International Journal of Pharmaceutical and Bio-Medical Science

ISSN(print): 2767-827X, ISSN(online): 2767-830X

Volume 02 Issue 08 August 2022

Page No: 233-248

DOI: https://doi.org/10.47191/ijpbms/v2-i8-02, Impact Factor: 5.542

Anti-Diabetic Potential of *Gymnema Sylvestre*: *In Vitro* and o*n Silico* Analysis

Zaharaddeen Abdullahi[1], Yakubu Magaji[2], Philip Anthony Vantsawa[3], Sadeeq Muhammad Sheshe[4], Jibril Abdullahi Alhaji[5]

[1,2,3]Department of Biotechnology, Faculty of Science, Nigerian Defence Academy, P.M.B. 2109, Kaduna State, Nigeria.

[4]Department of Biochemistry, Faculty of Science, Kano University of Science & Technology, P.M.B. 3244, Kano State, Nigeria.

[5]Department of Biochemistry, Faculty of Biomedical Science, Bayero University, P.M.B. 3011, Kano State, Nigeria.

ABSTRACT

Gymnema sylvestre (GS) is a powerful antidiabetic plant that has been utilized in ayurvedic, folk and homeopathic medicine for centuries. In this research, we evaluated the antidiabetic potential of methanolic leaf extract of *Gymnema sylvestre*. Fractionation was carried out using column chromatography and a total of twenty-eight (28) sub-fractions were obtained which were further screened and pooled into three (3) fractions (A, B and C) by thin layer chromatography based on their retention factor (Rf) values. The fractions were subjected to *in vitro* α-amylase and α-glucosidase inhibition activity. Some of the compounds identified from LC-MS were subjected to *in silico* analysis between all the ligands and the receptors with the aid of a docking software. Ligands were imported for visual screening into PyRx software while Biovia Discovery Studio Visualizer was used for protein preparation. Analysis of the properties of drug likeliness of the ligands was done via SwissADME online server according to Lipinski's Rule of Five. Final docking analysis was done through AutoDockVina and Biovia Discovery Studio client 2020. Fraction C showed the best IC_{50} of 0.84µg/ml α-glucosidase inhibitory activity when compared with fraction A and B, 2.00µg/ml and 1.58µg/ml (α-glucosidase), fraction A produced the best α-amylase activity among the fractions with IC_{50} of 16.78µg/ml, fraction B with 23.17µg/ml and fraction C with 28.22µg/ml. Molecular docking analysis of the ligands Orcinol (-5.5 kcal/mol) showed strong binding interaction with α-amylase, followed by 3-hydroxy-3'-methoxyflavone and Curcumin (-7.1 kcal/mol and -7.6 kcal/mol respectively) compared to acarbose (-8.0 kcal/mol) and Glyinflanin A (-8.4 kcal/mol) interactions. The binding affinity of Orcinol, 3-hydroxy-3'-methoxyflavone, Curcumin and Glyinflanin A (-5.7 kcal/mol, -8.0 kcal/mol, -7.6 kcal/mol and -9.1 kcal/mol respectively) were lower compared to acarbose (-9.7 kcal/mol) interaction with α-glucosidase. Thus, compounds identified from *Gymnema sylvestre* were found to have antidiabetic potentials with Orcinol displaying the most effective binding affinity in potential for drug development.

ARTICLE DETAILS

Published On:
03 August 2022

KEYWORDS: *Gymnema sylvestre* (GS), diabetes, alpha-amylase, alpha-glucosidase, *in vitro* analysis, *in silico* analysis.

Available on:
https://ijpbms.com/

INTRODUCTION

Diabetes mellitus (DM) is a long-term disorder of metabolism characterized by high level of blood sugar (hyperglycemia) due to insufficient secretion of insulin, insulin resistance, or both, as well as poor lipid, protein and carbohydrate metabolism (Piero *et al.*, 2014). These complications occur as a result of derangement in glucose storage for the regulatory system and metabolic fuel mobilization, including carbohydrate, protein and lipid anabolism and catabolism emanating from impaired action of insulin, secretion of insulin, or both (Votey and Peters, 2004, Piero *et al.*, 2014). As the condition advances, it causes vascular or tissue destruction, which can lead to serious complications of diabetes like renal disorder, ophthalmology, ulceration, coronary diseases and nervous disorder. Hence, diabetes encompasses a broad spectrum of diverse disorders (Salim, 2005).

Due to its high prevalence rates and high medical cost, diabetes has already become a concern to the global human population as well as individuals. Globally, diabetes has been reported as one of the generally known lifestyle-related non-infectious diseases with a permanent growth in the incidence. In most developed countries, it is one of the major causes of death and there is strong evidence that it is epidemic in many newly industrialized and economically developing countries, during the past two decades, the number of people diagnosed with diabetes has increased. In 2000, about 151 million people worldwide were diagnosed with diabetes (Zimet *et al.*, 2001; Wild *et al.*, 2004; Roglic *et al.*, 2005), in 2010, more than 221 million people were reported to be diabetic and by 2025, about 324 million people have been projected to be diabetic with an estimated global prevalence of diabetes at 9% in 2014 (WHO, 2014). By 2030, there is possibility that the total number of diabetics would rise to about 439 million worldwide (Upadhyay, 2016). International Diabetes Federation (IDF) reported that, there are over 415 million adults have been estimated to be diagnosed with diabetes in 2015, with the possibility of the figure rising to 642 million adults in 2040, with type 2 diabetes (T2D) accounting for roughly 91% of all the incidences of diabetes. It has also been estimated that 193 million people with diabetes are undiagnosed and about 318 million adults with impaired glucose tolerance (IDF Diabetes Atlas, 2015).

Globally, diabetic people are at high risk for premature death as a result of macrovascular and microvascular diseases; diabetes is primarily a major cause of sightlessness owing to retinopathy, a leading cause of chronic nephropathy as well as end-stage nephropathy that calls for dialysis, as well as other severe morbid conditions including amputation of the lower limb. Diabetes, if left untreated or handled ineffectively, can lead to death. Greater action is needed to improve diabetes outcomes in order to reduce the global burden of diabetes which is today affecting over 425 million people worldwide (IDF Diabetes Atlas, 2019).

Medicinal plants include various types of plants, shrubs or herbs containing phytochemicals that have profound physiological, pharmacological and biochemical effects (DEBTEC, 2003). Medicinal plants contain bioactive compounds that are primarily used for medicinal purposes. These compounds can act by interfering with the metabolism of infecting microbes and/or on different systems of animal models (Pandey and Madhuri, 2008; Kumar *et al.*, 2011). The identification of bioactive compounds in plant as well as their separation (isolation), purification and characterization of active components in crude extracts using various analytical methods is critical. The antimicrobial or antipyretic, antioxidant, antibacterial and antidiabetic effects of the medicinal plants could be based on the phytochemicals in them (Pandey and Madhuri, 2008; Kumar *et al.*, 2011).

Herbal Medicine phytomedicine or Botanical medicine is popularly called as "Herbal Medicine" because different parts of plants including stems, leaves, seeds, roots, berries, bark or flowers etc. are used for medicine preparation. The use of herbal medicine is increasingly becoming more popular as up-to date analysis and experimentation show their importance in the prevention and treatment of various diseases (ailments) due to their minimal side effect and natural origin (Laha and Paul, 2019).

The World Health Organization (WHO) has listed over 21,000 plants that are currently in use for medicinal purposes worldwide. This is scientifically validated due to its active phytochemical ingredients. Before the preparation or formulation of herbal medicine, necessary information about its cultivation, collections, processing, diagnosis, extraction of active phytochemicals, etc. India stands to become a significant and most successful exporter of herbal crude extract in the world. Indian herbal market is providing a good source of generating income to both farmers and traders. In developed countries, some precautions like well documentation about herbal medicine, single plant medicine that is free of pesticides, heavy metals, poisons and detrimental side effects should be followed in order to encourage the use of herbs (Laha and Paul, 2019).

The use of medicinal plants as a source of medicine started from ancient time in all most all cultures (Ghosh and Kishalay, 2014). Plants have historically been found to be the principal source of medications utilized as a preventive and therapeutic measures for different disorders (Mahammad and Amusa, 2005). They have some certain qualities or virtues which makes them to be medicinally valuable even though there are no apparent morphological characteristics growing within the medicinal plants. Plants that synthesize and accumulate secondary metabolites such as glycosides, tannins, alkaloids, as well as vitamins and minerals has been established to possess medicinal properties (DEBTEC, 2003). Many of the phytochemicals synthesized by plants have been found to offer long-term beneficial health effects when consumed by humans and can be effectively used in the treatment of human diseases. Traditional and folk medicines are widely used in health care system around the world (Gabhe *et al.*, 2006). Plants and their extracts are used in the treatment of many diseases by about three quarters of the world's population. Herbal treatments are used by a good number of our population, especially those who live in villages. For the treatment of allergy, cardiovascular, metabolic and a variety of other diseases, most of these herbal remedies have proven to be effective (Igoli *et al.*, 2005).

The extraction and development of medicine have been linked to a growing dependence on the use of medicinal herbs in the societies that have been industrialized (UNESCO, 1999). Both herbal and orthodox medicine are practiced in Africa, though trained medical experts are considering the former (Anani *et al.*, 2000). Despite the fact that traditional health care delivery systems are widely used and medicinal herbs are readily available in our local market, there are very few, if any, medicinal herbs available in government health centres. Therefore, it is believed that, there are problems due

to lack of proper information about the medicinal plant and the technique involved in traditional medicine (Anani *et al.,* 2000).

