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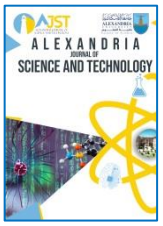

Article

In vitro and *in silico* antioxidant and antidiabetic properties of zinc oxide-chlorogenic acid nanoparticles composite

Aisha M. Albadawi [1], Amany A. Elkashef [2], Mohammed S. Nofal [1], Maysara S. El-Salakawy [1], Sofia A. Khalil [1], Shaymaa A. Abdulmalek [1], Doaa A. Ghareeb [1, *]

[1] *Bio-screening and Preclinical Trial Lab, Biochemistry Department, Faculty of Science, Alexandria University, 21511, Alexandria, Egypt.*

[2] *Chemistry Department, Faculty of Science, Alexandria University, 21511, Alexandria, Egypt.*

[*] *Correspondence Address:*

Doaa A. Ghareeb: Bio-screening and Preclinical Trial Lab, Biochemistry Department, Faculty of Science, Alexandria University, 21511, Alexandria, Egypt. Email: d.ghareeb@alexu.edu.eg.

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ABSTRACT: Despite the well-established antidiabetic effects of zinc oxide nanoparticles (ZnO), there remains a contentious debate surrounding their prooxidant and antioxidant properties. Chlorogenic acid (CGA), known for its potent antioxidant and antidiabetic effects, is hindered by its high cost and instability. In 2022, the synthesis of ZnO/CGA nanoparticles composite demonstrated remarkable antimicrobial and antioxidant properties, particularly effective against COVID-19 which is well Known as diabetes inducer. Consequently, the study's primary objectives were to employ a straightforward method to isolate CGA from coffee beans and subsequently synthesize ZnO/CGA nanoparticles. The secondary objective involved a comparative assessment of the antioxidant and antidiabetic effectiveness of ZnO/CGA-NPs against CGA, ZnO, and a ZnO/CGA mixture. Method: The extraction of CGA from green coffee seeds, utilizing a non-alcoholic method, yielded a notable 3.35% CGA. The resulting ZnO/CGA nanoparticles exhibited an aggregated structure, with CGA firmly attached to the ZnO surface through hydrogen bonds. Notably, while ZnO/CGA NPs displayed antioxidant properties slightly inferior to CGA alone, they successfully alleviated the prooxidant impact associated with ZnO. Moreover, the ZnO/CGA-NPs exhibited the highest inhibitory effects on α-amylase and α-glucosidase. This inhibitory action was attributed to the binding of ZnO/CGA on the allosteric sites of these enzymes, inducing alterations in their 3D geometric structures. In conclusion, the non-alcoholic CGA extraction method, yielding a substantial CGA percentage, presents an easily accessible procedure. The resulting ZnO/CGA-NPs emerge as a promising and effective antidiabetic compound with a moderate antioxidant effect, showcasing their potential in addressing diabetes and associated complications.

1. INTRODCTION

The daily physiological activities of the human body are usually accompanied by the production of free radicals. Then there is an abnormal series of changes in the body that are triggered once the free radicals significantly exceed the antioxidant tolerance range. When an imbalance in the antioxidant system occurs in the body that is connected with an increase of reactive oxygen species (ROS), this results in what is known as oxidative stress, Diabetes mellitus type 2 which is distinguished by insulin resistance, hyperglycemia, and hyperglycemia is directly linked with oxidative stress condition [1]. Chlorogenic acid (CGA) is a member of the hydroxycinnamic cinnamic ester family. This phenolic compound is composed of C_6-C_3 hydroxycinnamates (caffeic acid) and quinic acid, therefore it is also known as 5-caffeoylquinic acid (5 CQA). CGA has several biological activities, and it is employed in conventional medicine because it has hepato-/reno/neuro-protective effects besides that it is considered a hypolipidemic, hypoglycemic, cytotoxic agent.

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These actions may be related to its anti-inflammatory and antioxidant properties.[2].

Nevertheless, Chlorogenic acid has several intrinsic disadvantages, including its sensitivity to esterase-mediated degradation, limited lipid solubility, poor bioavailability, and chemical instability [3]. Several strategies can be used to solve this problem, one of them is transforming the compound into nanoparticles due to their advantages such as large surface area, solubility, and permeability [3]. Unroasted green coffee beans are considered a rich source of CGA, where they contain a high amount of Caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA), and feruoylquinic (FQA) acid which together form more than 80% of the total amount of chlorogenic acids (CGAs) in green coffee beans [4]. CGA has a comprehensive antioxidant mechanism because it has one carboxyl group and five active hydroxyl groups which interact with hydrogen free radicals superoxide anions and hydroxyl radicals [5]. Several different studies have shown the ability of CGAs to prevent oxidation of low-density lipoproteins (LDL) by various oxidants and are also able to prevent DNA damage in vitro. DNA damage resulting from oxidative stress is also protected by 5-CQA, which is the most important CGA in coffee and which can also scavenge hydroxyl radicals (OH) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, peroxynitrite ($ONOO^-$), and superoxide anions ($O2^{\bullet-}$) [6]. Other studies have also shown the ability of CGA during fasting to reduce the level of blood glucose (FPG), which helps reduce glucose accumulation to limit the development of diabetes, due to its ability to regulate genes poor glucose tolerance by increasing insulin secretion, which reduces their diabetes [5]. Moreover, CGA has in vitro inhibitory activity against amylase and glucosidase lower than caffeic acid and quinic acid indicating that esterification reduces the antidiabetic effect of CGA, these two enzymes are used to estimate the antidiabetic effect of compounds in vitro [7]. Zinc oxide is known as an inorganic chemical compound that is widely used in daily life, and the very increasing development in the use of nanotechnology recently, has helped to reveal new properties of zinc oxide nanoparticles [8]. Zinc oxide nanoparticles can be prepared easily, safely, and inexpensively, which is why it is the most abundant metal after iron dioxide [9]. Due to its small size, the human body can absorb it easily. So ZnO NPs can be used as anti-inflammatory, anti-aging, and antibacterial and also in wound healing and bio-imaging [10]. Also, ZnO NPs have antidiabetic action by increasing insulin secretion, improving glucose tolerance, and reducing the blood glucose level, furthermore, ZnO-NPs improve insulin signaling and sensitivity as well as glucose uptake by the liver, skeletal muscle, and adipose tissue, ZnO NPs inhibit gluconeogenesis in hepatic cells and lipolysis in adipocytes [11]. However, the use of high amounts of ZnO NPs or the administration of it for a long time led to tissue toxicity due to the activation of oxidative stress and inflammation axis leading to tissue damage by apoptosis [12]. Therefore, to increase the ZnO therapeutic index, the dose must be controlled, and its efficacy and potency must be increased, which could be done by complexity with another compound such as CGA. Abomughaid et al. (2022) synthesized ZnO/CGA NP composite and proved its antioxidant and antimicrobial activities. Furthermore, they proved that CGA eliminates ZnO-NP cellular oxidative stress and cytotoxic effects properties due to its antioxidant and anti-inflammatory properties [13]. Besides CGA being a very expensive compound, its extraction has certain disadvantages such as prolonged time for extraction, excess amount of solvent, unsatisfactory reproducibility, and poor extraction of polar substances [4].

