isolated compounds against glucose transporter 4 (GLUT4) from molecular docking approach are presented in Table S1 and Figure S6.
### Table 2. Antidiabetic effect of ethanol extract of *T. alnifolia* stems bark.

| Group          | Glucose (mg/dl)       |
|----------------|-----------------------|
|                | Day 0     | Day 3     | Day 7     | Day 14    | Day 21    |
| Group I        | 84.00 ± 2.51a | 87.40 ± 2.00a | 87.20 ± 3.53a | 85.62 ± 3.09a | 86.60 ± 2.31a |
| Group II       | 78.80 ± 1.06a | 320.50 ± 8.20a | 351.20 ± 5.20a | 326.00 ± 6.61a | 334.50 ± 4.26a |
| Group III      | 76.50 ± 1.50a | 308.20 ± 6.20a | 261.40 ± 7.2a | 93.00 ± 2.70a | 72.40 ± 1.2a |
| Group IV       | 80.20 ± 3.79a | 290.60 ± 5.80a | 252.00 ± 4.30a | 140.80 ± 2.20a | 90.20 ± 1.25a |
| Group V        | 81.11 ± 3.50a | 240.50 ± 4.50a | 190.00 ± 1.30a | 120.20 ± 2.05a | 70.20 ± 1.10a |

Values are expressed as mean ± SEM (*n* = 5); values with different alphabet letters are significantly different (*p* < 0.05) from one another.

*Mean is significant (*p* < 0.05) when compared with the diabetic control group. Group I (normal control)-rats given H2O only; Group II (diabetic control)-rats given STZ only; Group III (positive control)-STZ + 5mg/kg bw of aminoguanidine; Group IV (diabetic treated)- STZ + 250mg/kg bw of ethanol extract of *T. alnifolia* and Group V (diabetic treated)- STZ + 500mg/kg bw of ethanol extract of *T. alnifolia*.

![Figure 2](image1.png)

**Figure 2.** The time evolution of the root mean square deviation (RMSD) of 1-GLUT4 (blue), 2-GLUT4 (red) and 3-GLUT4 (black) after 100 ns run.

![Figure 3](image2.png)

**Figure 3.** A plot of the root mean square fluctuations (RMSF) of Ca atoms of each residue in 1 (blue), 2 (red) and 3 (black) in complex with Glucose Transporter 4 during 100 ns of MD.
Table S1 showed that kaempferol binds with higher binding energy compared to other compounds. This is consistent with the experimental antiglycation activity findings. To further understand the dynamics and binding interactions of these isolated compounds with the active site of the GLUT 4, molecular dynamics studies were carried out.

**Molecular dynamics analysis**

Molecular dynamics study shows a comparative analysis involving the thermodynamics of ligand-protein and inhibitory properties of isolated compared to experiment. A 100 ns production MD was carried out to investigate the conformational, structural flexibility, compactness, and inhibitory potential of compounds. These are discussed in sessions.

**Table 3.** Antiglycation activities of fractions of *T. alnifolia* stem bark.

| Samples         | % Inhibition | IC50 (mg/ml) |
|-----------------|--------------|--------------|
| Hexane fraction | 75.8 ± 0.05b | 0.83 ± 0.02  |
| DCM fraction    | 79.5 ± 0.03b | 0.06 ± 0.01  |
| Ethyl acetate fraction | 90.0 ± 0.25a | 0.011 ± 0.001 |
| Rutin           | 92.0 ± 0.21a | 0.04 ± 0.01  |

Values are expressed as mean ± SEM (*n* = 3). Values with different alphabet letters are significantly different (*p* < 0.05) from one another.

**Table 4.** Antiglycation activities of polyphenol compounds of *T. alnifolia* stem bark.

| Compounds      | % Inhibition | IC50 (mM) |
|----------------|--------------|-----------|
| Quercetin      | 91.9 ± 0.02b | 0.28 ± 0.03 |
| Kaempferol     | 92.0 ± 0.01b | 2.03 ± 0.32 |
| Gallic acid    | 74.2 ± 0.11b | 1.01 ± 0.02 |
| Rutin          | 92.0 ± 0.21a | 0.04 ± 0.01 |

Values are expressed as mean ± SEM (*n* = 3). Values with different alphabet letters are significantly different (*p* < 0.05) from one another.
Table 5. Calculated binding free energies (kcal mol\(^{-1}\)) of compounds 1, 2 and 3 in complex with Transporter 4 complex across last 10 ns of the 100 ns MD. Energy components are in.

| Energy components | 1    | 2    | 3    |
|-------------------|------|------|------|
| GBSA   | \(-45.6\) | \(-15.30\) | \(-12.02\) |
| \(\Delta E_{\text{elec}}\) | \(-3.4\) | \(-1.37\) | \(-11.94\) |
| \(\Delta E_{\text{vdw}}\) | \(-49.0\) | \(-16.67\) | \(-23.96\) |
| \(\Delta G_{\text{gel}}\) | 10.9 | 5.69 | 15.62 |
| \(\Delta G_{\text{ligand}}\) | \(-38.1\) | \(-10.98\) | \(-8.34\) |