Traditional medicine plants are expected to possess low toxic rate on the consumers. According to recent investigations, several therapeutic herbs employed in traditional medicine have some unfavourable consequences. Therefore, the utilization of any medicinal plant in a safe manner for medical reasons should be emphasized and elucidated. The results of the acute and sub-acute toxicity tests on the plants must be acquired in order to boost consumer belief in their safety, especially for use in the production of drugs. Evaluation of the toxic effects of any part of a medicinal plant extract is most critical, which necessitates a systemic approach to assessing the efficacy and safety profile of these plants (Shah *et al.,* 2016). In this research, we aimed to evaluate the antidiabetic potential of methanolic leaf extract of *Gymnema sylvestre*.

MATERIALS AND METHODS

Plant Material Collection, Identification and Processing

A fresh sample of the plant *Gymnema sylvestre* (GS) was collected from Shira Local Government Area of Bauchi State in Nigeria. It was identified at the herbarium unit of Department of Biological Sciences, Nigerian Defence Academy, Kaduna, with voucher specimen number 2017, and deposited for future reference. The leaves of the plant *Gymnema sylvestre* (GS) were removed off the stem, washed and dried under the shade. The dried sample was then crushed into powder using a pestle in a laboratory mortar. A closed plastic container was used to store the powdered sample.

Extract Preparation

About 350 grams of the plant sample was cold extracted by maceration with 1.5 litres of methanol. The extract was filtered using Whatman No. 1 paper. The methanolic extract filtrate was then evaporated initially, in a hot-air oven at 40^{o}C for 72 hours before being lyophilised under vacuum at -80^{o}C and a pressure of 0.06 mbar after which the dried/powdered extract and stored at -20^{o}C for further analysis (Kong *et. al.,* 2014).

BIOCHEMICAL ASSAY

Column Chromatography

Fractionation of *G. sylvestre* Leave

This was done in accordance with the work of Ode *et. al* (2011) with some little changes. In order to separate the extract into its component fractions, 15 gram of the *G. sylvestre* extract was subjected to column chromatography. The stationary phase was made up of 30 gram of Silica gel (60 - 120G), whereas the mobile phase was ethyl acetate: methanol in varying proportion (100:0, 70:30, 50:50, 30:70 and 0:100) of increasing polarity. The lower part of the glass column (5.5 x 80cm) was loaded with glass wool with the help of glass rod during column chromatography set up. The slurry, made by combining 30g of silica gel with 50 ml of ethyl acetate was gently poured down the column. The glass column's tap was left open to allow free flow of solvent into a conical flask below to ascertain whether the set–up was intact when the solvent drained freely without carrying either the silica gel or glass wool into the tap. The tap was locked at the end of the packaging process. The clear solvent on top of the silica gel was allowed to drip down to the silica gel meniscus after the column was allowed 24 hours to stabilize. Sample preparation was done by absorbing 15g of the extract to 20g of silica gel (60 - 120G) in ethyl acetate and allowed to dry in a ceramic mortar. The sample was continuously stirred to dryness with a spatula. Small amount of selide was added to the dry powder and stirred to mix very well, and carefully put on top of the column. The column tap was opened to allow the eluent to flow at the rate of 0.58ml per second. The extract was eluted using ethyl acetate and methanol as solvent systems with gradually increasing polarity. In the elution process, the following solvent ratios were used sequentially in the elution process; ethyl acetate: methanol 100:0, 70:30, 50:50, 30:70 and 0:100. Each solvent combination was collected gradually in a specified volume (100 ml) with a 100 ml measuring cylinder and poured uniformly into the column by the sides of the glass each time. This prevents solvent droplets from dropping straight onto the uppermost layer of the column and causing disturbance. If this layer is distorted, the fractions will drain in a non-uniform manner. The eluted fractions were collected in conical flasks in aliquots of 10ml, thus resulting in collecting 28 sub-fractions.

Thin Layer Chromatography (TLC)

Pooling and Screening of Fractions

Analytical TLC used pre-coated silica gel (Xtra SIL G/UV$_{254}$ on aluminium plate). Using a small chromatographic tank, the contents of each conical flask were spotted on pre-coated silica gel (Xtra SIL G/UV$_{254}$) aluminium plates in order to separate the different sub-fractions on the basis of their relative mobilities in solvent systems ethyl acetate: methanol (8:2). At about 1.0cm from the edge of the plates, a spot of the sub-fraction was applied and allowed to dry at room temperature (27^{0}C). The plates were place in a small chromatographic jar containing the solvent system ethyl acetate: methanol (8:2). A glass lid was placed on top of the jar. The solvent was allowed to migrate upward until the solvent front reach about ¾ of the plate length. The plates were then removed and allowed to dry at room temperature (27^{0}C). The plates were sprayed with a solution of freshly made 0.5ml p-anisaldehyde in 50ml glacial acetic acid and 1 ml 97% sulphuric acid and heated to 105°C until maximal spot visualization was achieved (mostly red, violet and purple as observed).

The visible spot was measured with a ruler and the relative Retention factor (Rf) value was determined using the formula:

$$Rf = \frac{\text{Distance travelled by the streak from the starting point}}{\text{Distance travelled by the solvent from the starting point to the solvent front}}$$

The sub-fractions (twenty-eight obtained) with close retention factor (Rf) values were pooled together into three fractions (fraction A, B and C) and kept at 4^0C in the refrigerator for further study (thus, *in vitro* and *in vivo* bioassay screening for antihyperglycemic/hypoglycemic activity). The weight of the fractions been pulled were also measured.

In vitro Bioassay

Alpha -amylase inhibition assay

The Oboh *et al*., (2010) method was used to perform the α-amylase inhibitory assay. There were three (3) tubes set up. The fraction (50µl) was diluted in 500µl of 20mM sodium phosphate buffer (pH, 6.9, with 6mM NaCl) containing porcine pancreatic α-amylase (0.5mg/ml) and incubated for 10 minutes at 25ºC. Each tube was then filled with 500µl of 1% starch solution in 20mM sodium phosphate buffer (pH, 6.9, with 6mM NaCl). The reaction mixtures were incubated at 25ºC for 10 minutes before being stopped with 1.0ml of 3,5-dinitrosalicylic acid (DNSA) colour reagent. After that, the mixture was incubated for 5 minutes in a boiling water bath before being cooled to room temperature (25ºC). After adding 10ml of distilled water to the reaction mixture, the absorbance was measured at 540nm. The α-amylase inhibitory activity was reported as percentage inhibition using the following expression with the control experiment performed without the test sample.

$$\text{Percentage of inhibition} = \frac{[(Abs_{control} - Abs_{sample})]}{\text{Abs of control}} \times 100$$

Where $Abs_{control}$ is the absorbance of the mixtures without the fraction, and Abs_{sample} is the absorbance of the mixture with the fraction.

Alpha-glucosidase inhibition assay

The assay was carried out according to an instruction by Apostolidis *et al.*, (2007). The fraction (50µl) and 100µl of α-glucosidase solution (1.0U/ml) were diluted in 0.1M phosphate buffer (pH, 6.9) and incubated for 10 minutes at 25^0C. (A single unit of the enzyme produce 1.0 µmol of D-glucose per minute from p-nitrophenyl-α-D-glucoside). This was then followed by adding 50µl of 5mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1M phosphate buffer (pH, 6.9). After 5 minutes of incubation at 25^0C, the absorbance was measured at 405nm. The α-glucosidase inhibitory activity was reported as percentage inhibition using the following expression with the control experiment performed without the test sample.

$$\text{Percentage of inhibition} = \frac{[(Abs_{control} - Abs_{sample})]}{\text{Abs of control}} \times 100$$

Where $Abs_{control}$ is the absorbance of the mixture without the fraction, and Abs_{sample} is the absorbance of the mixture with the fraction.

Identification of Compounds

Ultra-High-Performance Liquid Chromatography-Electrospray Ionization- Ultra-High-Resolution-Quadrupole-Time-of-Flight Mass Spectrometer (UHPLC-ESI- Q-TOF-MS/MS).

The (UHPLC) system (Agilent infinity 1290, USA) was integrated with a binary pump (G4220A), an auto sampler (HiP - ALS, G4226A), degasser (G1322A), Thermostatted Column Compartment (G1316C) and a Diode-Array Detector (DAD) (G4212A) with a light-pipe flow cell (recording at 280nm and 320nm). This was connected to an Ultra-High-Resolution-Quadrupole-Time-of-Flight Mass Spectrometer (G6550A) (USA) fitted with an ESI source (DUAL AJS ESI) operating on Auto-MS/MS mode. The study was carried out in the negative ion mode through a mass range from m/z 100-1250. Capillary voltage 3.5 KV; iFunnel 1RF750.0 V; nebulizing gas pressure 2.4 bar; drying gas flow 14.0 l/min; transfer time 50.0µs; pre-pulse storage 2.0µs and drying gas temperature 200.0°C were the ESI source settings. Agilent Mass Hunter Qualitative Analysis software (B.06.00) was used to analyse the MS data. The UHPLC separation was performed on a ZORBAX Eclipse Plus C18, Rapid Solution, 4.6x100mm, particle size 3.5micron column (Agilent Technologies, USA). Water: methanol (60:40 v/v) was used as solvent A, while acetonitrile (100%) was used as solvent B. A total flow rate of 0.5 ml/min was used to deliver the solvents. Within 50 minutes, the profile gradient changed from 0% B (100% A) to 100% B (0% A). The injection volume was 2.00µl. (Satheeshkumar *et. al.*, 2014; Karar and Kuhnert, 2015 – with modifications).