Therefore, this study was designed to solve these problems, where CGA was extracted from coffee beans by an easy procedure, and then ZnO/CGA NPs were prepared according to Abomughaid et al. (2022) [13]. Then the antioxidant and anti-diabetic properties of ZnO/CGA NPs were confirmed and compared with ZnO-NPs, CGA, and ZnO/CGA-NPs.

2. Materials and Methods

2.1. Materials

Powdered green coffee beans (500 g), Zinc oxide nanoparticles, and Hexan were purchased from an (Egyptian local marker, Nano research lab, India, and biochem, Egypt), respectively. Chloroform (Isochem, France), Ethyl acetate, Dextrin, Maltose and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, United States), α-amylase (Fluka, Switzerland), Pancreatin enzymes (Loba Chemie, India), trichloroacetic acid (TCA) (Smart lab, Indonesia), Ferricyanide (wintchem, India), Ferric sulfate (Nasr company, Egypt), sodium nitroprusside (Research lab fine chem industries, India), N-(1-Naphthyl)-ethylenediamine (Oxford lab fine chem LLP, India) were used in this work. Other solvents and chemicals were analytical grade.

2.2. CGA extraction

The method of extraction was done according to Chien et al. (2017) [14], with some modifications. Powdered coffee beans (100 grams) were mixed with 1 liter of acidic water at 50 °C, and the temperature was gradually increased to 55° C, with constant stirring for 4 hours. The filtrate was then washed with 250 mL hexane two times, to remove fats, and then washed with 250 mL chloroform two times, to remove caffeine. The CGA in the filtrate was extracted with 500 mL of ethyl acetate two times. The extracts were combined and concentrated to dryness to obtain the final green coffee bean CGA powdered fraction (3.35 g).

2.3. ZnO/CGA nanoparticles preparation

By using ultrasound, ZnO-NPs dispersed in Milli-Q water at a concentration of 100 µg/mL were mixed with 10 mL of aqueous CGA solution of 55 µg/mL for 30 minutes. Then this mixture was stirred until the suspension turned from cloudy ZnO-NPs into pale yellow [15]. The characterization of formed ZnO/CGA NPs was performed by UV-spectrophotometer, scanning electron microscope, EDX and transmission electron microscope as described in our previous work [13].

2.4. Compound serial dilution preparation

(ZnO/CGA NPs) a stock solution of 2mg/mL was prepared in which the ratio CGA and ZnO was 0.5: 1. The serial dilution of ZnO, CGA, and their mixture were prepared with concentrations equivalent to that in ZnO/CGA NPs different concentrations used.

2.5. Antioxidant assays

2.5.1. DPPH scavenging activity

DPPH free radicals are used to measure antioxidant activity. Antioxidants prevent the autooxidation of DPPH by donating protons to stabilize it in a reduced form. Based on the method of Brand-Williams et al. (1995) [16] with some modifications, the free radical scavenging activity of the prepared compounds was estimated. A solution of 0.2 mM DPPH in methanol (0.0078 g/100 mL) was prepared, and then 100 µL of this radical solution was added to 100 µL of each compound at different concentrations, and incubated at room temperature for 30 minutes. A control sample (no radical scavenging activity) was prepared, using 100 µL of distilled water instead of the compounds that were determined. By using a microplate reader, the absorbance of samples (Abs) and control (Abc) was measured at 517 nm wavelength [17]. The following equation (1) was used to calculate the percentage of radical scavenging activity:

$$\text{DPPH radical scavenging \%} = [(Abc - Abs) / Abc] \times 100 \quad (1)$$

Where; Abc = The absorbance of control (DPPH solution), Abs = The absorbance of sample [18].

2.5.2. Nitric oxide radical scavenging assay (NO°)

The reaction of Griess_Illosvoy reagent which is composed of 2.5% phosphoric acid, 1% sulfanilamide, and 0.1% N-(1-Naphthyl)-ethylenediamine, and sodium nitroprusside (10 mM) is used to estimate NO° inhibition. The absorbance of the pink color was read at 540 nm wavelength [19].

2.5.3. Ferric Reducing Antioxidant Power assay (FRAP)

The FRAP test is a redox-dependent colorimetric test that is dependent on the molar concentrations of antioxidants present in the sample. FRAP is a highly repeatable and cost-effective test to evaluate the antioxidant activity of compounds [20]. The FRAP method was used to estimate the antioxidant activity of ZnO/CGA NPS and different parent compounds in this study, according to the methodology proposed by Celik et al, (2014) [21] with some modifications. A volume of 200 µL of different compounds was mixed with 120 µL of phosphate buffer (pH 6.6). Then, 500 µL of potassium ferricyanide ($C_6FeK_4N_6$) (1%) was added to the reaction mix, and incubated at 50 °C for 20 min. Next, 500 µL trichloroacetic acid (TCA) (10%) was added, and then was centrifuged at 3000 rpm for 10 min, after that 500 µL of supernatant was mixed with 500 µL of distilled H_2O, and 100 µL of ferric chloride (0.1%) and incubated for 15 minutes at room temperature. The absorbance was recorded at a wavelength of 734 nm [22].

2.6. Antidiabetic assays

2.6.1. Effect on α-Amylase activity

The inhibitory/stimulatory effect of ZnO, CGA, their mixture, or ZnO/CGA NPs toward α-amylase activity was assessed according to Kusano et al. (2011) [23] with some modification. The enzyme solution was prepared by dissolving α-amylase (1%) in 10 mL phosphate buffer (pH 6.9), then 1 mL of this solution diluted with 10 mL buffer again. The substrate solution was prepared by dissolving dextrin (1%) in 10 mL distilled water. 110 µL of the enzyme solution was pre-incubated with 10 µL of varying concentrations of different compounds at 37°C for 30 min. After this, 60 µL of substrate solution was added to each of them and then again incubated at 37°C for 20 min. Then, 100 µL of color reagent was added. The absorbance was measured at 490 nm [18]. The IC50 was determined graphically as the concentration of the inhibitor at which 50% of the α-amylase activity was inhibited.