**Conformational and flexibility dynamics**

The structural dynamics and atomic fluctuation across tractors are given by RMSD and the RMSF diagram (Ibeji, 2020; Martinez, 2015). To ascertain the evenness of the 100 ns MD, the RMSD with respect to the \(C\alpha\) atoms (heavy atoms) of the protein backbone were analyzed after 100 ns MD (Figure 2). It has been reported that an average RMSD value less than 2.5 Å describes a stable simulation (Honarpourvar et al., 2014; Tolufase et al., 2018). The obtained average RMSD values for 1, 2 and 3 are 1.98, 1.84 and 2.06 Å respectively with compounds 2 and 3 being more stable than 1. The average RMSD of 1, 2 and 3 were stable throughout the 100 ns run, while 3 was unstable between 80 ns and 90 ns but became stable after 80 ns simulation. The RMSD is used in understanding the fluctuations of the whole system; nevertheless, it also helps in understanding the components of the proteins where more flexibility is high. The per-residue root mean square fluctuation (RMSF) of the protein backbone were analyzed after 100 ns MD (Figure 2) revealed that compounds 1 and 2 were stable throughout the 100 ns run, while 3 was unstable between 80 ns and 90 ns but became stable after 80 ns simulation. The RMSD obtained from molecular dynamics corroborate with the in vitro antiglycation activity where kaempferol showed the highest activity and actinic acid having the least antiglycation effect. Therefore, the sequence of stability agrees with the antiglycation activity: kaempferol > quercetin > gallic acid.

**Structural flexibility and fluctuation**

To gain insight into the flexibility and structural fluctuations of the amino acid residues around the active site region (Arodola & Soliman, 2016; Fakhar et al., 2017; Minkara et al., 2014) the per-residue root mean square fluctuation (RMSF) of the \(C\alpha\) atoms were analyzed after the 100 ns MD. The RMSF is useful in giving an understanding of the dynamics of the whole system; nevertheless, it also helps in understanding the components of the proteins where more flexibility is high and the concerned binding mode of ligands. The binding of 1 and 2 to the active site of the protein imposed structural flexibility and residue fluctuation. Compound 3 showed more flexibility compared to 1 and 2 (Figure 3). The average RMSF of 1, 2 and 3 are 8.59, 8.48 and 12.32 Å respectively with 3 showing lesser rigidity which agrees with the intro antiglycan activity and significant IC\(_{50}\) value. The same trend follows for RMSF and RMSD. There exist two substantial fluctuations that occurred in 1, the first involving residues ASP75-ASP99 around the \(\beta\)-hairpin flap region (Figure 3 and 4) and the most significant with low rigidity around ASP100 and LEU120.

**The radius of gyration (RoG)**

RoG determines the compactness of the protein-ligand complex (Lobanov et al., 2008) which gives insight into the molecular shapes. Compounds 1, 2 and 3 showed an average RoG of 17.11, 17.04 and 17.23 Å respectively. A high Rg value suggests a less tightly packed system and less stable conformation (Ibeji, 2020; Kumalo & Soliman, 2016). Figure 5 indicates that 2 had more structural compactness compared to 1 and 3 with the least average RoG value suggesting more stability.

**Inhibitory potential of compounds using MM/GBSA analysis**

MM/GBSA has been reported to be a standard computational tool for evaluation of the binding tendency of protein-ligand interactions (Tolufase et al., 2018). Analysis of the binding free energy provides an understanding of protein-inhibitor complex energies and interactions (Ibeji, 2020). MM/GBSA (Shinivasan et al., 1998) free energy method was calculated and the average thermodynamic energy contributions are generated from over the last 10 ns run. \(\Delta E_{\text{elec}}\) and \(\Delta E_{\text{vdw}}\) parameters depict the van der Waals intermolecular and electrostatic interacting components respectively (Caravella et al., 1999; Cottrell & Tupper, 2007). Table 5 revealed that compounds 1 and 2 possessed the highest binding energies with 3 displaying the lowest MM/GBSA energy. The obtained MM/GBSA energies are consistent with the anti-diabetic and antioxidant activities experimentally reported in this study. The high binding energy of quercetin is indicative of the necessity for further studies to find the distinctive moieties responsible for high energetic.

**Conclusion**

This study reports for the first time the anti-diabetic, antioxidant efficacy, antiglycation activity and computational analysis of compounds isolated from the T. alnifolia stem bark. This study revealed that T. alnifolia stem bark extract possesses strong anti-diabetic and antioxidant activities, and the extract was able to prevent AGE formation in vitro. Quercetin, kaempferol, and gallic acid; ethanol extract of T. alnifolia stem bark isolate of ethyl acetate fraction exhibited significant antiglycation activity. Molecular dynamics study agreed with the in vitro antiglycation activity with kaempferol showing the highest activity. MM/GBSA binding free energies showed that the isolated compounds in complex with glucose transporter 4 protein exhibited good binding energies and further studies could be investigated to determine distinctive moieties responsible for the high energetics and interactions with glucose transporter 4 protein.

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