MOLECULAR DOCKING

Drug Likeliness Property Analysis

The properties of drug likeliness were analyzed using SwissADME online server. The ligands screened were analyzed for their property on drugs. SMILE screened ligand notations were copied from PubChem and pasted on SwissADME online web server (Zhang and Wilkinson, 2007). Drugs for the five-fold Lipinski rule were analyzed. The five points of the Lipinski rule are as follows: -

1) The molecular weight should be less than five hundred (500) Dalton.
2) The LogP partition coefficient should be less than five (5).
3) The number of hydrogen bond donors should be less than five (5).
4) The number of hydrogen bond acceptors should be less than ten (10).
5) Not more than one (1) rule can be violated.

Four (4) of the identified ligands which followed the above Lipinski rule of five were selected for final docking through AutoDockVina and Biovia Discovery Studio Client 2020.

Ligand Structures

Optimization of all ligand structures in order to remove all strain from the molecular structure was done using the Merck Molecular Force Field (MMFF) and the semi-empirical Austin Model (AM1) methods, both of which are implemented in Discovery studio visualiser (v20.1.0.19295, BIOVIA Software, http://www.3dsbiovia.com/product/collaborative-science/biovia-discovery-studio/). Furthermore, this will ensure that the study's compounds have a well-defined conformer connection (Viswanadhan *et al.*, 1989). The calculation was set to equilibrium geometry at the ground state using density functional theory at B3LYP (Becke88 three-parameter hybrid exchange potentials with Lee-Yang-Parr correlation potential) level of theory and 6-311G (d) basis set for the geometrical optimization of the cleansed structures i.e. B3LYP/6-311G (d) level of theory using the setup calculation option on Discovery studio visualise v20.1.0.19295. The display-output and display-properties options on Discovery studio visualiser v20.1.0.19295 were used to obtain the Discovery studio visualiser descriptions after optimization. Through the file option on the Discovery studio visualiser v20.1.0.19295, the completely optimized 3D structure without symmetry restrictions, was saved as an SD file.

Enzyme Structure

The X-ray crystal structures of α-Glucosidase (PDB ID:3WY1) and Human pancreatic α -Amylase (PDB ID: 3BAW) with resolutions of 2.15Å and 2.00Å respectively were downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Database Bank (PDB) (http://www.rcsb.org/pdb).

Docking Simulations

All proteins preparation and minimization were done with the Discovery studio visualizer's (v20.1.0.19295) tools and protocols. The structure was optimized using a force field developed by Harvard Macromolecular Mechanics (CHARMm). Hydrogen atoms were added to the complex throughout the protein preparation technique, after which water molecules were removed and the pH of the protein was set to nearly neutral value. To identify the binding site of the protein structure, a sphere binding site with a radius of nine Armstrong (A˚) was defined around the attached ligand. The ligands' SD files of were then imported into PyRx-virtual screening tool, where they were utilized to dock the receptors that have been prepared. The ligands are scored on the basis of biased probability Monte Carlo (BPMC) method, which selects a conformation in the internal coordinate space at random and then moves to a new random position that is independent of the previous one but follows a specified continuous probability distribution. The results of the best scored, binding energy and inhibition constants of all the ligands were reported on a table.

RESULTS

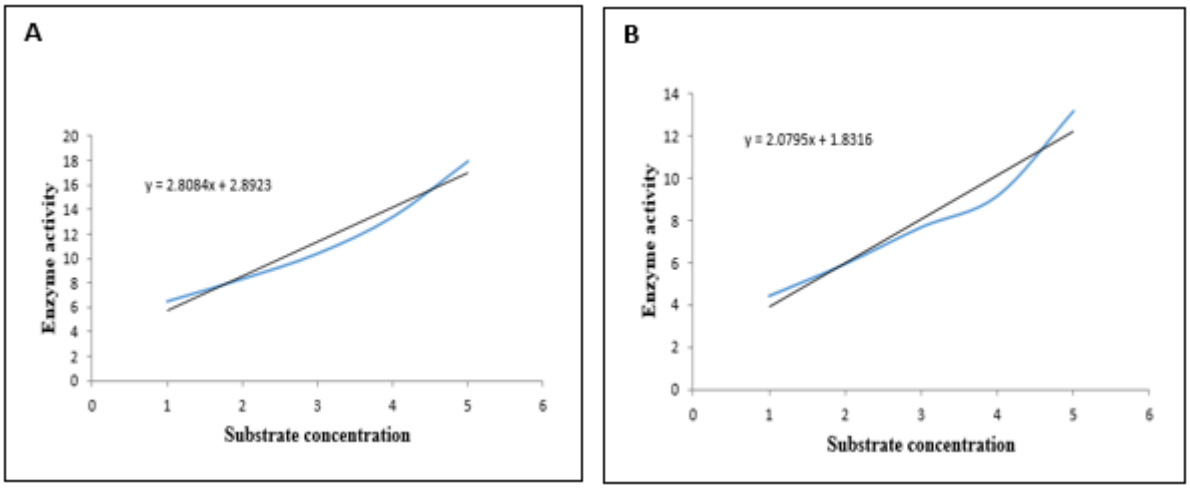
Bioassay – Guided Studies

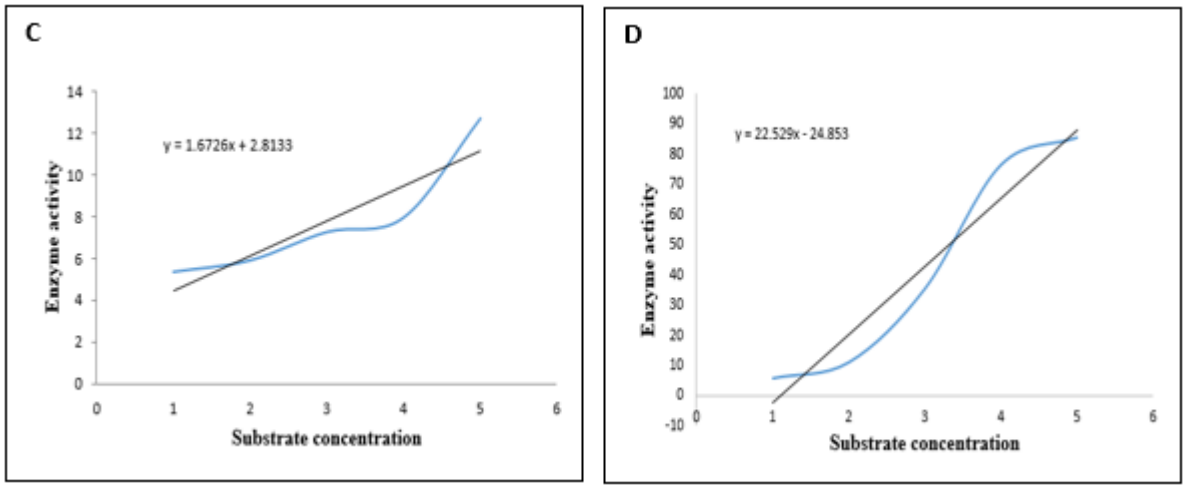
Fractionation by column chromatography guided by TLC

Fifteen grams (15g) of the *G. sylvestre* methanolic extract was subjected to column chromatography. Twenty-eight (28) sub-fractions were collected for *G. sylvestre* which were pooled based on their TLC profile, into three (3) fractions. The total recovery (i.e. eluted amount) for fraction A was 5.44g (i.e. 36.27%), fraction B was 3.44g (i.e. 22.93%) and fraction C was 2.70g (i.e. 18.00%).

Alpha-amylase and Alpha-glucosidase in vitro Inhibition Activities

G. sylvestre leaf extract showed inhibitory activities against α-amylase and α-glucosidase. IC_{50} calculations were performed with Microsoft Excel (figure 1 and 2) using a linear regression graph.





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Figure 1: Linear regression graphs for determining the half maximum inhibitory concentration (IC_{50}) of *Gymnemavestre* fractions and acarbose against alpha-amylase activity. (A) fraction A inhibitory activity, (B) fraction B inhibitory activity, (C) fraction C inhibitory activity and (D) acarbose inhibitory activity.