2.6.2. Effect on α-Glucosidase activity

According to the method of Zhang et al. (2019) [24], the α-glucosidase inhibitory/stimulatory activity of the tested compounds was estimated with some modifications. An enzyme solution was prepared by adding 10 mL of phosphate solution (pH 7.4) to 0.1 g of α-glucosidase (1%), and then 1 mL of this solution was diluted by adding 10 mL of phosphate solution again. While the substrate solution was prepared by adding 10 mL of phosphate solution to 0.1g of maltose (1%), then it was diluted in the same way by adding 10 ml of phosphate solution. Then at 37 °C for 30 minutes, 110 µL of enzyme solution was pre-incubated with 10 µL of different concentrations of different compounds, and then 60 µL of substrate solution was added to each of them and then incubated again at the same temperature of 37°C for another 30 minutes. Then 100 µL of colorimetric reagent was added and the absorbance was measured at 490 nm wavelength [25].

2.7. Docking analyses

The inputs of the molecular docking simulation are the drug-like compounds and the suitable determined receptors. First, the chemical structures were drawn by chemsketch programme (ACD/lab release, version 12.01) and saved as MOL file formats. Open babel software (version 2.3.2) was used to convert the MOL file formats of the drug-like compounds into PDBQT file formats. Second, the suitable PDB file formats of the chosen receptors were downloaded from www.rscb.org. the SWISS-PDBVIEWER (version 4.1.0) (SPDBV) was used to minimize the energy of the downloaded proteins. At this stage, both prepared inputs were ready to be submitted to blind molecular docking process which accomplished using Autodock 4 (Autodock tool, version 1.5.6rc3) and ligplus (LigPlot+ v.2.2.8) softwares. The ligands under study were the nanosized (ZnO, CGA and ZnO/CGA) particles which docked into the proteins (1, 4 alpha glucosidase ID: 7KBR, and alpha-amylase ID: 8OR6). The resulted free energy of binding represented by the non-covalent interactions (NCIs), and the inhibition constant Ki of these three ligands under study were compared to the reference drug (acarbose) which docked into these chosen receptors as well. Biovia discovery studio analysis visualized the best poses of the ligands docked into the two proteins in addition to the 3D images that clarified the best interactions. Finally, the ligand-receptor complexes were obtained from PyMol software then were ran by ligplot+ to illustrate the hydrophobicity of the docked drug-like molecules into the proteins.

2.8. Statistical analysis

Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test using SPSS 22.0. All data were expressed as the means ± SD, and significant differences were confirmed when $P < 0.05$.

3. Results and discussion

3.1. Characterization of ZnO nanoparticles and ZnO/CGA NPs

Two CGA characteristic absorption peaks were shown at 217 and 324 nm while coffee bean CGA fraction Extract showed its characteristic peaks at 216 and 323 nm. Therefore, the coffee bean extract contains a high percentage of CGA (**Figure 1A**). Energy Dispersive X-ray spectrum showed that carbon and oxygen weight was 34.4% while Zn weight was 65.6% which indicated the CGA binds over ZnO (**Figure 1B**). **Figure** (**2B**) shows that the presence of CGA over ZnO-NPs leads to the formation of aggregation. TEM analysis confirmed that SEM results where CGA fraction is linked on the surface of ZnO-NPs lead to the formation of aggregated clusters of ZnO-CGA (**Figure 2D**) fraction nanoparticles which is due to hydrogen bonding that took place. These results are in alignment with our previously obtained results in 2022[24].

3.2. Antioxidant activity

Overproduction of ROS can lead to cellular malfunction and disruption of regular physiological processes by causing damage to proteins, lipids, and DNA, which can ultimately aid in the onset and advancement of several diseases, including diabetes [26].

Our study was designed to synthesize ZnO/CGA NPs and determine their efficacy as antioxidant and antidiabetic in comparing with ZnO, CGA, ZnO/CGA mixture. The antioxidant activity of ZnO, CGA, ZnO/CGA mixture, and ZnO/CGA NPs were estimated in a set of *in vitro* assays involving DPPH radicals, Ferric reducing power, and nitric oxide scavenging assay.

3.2.1. DPPH Radical Scavenging Activity

As the concentration of ZnO increased from 6.6 to 1320 (µg/mL), the DPPH scavenging activity decreased from 67.82 to 2.2 (**Figure 3A**) because it is well known that ZnO at high concentration could be acted as prooxidant because it is considered as 2e-/2H+ acceptors that can accelerate the oxidation of DPPH [27]. Unlike, CGA acts as an antioxidants where the antioxidant properties increased from 23.91304 to 72.17391% as the CGA concentration increased from 3.3 to 660 (µg/mL) (**Figure 3B**), In the case of ZnO/CGA mixture, when the concentration increased from 10 to 2000 (µg/mL), the DPPH scavenging activity increased from 13.5 to 78.7 % (**Figure 3C**), while in the case of ZnO/CGA NP, the DPPH scavenging activity increased from 17.3913 to 90.1% (**Figure 3D**). The most potent one was CGA because it has the lowest IC50 (27.6 µg/mL) followed by ZnO/CGA NPs (71.26 µg/mL that contained 47.5 µg/mL ZnO and 24.1 µg/mL CGA), ZnO/CGA mixture (221.87 µg/mL that contained 147.9 µg/mL ZnO and 73.97 µg/mL CGA) as shown in Table (1). CGA exerts its action through the direct hydrogen atom transfer (HAT) reaction from chlorogenic acid to free radicals, or the formation of a radical intermediate [28]. During the mixing of CGA with ZnO, CGA could eliminate the prooxidant properties of ZnO. It was noticed that the antioxidant properties of composite is threefold higher than ZnO and CGA mixture this mainly due to the fact of nanoparticles have large surface area that potentiated and unleashed the antioxidant properties of composite when compare to mixture.