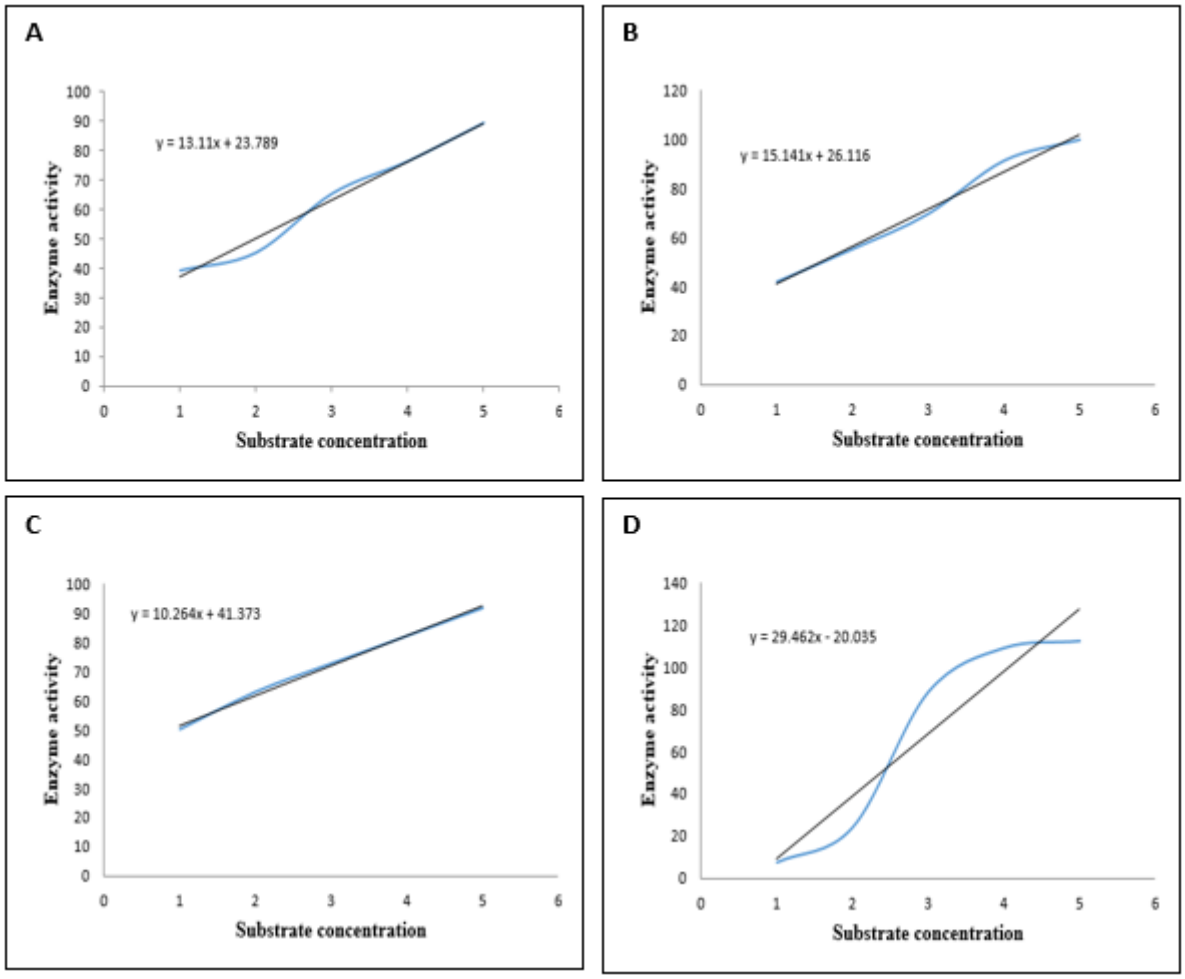


Figure 2: Linear regression graphs for determining the half maximum inhibitory concentration (IC_{50}) of *Gymnema sylvestre* fractions and acarbose against alpha-glucosidase activity. (A) fraction A inhibitory activity, (B) fraction B inhibitory activity, (C) fraction C inhibitory activity and (D) acarbose inhibitory activity.

Comparing *G. sylvestre* fractions inhibition activity against α-amylase (Table 1), statistical analysis between acarbose as control and fraction; A, B, and C revealed a significant difference ($p<0.05$) at 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml concentration of substrate respectively. When compared to the standard, statistical analysis revealed a statistical significant difference ($p<0.05$) between all fractions at substrate concentration of 40, 60, 80 and 100 µg/ml respectively when, but no statistical significant differences ($P>0.05$) at substrate concentration of 20µg/ml between all the groups, and no significant difference between fraction B and C at all the substrate concentrations. Statistical

significance difference was found between acarbose and fraction; A, B and C at all substrate concentration (20, 40, 60, 80, and 100 µg/ml respectively) of *G. sylvestre* fractions against α-glucosidase (Table 2). All the fractions show statistical difference with the standard acarbose respectively at 20µg/ml, 40µg/ml, 60µg/ml, 80µg/ml and 100µg/ml. Considering the alpha glucosidase inhibitory activity at 60µg/ml, there were no statistical significance differences (P>0.05) between all the fractions (A, B, and C) and also between B and C at 20µg/ml. However, there is statistical difference (p<0.05) between A and C at 20µg/ml substrate concentration.

Table 1: Effect of *G. sylvestre* Methanolic Leaf Extract Fractions on Alpha-amylase Inhibitory Activity.

	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml	IC_{50} µg/ml
Fraction A	6.48±0.02	8.32±0.00*^{abc}	10.40±0.00*^{abc}	13.14±0.03*^{abc}	17.98±0.05*^{abc}	16.78
Fraction B	4.40±0.00	5.93±0.02*^{a}	7.67±0.06*^{a}	9.12±0.00*^{a}	13.19±0.00*^{a}	23.17
Fraction C	5.35±0.00	5.90±0.00*^{a}	7.26±0.00*^{a}	7.95±0.17*^{a}	12.69±0.10*^{a}	28.22
Acarbose	5.58±0.72	10.89±0.00*^{abc}	35.32±1.37*^{abc}	76.41±0.14*^{abc}	85.46±0.53*^{abc}	3.32

Values are expressed as mean ± standard error of mean with those bearing same superscripts under the same column are significantly different (P< 0.05) using the Bonferroni correction; N = 5. Fractions A, B and C = sub-fractions obtained from column chromatography pooled by TLC on the basis of their closed retention factor (Rf) values.

Table 2: Effect of *G. sylvestre* Methanolic Leaf Extract Fraction on Alpha-glucosidase Inhibitory Activity.

	20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	100 µg/ml	IC_{50} µg/ml
Fraction A	39.27±0.02*^{abc}	45.34±0.01^{a}	66.33±0.02	76.34±0.01^{abc}	89.33±0.02*	2.00
Fraction B	41.70±0.00*^{abc}	55.25±0.28 ^{a}	69.58±0.39	91.20±0.12^{abc}	99.70±0.05*	1.58
Fraction C	50.36±0.00*^{abc}	63.16±0.00 ^{a}	72.92±0.01	82.42±0.10^{abc}	92.00±0.17*	0.84
Acarbose	7.62±1.33*^{abc}	24.20±0.00 ^{a}	88.33±3.43*	109.16±0.21^{abc}	112.45±0.70*	2.38

Values are expressed as mean ± standard error of mean with those bearing same superscripts under the same column are significantly different (P< 0.05) using the Bonferroni correction; N = 5. Fractions A, B and C = sub-fractions obtained from column chromatography pooled by TLC on the basis of their closed retention factor (Rf) values.

Identification of Compounds

Table 3 shows some compounds identified in *G. sylvestre* methanolic leaf extract through liquid chromatography-mass spectroscopy (LC-MS) technique using the m/z values. The compounds were automatically profiled through UHPLC-Q-TOF-MS analysis which were further identified based on their retention times and MS/MS spectra including characteristic MS/MS fragmentation ions. For the molecular ions, the differences in mass between the observed and theoretical (calculated) values were less than 4 ppm. Four compounds namely: Orcinol, 3-hydroxy-3'-methoxyflavone, Curcumin, Glyinflanin A and a standard drug acarbose were selected for the molecular docking analysis. The compounds were analysed for drug likeliness property analysis using the pkCSM and SwissADME online servers and screened using the qualifying Lipinski Rule of five. The compounds were further analysed for its Hydrogen bond acceptor, Rotatable Hydrogen bonds, Hydrogen bond donor, Molecular weight, LogP and Surface area. Acarbose was found to have three (3) violations according to Lipinski Rule of five.

Table 3: Drug Likeliness Property Analysis of some Identified Compounds and acarbose (standard).

Ligands	Binding affinity (Kcal/mol)	Number of Hydrogen bond between ligand and receptor	Hydro-phobicity	Interpol-ated charge	Solvent accessibility surface (SAS)	RMSD lower bound	RMSD upper bound
Orcinol	-5.5	2	-1.00	-0.033	10.0	0.008	2.767
3-hydroxy-3'-methoxyflavone	-7.1	4	-2.00	-0.033	12.5	20.650	21.478
Curcumin	-7.6	3	-1.00	-0.033	12.5	0.412	4.932
Glyinflanin A	-8.4	3	-1.00	-0.033	12.5	2.651	6.049
Acarbose	-8.0	9	-1.00	-0.033	15.0	27.710	32.481

In silico analysis

All the ligands were subjected for virtual screening through PyRx software. Docking analysis showed the compounds from *Gymnema sylvestre* to have inhibitory activities against α-amylase (PDB ID = 3BAW) with Orcinol having a total score of binding affinity of –5.5 Kcal/mol, root mean square deviation (RMSD) lower bound of 0.008 and RMSD upper bound of 2.767, that of 3-hydroxy-3'-methoxyflavone was –7.1 Kcal/mol, with root mean square deviation (RMSD) lower bound of 20.650 and RMSD upper bound of 21.478, , Curcumin showed a binding affinity of –7.6 Kcal/mol, root mean square deviation (RMSD) lower bound of 0.412 and RMSD upper bound of 4.932, Glyinflanin A displayed a binding affinity of –8.4 Kcal/mol, root mean square deviation (RMSD) lower bound of 2.651 and RMSD upper bound of 6.049 and that of Acarbose was –8.0 Kcal/mol, with root mean square deviation (RMSD) lower bound of 27.710 and RMSD upper bound of 32.481 (Table 4).

Docking with the protein α-glucosidase (PDB ID= 3WY1) revealed the total binding affinity for Orcinol –5.7 Kcal/mol, root mean square deviation (RMSD) lower bound of 0.022 and RMSD upper bound of 2.767, the binding affinity of 3-hydroxy-3'-methoxyflavone was –8.0 Kcal/mol, with root mean square deviation (RMSD) lower bound of 1.138 and RMSD upper bound of 2.050, that of Curcumin was found to be –7.6 Kcal/mol, root mean square deviation (RMSD) lower bound of 2.727 and RMSD upper bound of 6.928, it also revealed the binding affinity of Glyinflanin A to be –9.1 Kcal/mol, root mean square deviation (RMSD) lower bound of 2.651 and RMSD upper bound of 7.950 and Acarbose showed a binding affinity of –9.7 Kcal/mol, with root mean square deviation (RMSD) lower bound of 1.476 and RMSD upper bound of 2.272 (Table 5).