3.2.2. Nitric oxide scavenging activity

Nitric oxide scavenging assay is another method used to assess the capability of compounds to eliminate the reactive nitrogen species. For ZnO, the percentage of inhibition at the concentration 6.6 µg/mL was (47%), and at 66 µg/mL was (16%), therefore, ZnO-NPs must be used in low concentrations to exert its antioxidants properties because at high concentrations it acts as a prooxidant (**Figure 4A**). While CGA has an inhibitory effect toward NO° formation, where at a CGA concentration 3.3 µg/mL, NO° production was decreased by (2.5 %) while a CGA concentration 660 µg/mL decreased the NO° production by (44.5 %) (**Figure 4B**). The CGA, IC50 was (833.35 µg/mL). The mixture of both ZnO and CGA showed low antioxidant properties (**Figure 4C**) where the IC50 was 3146.98 µg/mL that composed of 1048.99 µg/mL CGA and 2097.98 µg/mL ZnO. The NO° scavenging activity of ZnO/CGA-NPs was ranged from 3.5% to 55.3% as the concentration was increased from 10 µg/mL to 2000 µg/mL (**Figure 4D**) with IC50 (1722.52 µg/mL) which composed of 574.17 µg/mL CGA and 1148.34 µg/mL ZnO, this data showed that the amount of CGA in the composite is lower than CGA IC50. Therefore, this data confirmed that when CGA binds on the surface of ZnO by hydrogen bonds during the formation of nanoparticles strongly eliminates the prooxidant properties of ZnO and diminishes its proton acceptor properties.

3.2.3. Ferric Reducing Antioxidant Power (FRAP). Each point is the mean of three independent measurements (n=3).

Figure (5A) shows that the FRAP of ZnO had decreased from 12.06 to 7.57 as the concentration increased from 6.6 µg/mL to 1320 µg/mL. This data confirmed the prooxidant ZnO properties at high concentrations. The FRAP of CGA was increased from 12.6 to 55.5 as the concentration increased from 3.3 to 660 µg/mL (**Figure 5B**) with EC50 of 464.39 µg equivalent to ascorbic acid/mL. The same pattern was shown in the case of mixture of CGA and ZnO and ZnO/CGA-NPs (**Figure 5 C and D**, respectively) with EC50 of 4437.96 µg equivalent to ascorbic acid/mL for mixture and 480.55 µg equivalents to ascorbic acid/mL for ZnO/CGA NPs Table (1).

Our research results show that ZnO can function as an antioxidant at low concentrations, but at greater concentrations it becomes a prooxidant, because it can accelerate the DPPH oxidation, and NO° production and preventing ferric reduction by accepting 2e-/2H, while CGA acts as antioxidant because it can donate 2e-/2H for free radical and convert it to more stable reduced form. The phenolic hydroxyl structure readily reacts with free radicals and forms hydrogen free radicals with an anti-oxidant effect [5].

In the case of ZnO and CGA mixture, the functional groups of chlorogenic acid were more than ZnO, this led to competition between compounds which caused ZnO to be masked and its prooxidant activity to be reduced. In the case of ZnO/CGA-NPs, CGA adsorbed on ZnO-NPs surface by forming interaction between phenolic and carboxylate group of CGA with Zn (non-covalent interaction), This interaction formed a ground-state complex, increased CGA's surface area, and caused the functional groups of ZnO to disappear from each other, thereby increasing the antioxidant activity.

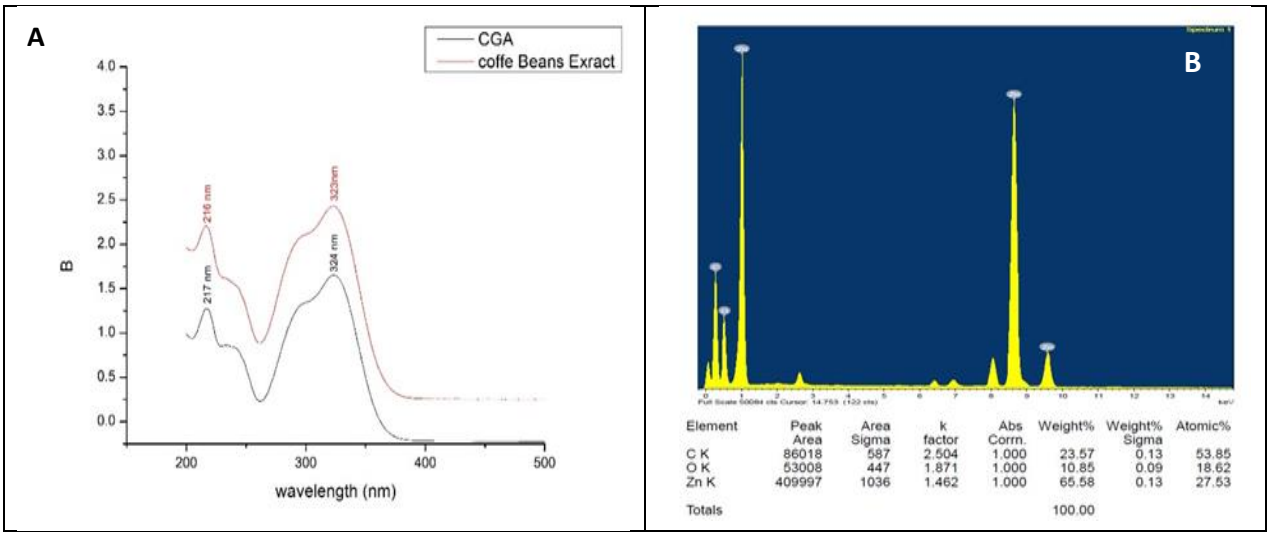


Figure 1. The UV spectrum of CGA and coffee bean CGA fraction (A) and EDX spectrum of ZnO/CGA fraction (B).