Table 4: Molecular Docking Results of Receptor (α-amylase) with Ligands (Orcinol, 3-hydroxy-3'-methoxyflavone, Curcumin, Glyinflanin A and Acarbose).

Compound Names	Molecular Weight (g/mol)	Number of Hydrogen Donor	Number of Hydrogen Acceptor	Number of Rotatable Bonds	LogP	Surface Area	Violations
Orcinol	124.14	2	2	0	1.4062	53.385	No violation
3-hydroxy-3'-methoxyflavone	268.26	1	4	2	3.1742	114.409	No violation
Curcumin	368.13	2	6	8	3.3699	156.532	No violation
Glyinflanin A	408.50	4	7	5	5.6026	176.502	No violation
Acarbose	645.60	14	19	9	-8.5645	250.228	Yes; 3 violations: MW >500, N or O >10, NH or OH >5

Table 5: Molecular Docking Results of Receptor (α-glucosidase) with Ligands (Orcinol, 3-hydroxy-3'-methoxyflavone, Curcumin, Glyinflanin A and Acarbose).

Ligands	Binding affinity (Kcal/mol)	Number of Hydrogen bond between ligand and receptor	Hydro-phobicity	Interpol-ated charge	Solvent accessibility surface (SAS)	RMSD lower bound	RMSD upper bound
Orcinol	-5.7	3	-1.00	-0.033	17.5	0.022	2.767
3-hydroxy-3'-methoxyflavone	-8.0	3	-1.00	-0.033	22.5	1.138	2.050
Curcumin	-7.6	5	-2.00	-0.033	15.0	2.727	6.928
Glyinflanin A	-9.1	6	-1.00	-0.033	12.5	2.651	7.950
Acarbose	-9.7	6	-2.00	-0.033	12.5	1.476	2.272

Molecular docking has shown the binding affinities of all the ligands to α-amylase catalytic residues. Other interactions such as hydrogen bonds, van der Waals interactions, hydrophobicity as well as pi-bonds cannot be discarded in the inhibitory activities of phenols against alpha-amylase (Parveen *et al*., 2019). Four hydrogen bonds interaction were found between 3-hydroxy-3'-methoxyflavone and α-amylase catalytic residues: PRO332, ARG421, ARG398 and GLY334. Two hydrogen bonds interaction between Orcinol and α-amylase catalytic residues: GLU233 and LYS200 were present. Curcumin was found to interact with GLN63, ASP197 and GLU233 through three hydrogen bonds interaction. Three hydrogen bonds interaction were found between Glyinflanin A and the α-amylase catalytic residues: GLU233, ASP197 and GLN63. Nine hydrogen bonds interaction were found between Acarbose and the α-amylase catalytic residues: ASN150, ASN152, GLY 239, GLU240, ILE235, LYS200, GLU149, HIS201 and TYR151 (Figure 3).

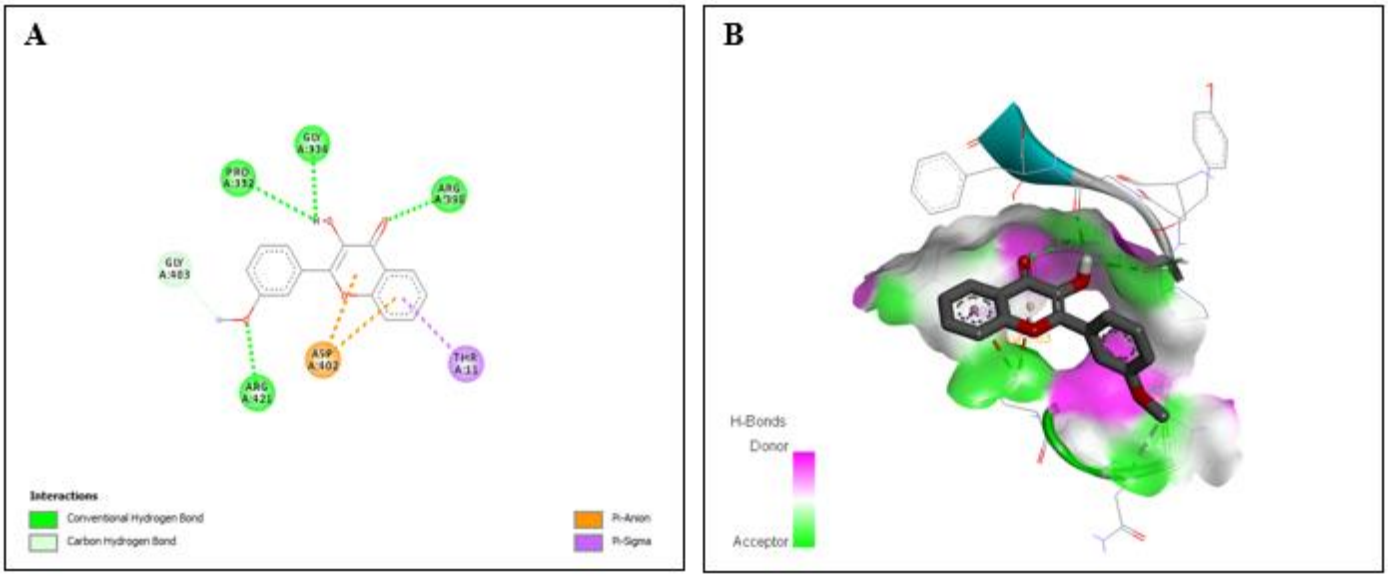


Figure 3a: Molecular docking of a receptor α-amylase with 3-hydroxy-3'-methoxyflavone, (A) 2D structural interaction, (B) Hydrogen bond distance.

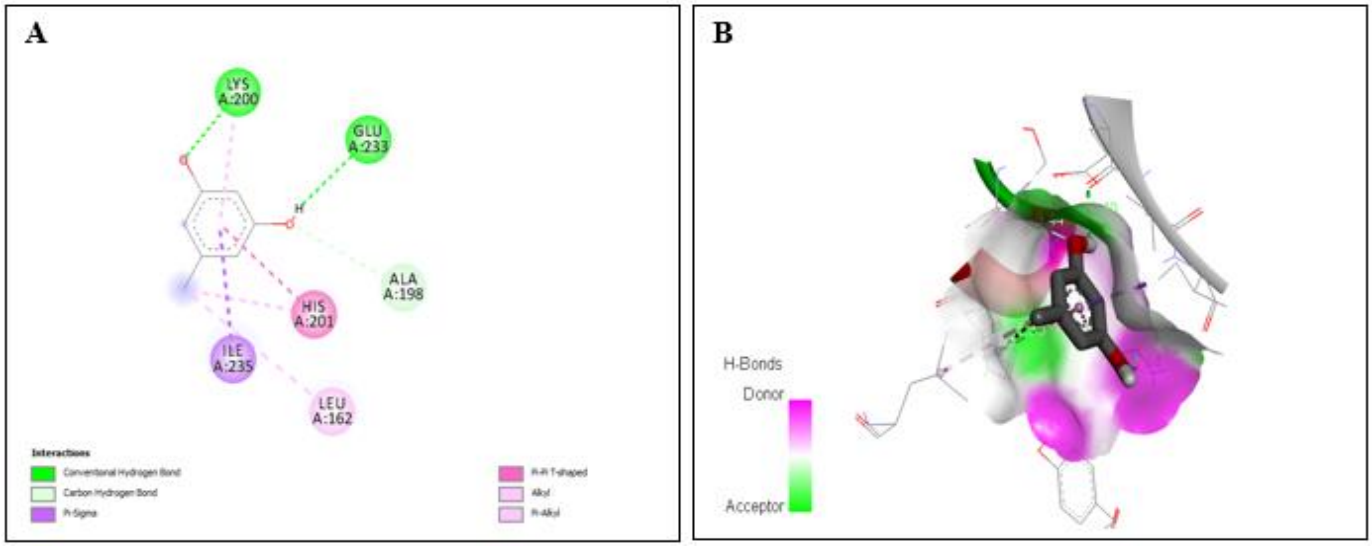


Figure 3b: Molecular docking of a receptor α-amylase with Orcinol, (A) 2D structural interaction, (B) Hydrogen bond distance.

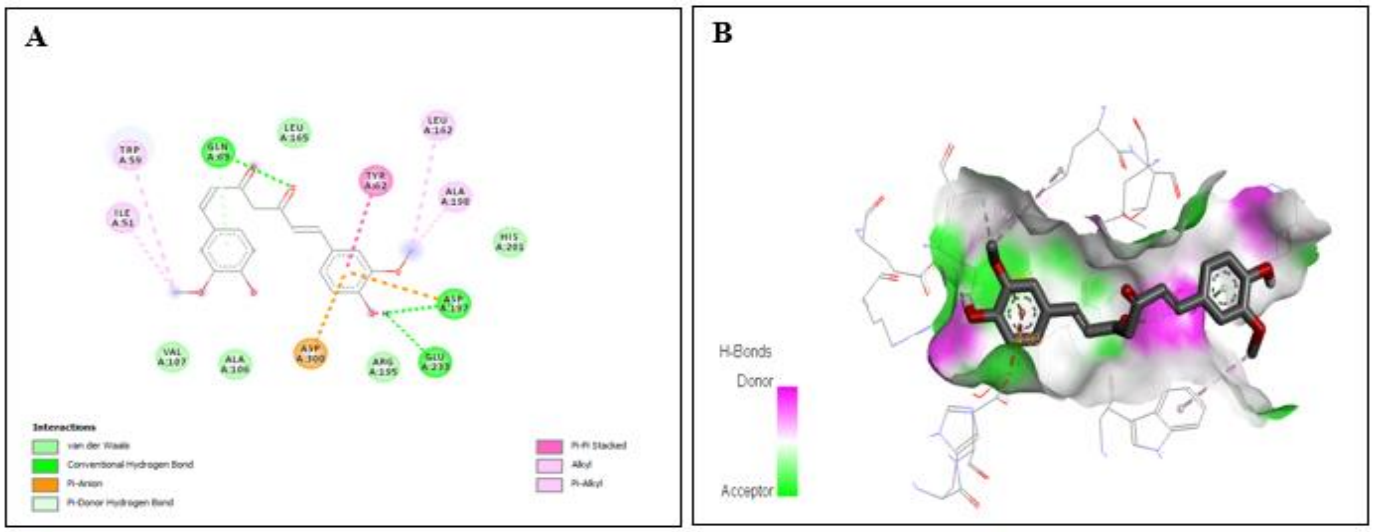


Figure 3c: Molecular docking of a receptor α-amylase with Curcumin, (A) 2D structural interaction, (B) Hydrogen bond distance.