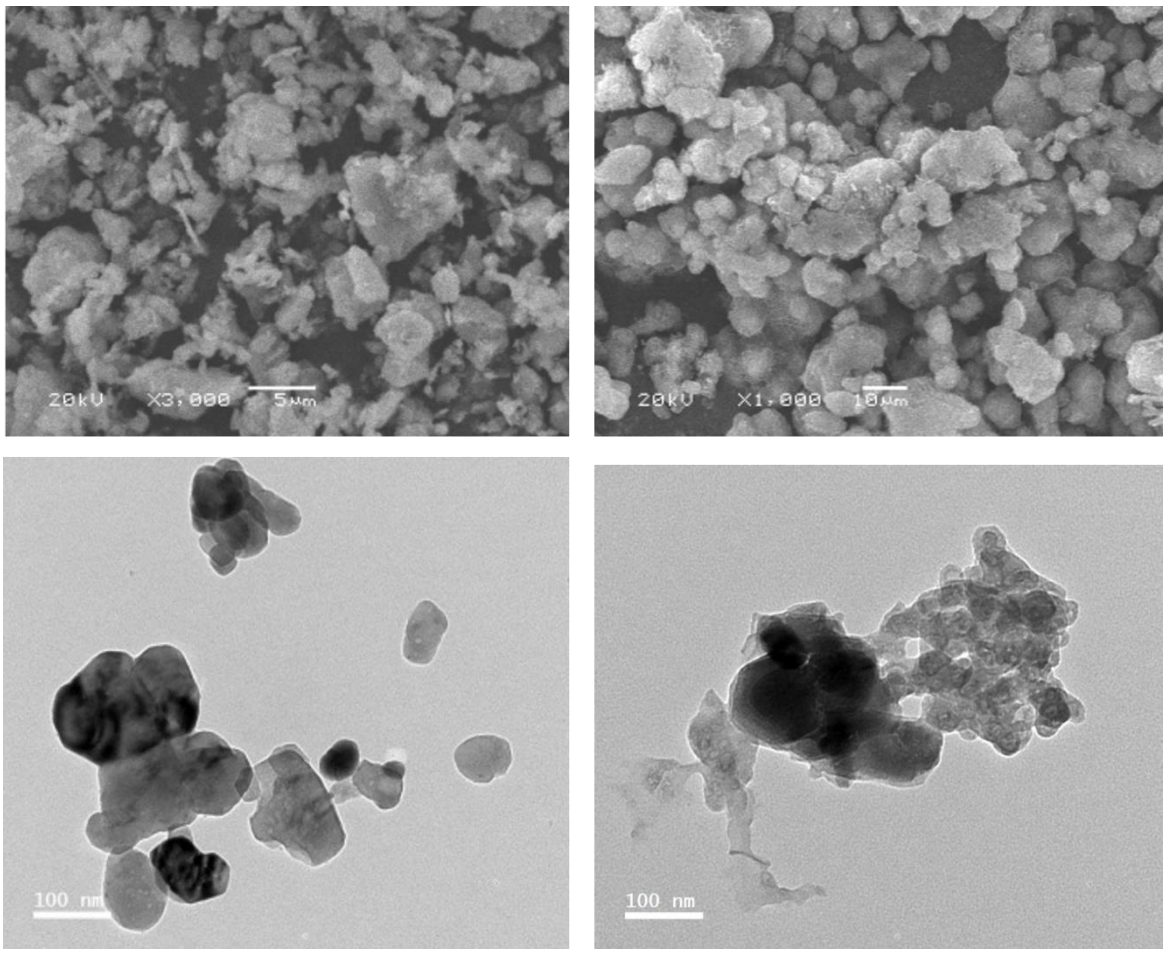


Figure 2. SEM (A and B) TEM (c and d) analysis of ZnO-NPs and ZnO/CGA, respectively.

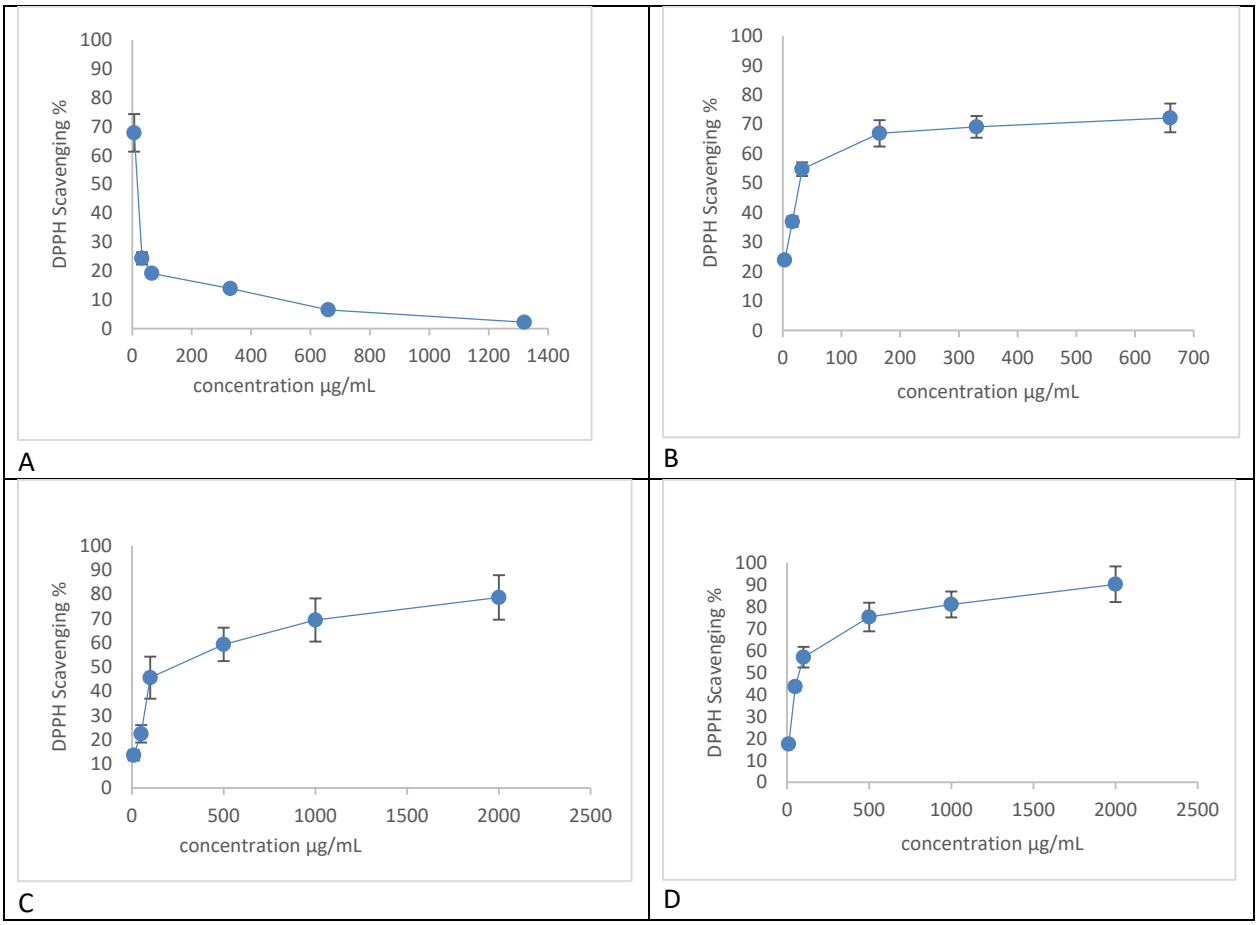
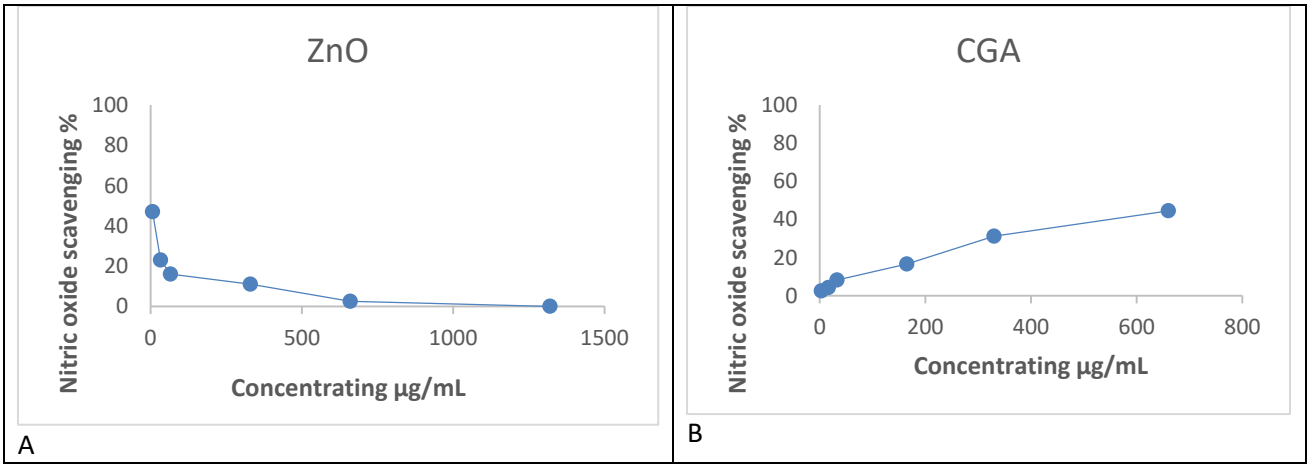


Figure 3. DPPH Radical Scavenging Activity of (A) ZnO, (B) CGA (C) ZnO/CGA mixture, (D) ZnO/CGA NPs. Each point is the mean of three independent measurements (n=3).



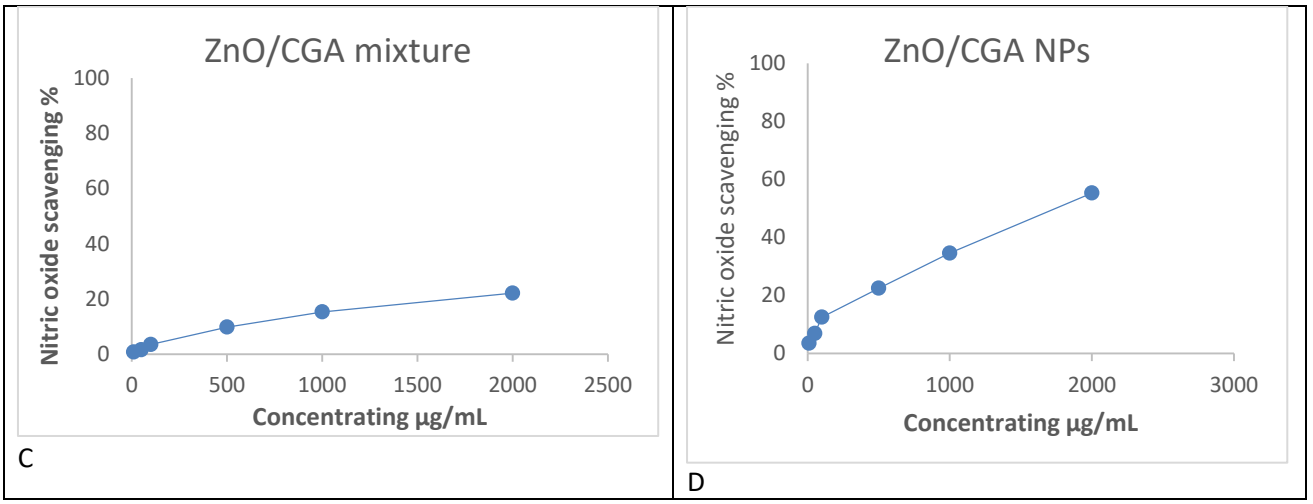


Figure 4. Nitric oxide scavenging activity of (A) ZnO, (B) CGA (C) ZnO/CGA mixture, (D) ZnO/CGA NPs.