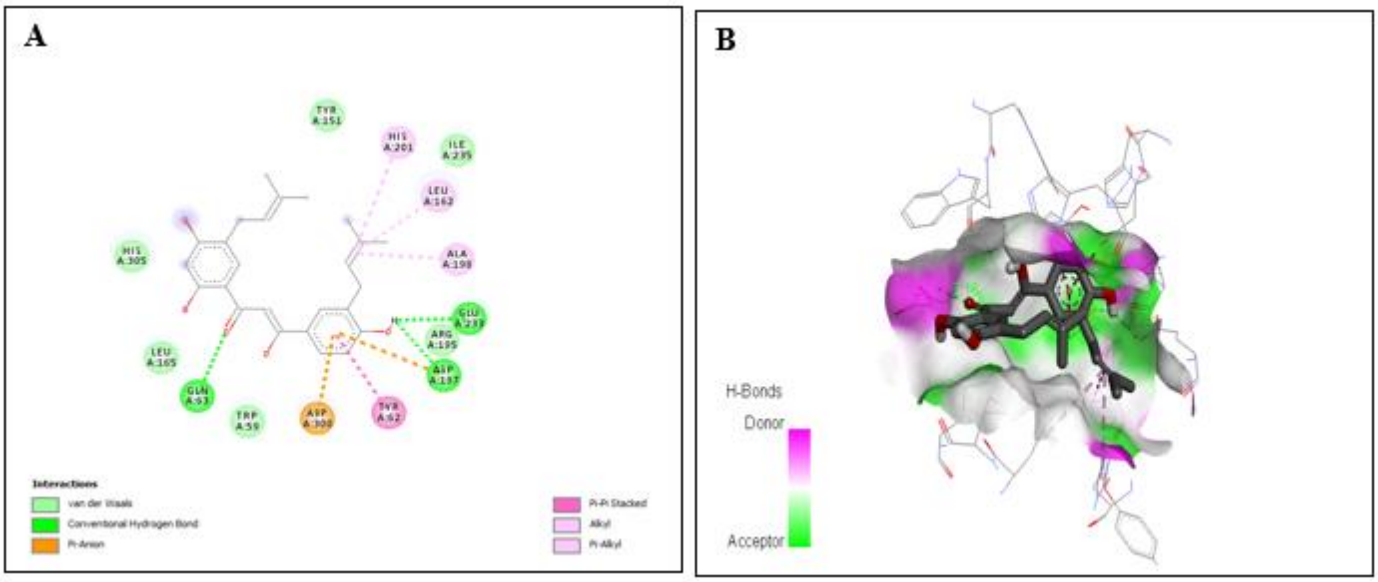


Figure 3d: Molecular docking of a receptor α-amylase with Glyinlanin A, (A) 2D structural interaction, (B) Hydrogen bond distance.

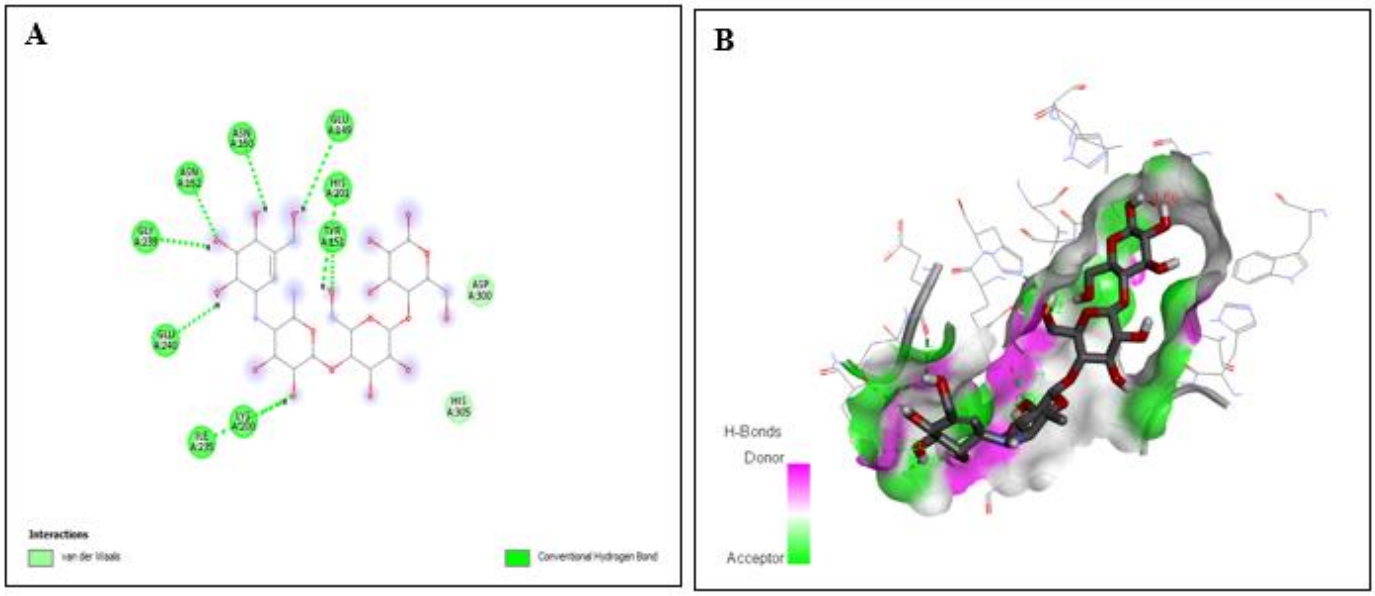


Figure 3e: Molecular docking of a receptor α-amylase with Acarbose, (A) 2D structural interaction, (B) Hydrogen bond distance.

The subjects under study have indicated a significant inhibitory mechanism against α-glucosidase. Two hydrogen bonds were found between 3-hydroxy-3'-methoxyflavone and α-glucosidase catalytic residues: two with ASN301 and one with LEU227. Orcinol docked with α-glucosidase also produced three hydrogen bond interactions involving GLY402, LYS398 and VAL380. Curcumin was found with five hydrogen bond interactions involving ALA229, LEU227, ASN301, ALA378 and VAL380 of α-glucosidase binding site, Glyinflanin A was found with six hydrogen bond interactions involving two residues of GLY402, LYS398 and one residue each of VAL380 and GLY399 of α-glucosidase binding site. Acarbose was found to have six hydrogen bond interactions involving GLU432, two residues ARG437, ASN443, ALA514 and GLN531 of α-glucosidase binding site (Figure 4).

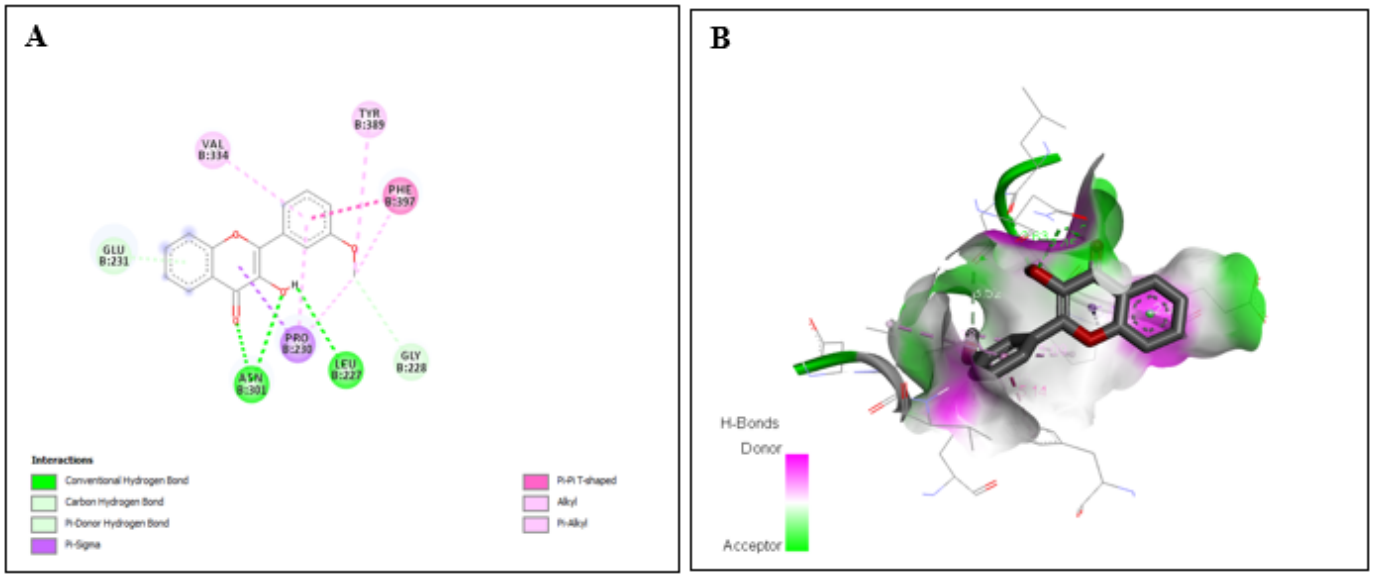


Figure 4a: Molecular docking of a receptor α-glucosidase with 3-hydroxy-3'-methoxyflavone, (A) 2D structural interaction (B) Hydrogen bond distance.

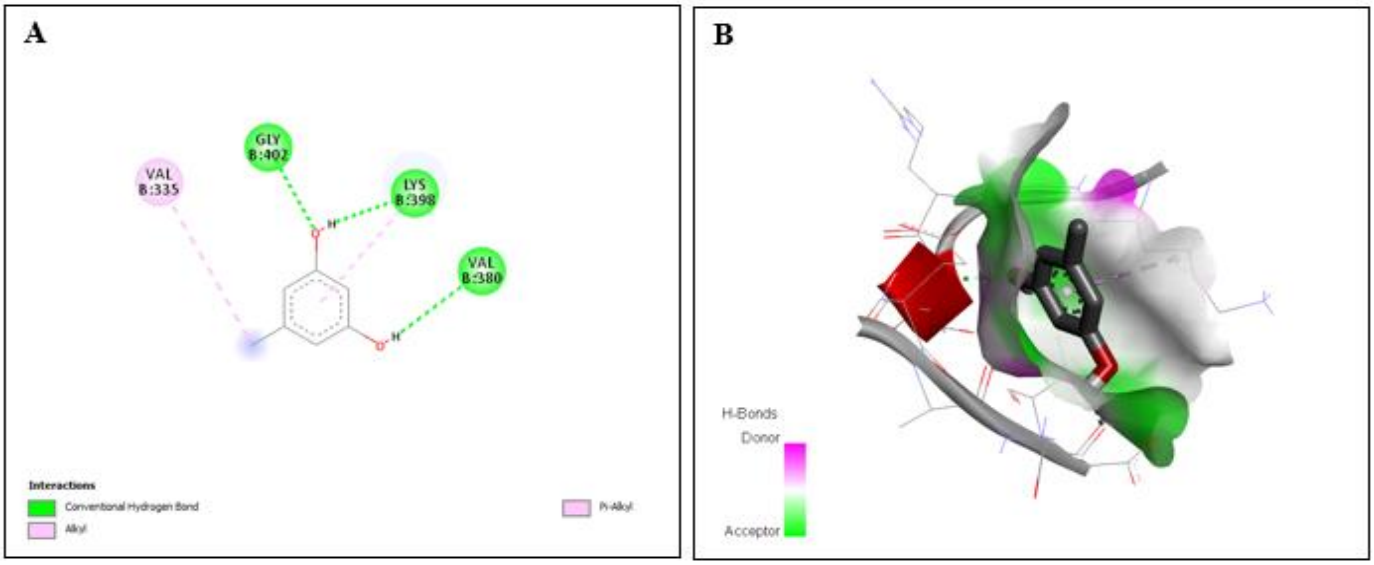


Figure 4b: Molecular docking of a receptor α-glucosidase with Orcinol, (A) 2D structural interaction (B) Hydrogen bond distance.

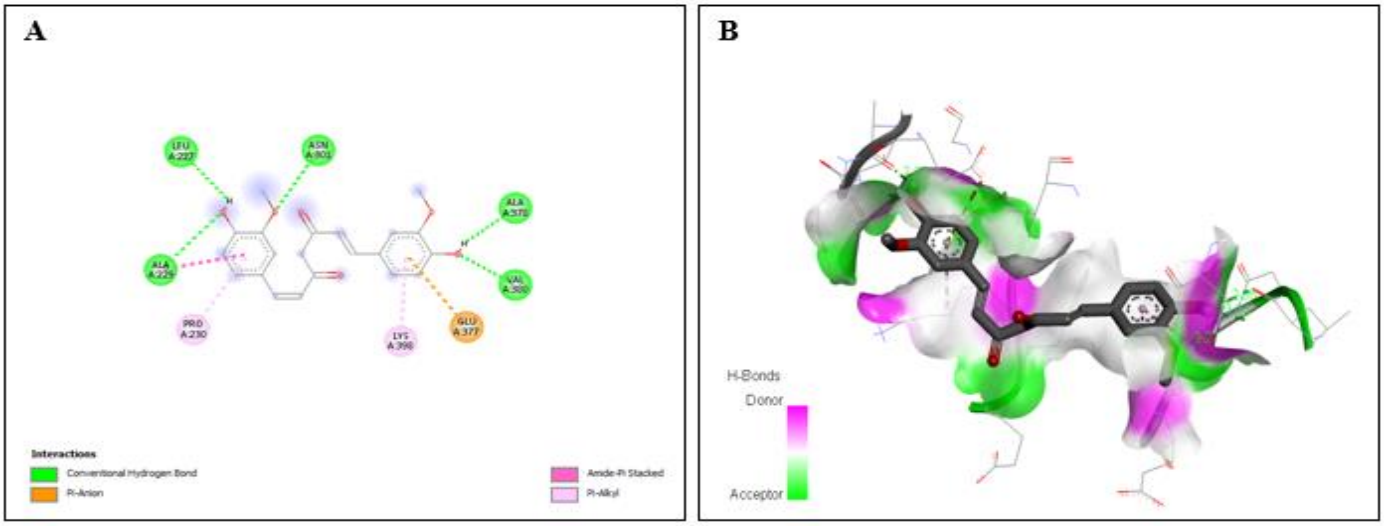


Figure 4c: Molecular docking of a receptor α-glucosidase with Curcumin, (A) 2D structural interaction (B) Hydrogen bond distance.

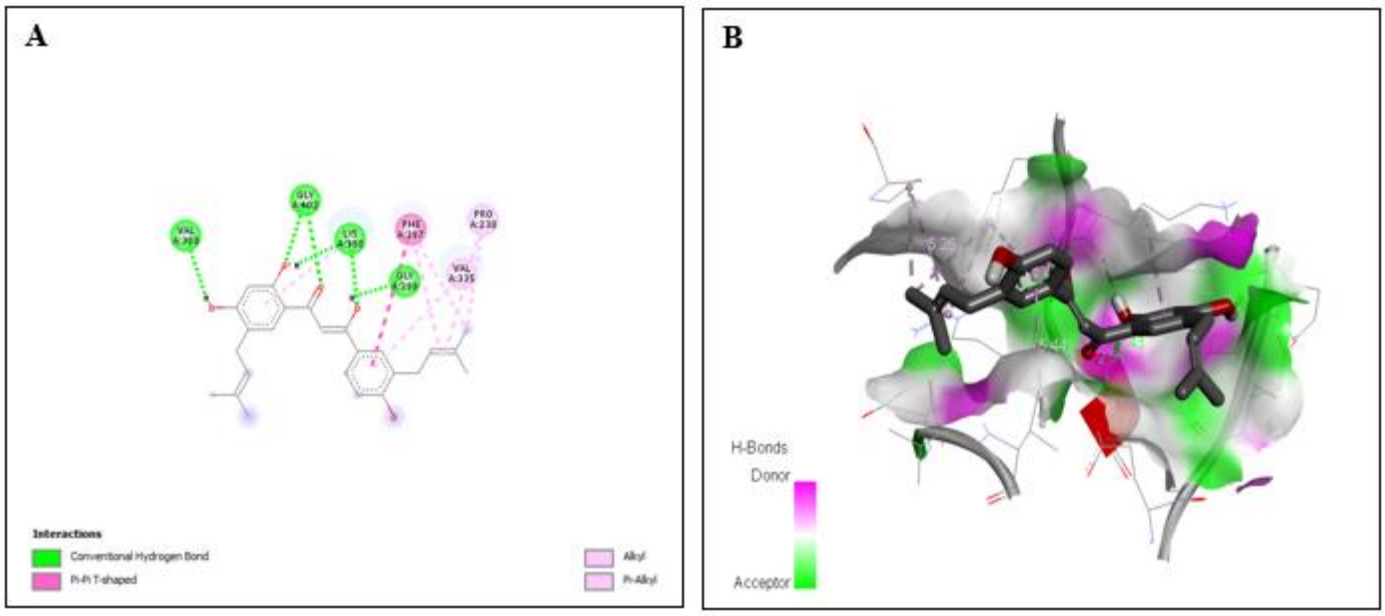


Figure 4d: Molecular docking of a receptor α-glucosidase with Glyinflanin A, (A) 2D structural interaction (B) Hydrogen bond distance.