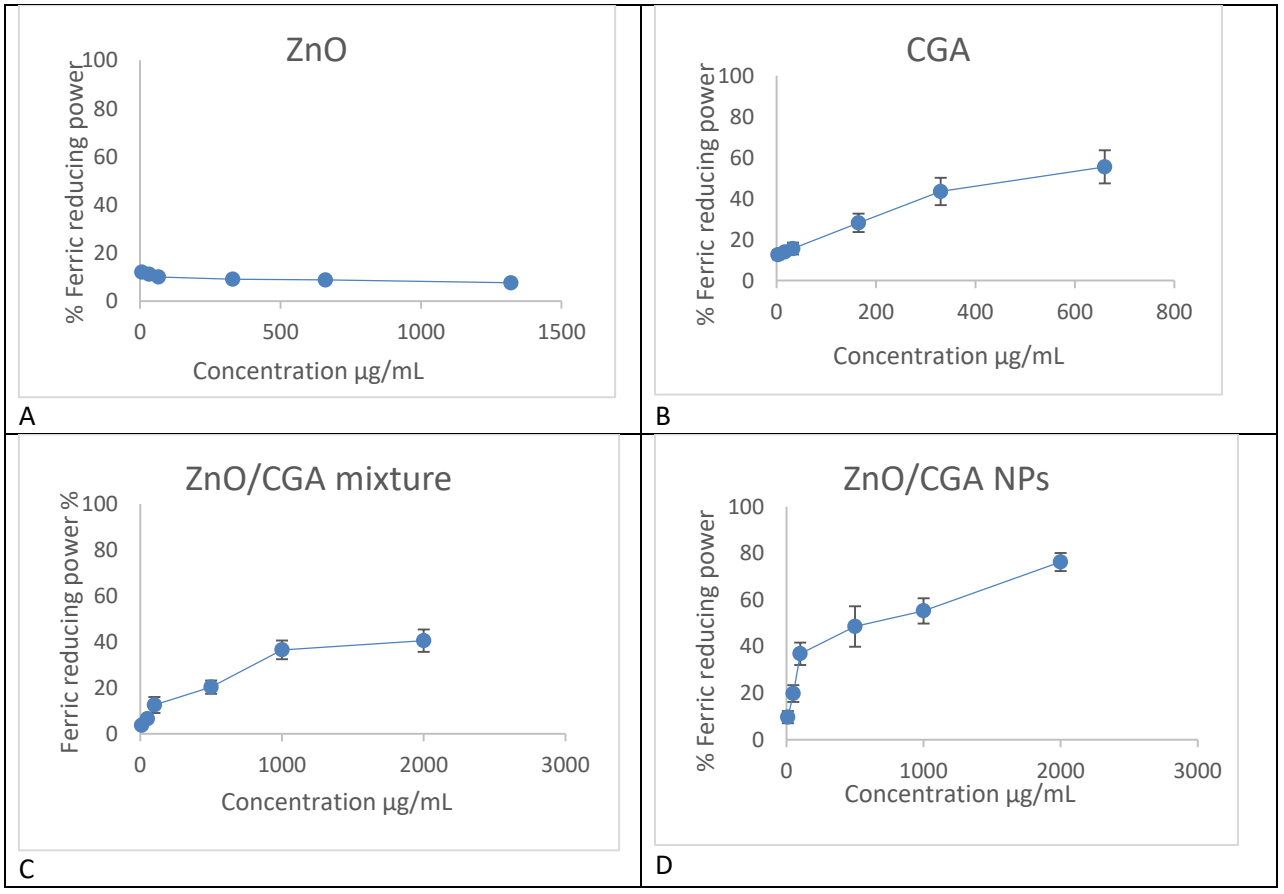


Figure 5. Ferric reducing antioxidant power for (A) ZnO, (B) CGA (C) ZnO/CGA mixture, (D) ZnO/CGA NPs. Each point is the mean of three independent measurements (n=3).

3.3. Antidiabetic activity

3.3.1. α-Glucosidase inhibition effect

One of the basic digestive enzymes that are located within the mucosal brush of the small intestine and whose role is to break down and process complex carbohydrates into simple and absorbable ones is the α-glucosidase enzyme.

The development of diabetes can be reduced by inhibiting this enzyme, which prevents the rise in blood glucose levels after eating, as its inhibition causes a delay in the absorption of glucose in the body [20]. The data represented in **Figure (6 A-D)** indicates that all tested compounds had anti- α-glucosidase effect. The most effective compound was ZnO/CGA NPs, followed by ZnO, CGA, and finally ZnO/CGA mixture, where ZnO/CGA NPs has the lowest IC50 (402.73 µg/mL) which composed of 134.24 µg/mL CGA and 268.48 µg/mL ZnO while ZnO/CGA mixture had the highest IC50 (744.49 µg/mL) as shown in Table (1). This data confirmed the synergistic effect that takes place between ZnO and CGA in composite besides the high polar surface area that potentiates the composite effect. This effect was not shown in the case of the ZnO/CGA mixture which had IC50 (744.49 µg/mL) higher than that could have occurred due to the additive effect of both compounds (630 µg/mL).

3.3.2. α-Amylase inhibition effect

One of the most important enzymes in the digestive process, which has a fundamental role in the breakdown of sugars, is alpha-amylase , which is usually found in saliva and pancreatic juice. Therefore, when eating and wanting to prevent a rise in blood glucose levels afterward, inhibiting the alpha-amylase enzyme is used as one of the solutions [24].

As shown in **Figure (7A-D)**, the α-amylase inhibitory activity increased as the concentration of each compound was increased. The most potent inhibitor was ZnO/CGA NPs because it has IC50 of 132.08 µg/mL, followed by ZnO/CGA mixture (IC50=381.56), then CGA (IC50= 581.58 µg/mL) and finally, ZnO (IC50=721.68 µg/mL), as shown in Table (1). Both the CGA/ZnO mixture and ZnO/CGA-NP showed synergistic effects toward amylase when compared to each sole effect. Our results are consistent with other studies that have reported the antidiabetic effect of ZnO NPs and CGA. Zinc nanoparticles are particularly interesting since (Zn^{2+}) ions are involved in insulin secretion by the pancreatic islets of Langerhans and they are beneficial for insulin function and carbohydrate metabolism [29], in a study conducted by (Nasab et al, 2020) on α- amylase inhibition by ZnO-NPs, 1000 µg/mL zinc oxide nanoparticles gave 78% inhibition with (IC50 = 340 µg/ mL) [29], also in another study by (Abd El-Rahman et al,2021), ZnO- NPs showed highly significant inhibition activity (38.11 ± 1.33 and 88.32 ± 0.73)% at (7.81 and 1000) µg/mL concentration, with (IC50 = 25.54 µg/ mL) [30]. Furthermore, CGA can act as antidiabetic because its hydroxyl group bind with amylase active site and blocks its action [4]. (Zheng et al,2020) proved that CGA has an inhibitory effect on α- amylase with IC50 of 0.498 µg/mL [31]. In agreement with our results, (Shan Wang et al,2022) found that CGA had α-glucosidase inhibition activity with IC50 (29.93 ± 0.73 µmol/mL) [32]. Due to the differences in the structure of α-glucosidase and α-amylase, there were different CGA inhibitory pattern toward both α-glucosidase and α-amylase, where in the case of α-amylase, the inhibition type is uncompetitive, while in the case of α- glucosidase, the type of inhibition is competitive [32]. Notably, both ZnO and CGA have an anti-diabetic effect. Hence, the combination of ZnO with CGA might produce a synergistic interaction in increasing the antidiabetic action of ZnO-CGA NPs.