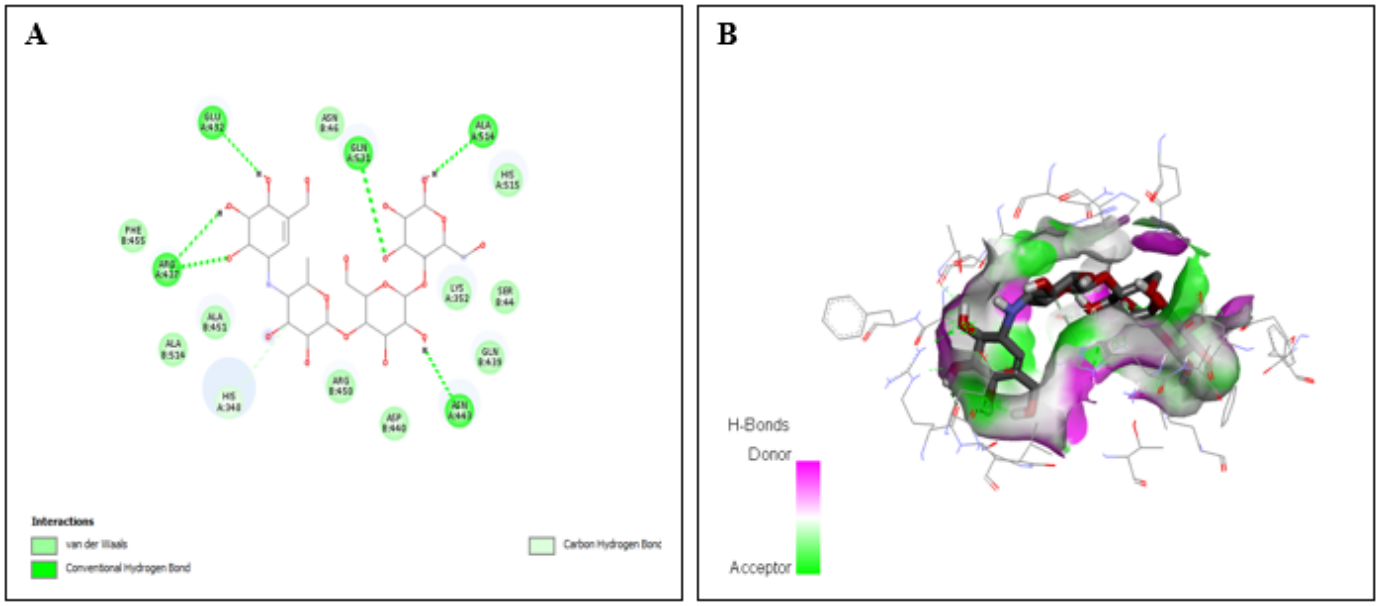


Figure 4e: Molecular docking of a receptor α-glucosidase with Acarbose, (A) 2D structural interaction (B) Hydrogen bond distance.

DISCUSSION

The key enzymes in dietary carbohydrate digestion are Pancreatic *α*-amylase and intestinal *α* glucosidase and inhibitors of both enzymes have been proven to be efficient in delaying glucose absorption and suppressing hyperglycemia. Findings of this work correlates well with previous studies conducted using *G. sylvestre*, which reported lower blood glucose level and higher level of plasma insulin in both type 1 and type 2 diabetic rats (Shanmugasundaram *et. al.*, 1990; Ramkumar *et. al.*, 2010; El Shafey *et. al.*, 2013). Controlling these major enzymes involved in starch breakdown is seen as a model pathway for developing new drugs for the treatment or prevention of diabetes. These enzymes are capable of breaking down carbohydrate into glucose, which can then be further absorbed by the body (Suttisansanee *et al*., 2016). Alpha-amylase and alpha-glucosidase have been found to work synergistically to hydrolyse carbohydrate. As a result, a molecule that targets both enzymes could be a superior option in the management of diabetes (Kaur and Singh, 2015).

The *in vitro* results revealed that all the fractions of *G. sylvestre* possess α-amylase and α-glucosidase inhibitory activity, which correlates with the findings of previous studies by Ibrahim *et al*., (2017) and Parveen, *et al*., (2019). All the fractions inhibit both α-amylase and α-glucosidase enzymes in dose-dependent manner at lower IC_{50} values of 16.78µg/ml, 23.17µg/ml, 28.22µg/ml of fractions A, B and C respectively for α-amylase (Table 1) and 2.000µg/ml, 1.58µg/ml, 0.841µg/ml of fractions A, B and C respectively for α-glucosidase (Table 2). Although, acarbose has lower IC_{50} of 3.32µg/ml for α-amylase when compared with all the fractions, it has a higher IC_{50} value of 2.38µg/ml for α-glucosidase when compared with all the fractions. Fraction A has the lowest IC_{50} of 16.78µg/ml for α-amylase inhibitory activity between the fractions, but fraction C has the best IC_{50} of 0.84µg/ml for α-glucosidase inhibitory activity between both the fractions and the acarbose (standard). The results clearly showed that the extract of *G. sylvestre* had a potent inhibitory effect and showed better activity than acarbose as a standard drug in the *in vitro* *α*-amylase and *α* glucosidase inhibition test. This *in vitro* finding suggested that the extract of *G. sylvestre* could reduce blood glucose level by inhibiting the activity of *α*-amylase and *α*-glucosidase, two important enzymes involved in the digestion of complex carbohydrates into absorbable monosaccharides units.

The *in silico* analysis in this study revealed many hydrogen bond interactions, hydrophobic interactions, interpolated charges, solvent accessibility surface, binding energy and bond length, all of which are critical for optimizing the activity of the active compounds upon or against any biological targets between all the subject under study (Meng *et al*., 2011). The proposed action's mechanism of the alpha-amylase inhibitory activity is connected to its ability to produce a sliding barrier by establishing a hydrogen bond interaction with the residues of the active or substrate binding (catalytic) region (Vilcacundo *et al*., 2017). The subjects under study were found to have inhibitory mechanism towards α-amylase.

Acarbose was found to interact with 9 and 6 amino acids in the binding sites of α-amylase and α-glucosidase respectively, however the majority of interactions occur with water molecules, with very few hydrogen bond interactions (Senthil *et al*., 2019). The superior atomic structure of acarbose (with 14 hydrogen bond donors and 19 hydrogen bond acceptors (CID 444254- National Centre for Biotechnology Information, PubChem Compound Database)) when compared to the identified ligands hydrogen bond donors and hydrogen bond acceptors (Table 3) may also obscure the true inhibitory potential of these compounds as well. Although, other factors including molecular weight,

bond length, binding affinity and hydrogen bond interactions are very important for improving active compound activities against biological targets (Meng *et al*., 2011).

In ligand-protein binding, hydrophobic interactions are critical (Ajay and Murcko, 1995). The majority of ligand binding sites have at least one hydrophobic (nonpolar) region, and many of them show a distinct preference for non-polar ligands. Hydrophilicity and loss of hydrophobicity are indicated by the negative values of logP (Table 3). As a result of which there must be a relationship between pharmacological activity and the hydrophobicity (logP). The hydrophobicity (log P) of the compounds has a direct relationship with their activity, as log P declines, activity also diminishes (Sahu *et al.,* 2008).

The solvent accessible surface (SAS) is also a valuable tool for determining the overall extent of a hydrophobic region on a molecule or at the binding site of a protein, but does not take into account the specific atom types that make up the binding site or their relative positions (Sahu *et al.,* 2008). There is a direct link between the activity of the compounds and SAS, and as the SAS declines, so does the activity. It has become impossible to sustain a hydrogen-binding network in the proximity of a huge hydrophobic item, causing the structure of water to be disrupted as well as a stronger hydrophobic interaction. The change that occurs from the hydrophobic hydration of small non-polar solutes to a high tendency for water depletion on extended nonpolar nanometer-scale length surfaces, such as those in proteins can be accounted by the Lum-Chandler Weeks theory of hydrophobicity (Lum and Weeks, 1999; Huang and Chandler, 2000).

The computer simulations and subsequent theoretical advancements have consequently revealed that capturing the increased hydrophobic attraction that would exist between a ligand and a protein with a broad or concave nonpolar surface is necessary. The shape and extent of the exposed molecule surface, as well as the polarity, determine the strength of the hydrophobic interaction. For many drug-receptor interactions, hydrogen bonding is very certainly an essential requirement. A single hydrogen bond is relatively weak and would not be anticipated to support a drug-receptor interaction alone; nevertheless, when many hydrogen bonds are established between drugs and receptors, as is usually the case, the drug-receptor interaction gain a significant amount of stability (Sahu *et al.,* 2008).

Higher molecular weight active compounds are likely to be absorbed less and reach the target more slowly, thus, delaying their effects (Senthil *et al*., 2019). The low molecular weight of these phenols (Table 3) when compared to Acarbose (645.6g/mol) adds to the potential dual inhibitory effects these compounds and fulfils the recommended criterion (< 500g/mol) for oral small molecule drug candidates (Lipinski, 2004). Furthermore, if the molecular weight is less than 500g/mol, clinical attrition rates are much lower. Indeed, adverse effect such as diarrhea, meteorism, abdominal distention and flatulence have all been recorded as side effects of acarbose (Senthil *et al*., 2019).

Therefore, molecular docking study radically confirms the inhibition of α-glucosidase and α-amylase enzymes and their binding affinity (Ahmed *et. al.,* 2014) and further substantiates the insulin-mimicking ability of compounds present in *G. sylvestre* which coincides with the findings of (Alexiou and Demopoulos, 2010; Azeez *et. al.,* 2010; Yadav *et. al.,* 2010; Okoro *et. al.,* 2014).

CONCLUSIONS

The findings of this study revealed that compounds from methanolic leaf extract of *G. sylvestre* could reduce glucose level by inhibiting the activity of *α*-amylase and *α*-glucosidase, two important enzymes involved in the digestion of complex carbohydrates into absorbable monosaccharides units, confirming the antidiabetic potential of *G. sylvestre* leaves, which have long been used for diabetes management. An *in silico* analysis suggested the mechanism behind the extract's antidiabetic properties. Therefore, phytopharmaceuticals can be developed from the plant extract.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-sectors.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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