3.4. The molecular docking study.

In silico analyses of the nanosized ligands coped with the experimental work and proved that the nanosized ZnO/CGA-NPs bonded with glucosidase (7KBR) and amylase (8OR6) receptors better than the reference drug, acarbose. Acarbose is authorized for treating adults with type 2 diabetes mellitus as an adjunct to diet only or diet and exercise. In the case of 7KBR, the binding energy of the best conformer of the acarbose was -6.66 Kcal/mol. The best conformer's free energy of binding was (-6.90 Kcal/mol of ZnO/CGA NPs) and (-5.36 Kcal/mol of CGA-NPs), and (-2.78 Kcal/mol of ZnO-NPs). Further for 8OR6, the binding energy of the best conformer of the acarbose was -6.12 Kcal/mol. The best conformer's free energy of binding was (-7.27 Kcal/mol of ZnO/CGA -NPs) and (-5.13 Kcal/mol of CGA-NPs), and (-2.76 Kcal/mol of ZnO-NPs) as shown in Table 2. Additionally, Table 2 shows that ZnO/CGA-NPs had the lowest estimated Ki of the two examined proteins and had the highest stability as it showed the lowest binding energy values that confirmed by in vitro assay where ZnO/CGA had inhibitory activity toward amylase (lower IC50) higher than that of glucosidase (Table 1). **Figure 8** illustrates the non-covalent interactions and hydrophobicity of the docked best poses of the under-study nanosized ligands. Table 2 listed the H-bonding between the examined nanosized ligands and the receptors. The multiple interactions with the same amino acids differentiated by mentioning their bond lengths in Angstrom unit. It is worth mentioning that some unfavourable interactions, red circled (Acceptor-Acceptor repulsion) and (positive-positive repulsion) were detected from the interaction of both CGA-NPs and ZnO-NPs with 8OR6 receptor showed in **Figure 7** which decreased the effectiveness of them to be considered as drugs. All these ligands showed up hydrophobic interactions to varying degrees when docked into 7KBR and 8OR6 receptors as shown in **Figure 8** and **Figure 9**. **Figure 10** clarified that each ligand behaved differently and occupied a different docked position compared to the other ligands. Every ligand showed multiple interactions but with different amino acids so there was no representation of circled equivalent amino acid chains.

4. Conclusions

This study highlights the successful extraction of chlorogenic acid (CGA) from green coffee beans using a non-alcoholic method, yielding a significant CGA percentage. The synthesized ZnO/CGA nanoparticles (NPs) successfully mitigated the prooxidant effect associated with ZnO and showed promising antioxidant properties. Furthermore, ZnO/CGA-NPs exhibited superior inhibitory effects on α-amylase and α-glucosidase, crucial enzymes in diabetes management. These inhibitory effects are due to the binding of all compounds with enzymes allosteric site, leading to alteration in active site 3D structure and prevent the binding of substrate. In silico data confirmed the specificity and stability of ZnO/CGA-NP to both enzymes comparing to CGA and ZnO which showed the formation of unstable bonds. Therefore, the ZnO/CGA NPs present a compelling case as an effective

antidiabetic compound with a moderate antioxidant effect. But taking into consideration, this *in vitro* study did not give any indication about compound toxicity, its pharmacokinetics, pharmacodynamics, blood brain barrier penetration ability.

Even so, this study proved that the simplicity of the non-alcoholic CGA extraction method beside the formation of ZnO/CGA-NP, making this composite a feasible and promising avenue for the development of therapeutic interventions in the context of diabetes and related complications.

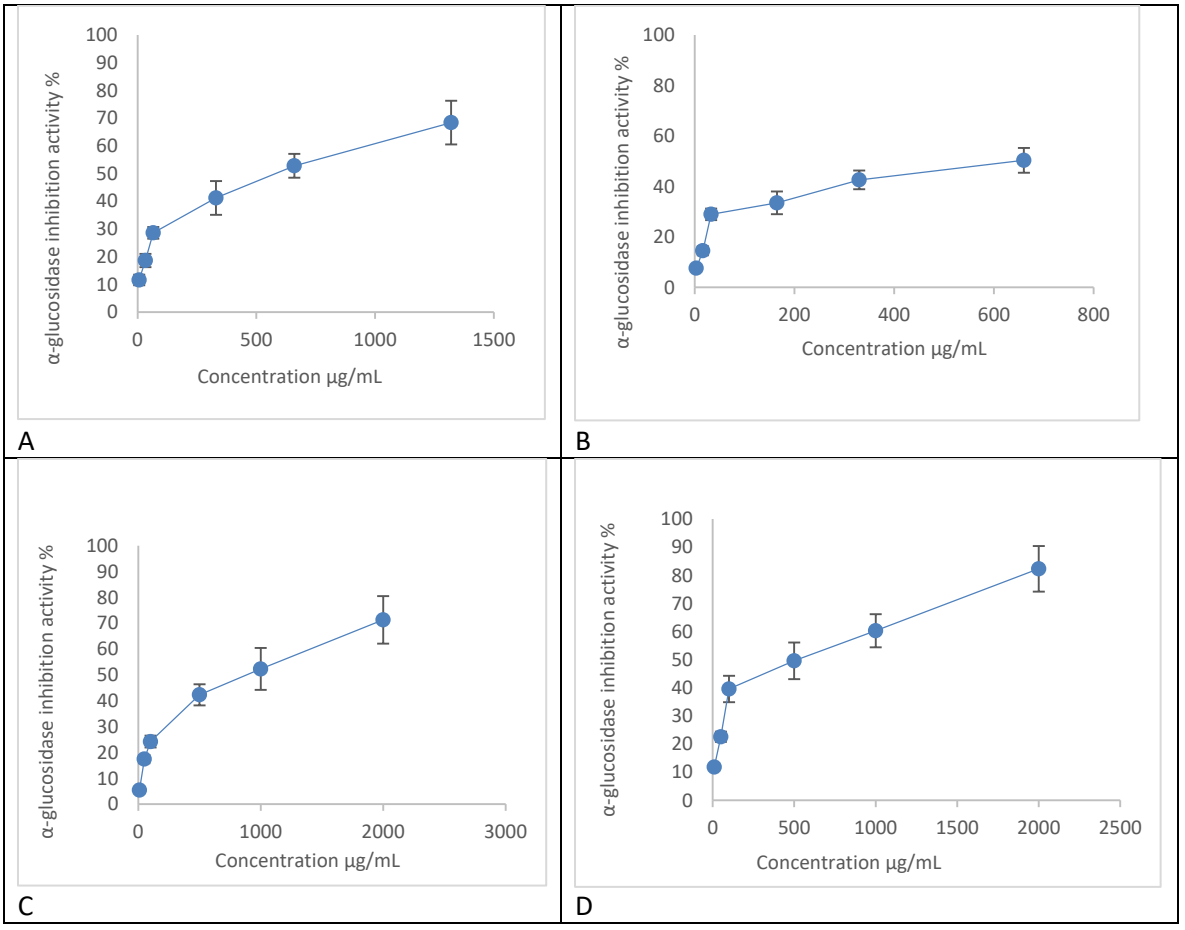


Figure 6. α-Glucosidase inhibitory activities of (A) ZnO, (B) CGA (C) ZnO/CGA mixture, (D) ZnO/CGA NPs. Each point is the mean of three independent measurements (n=3).

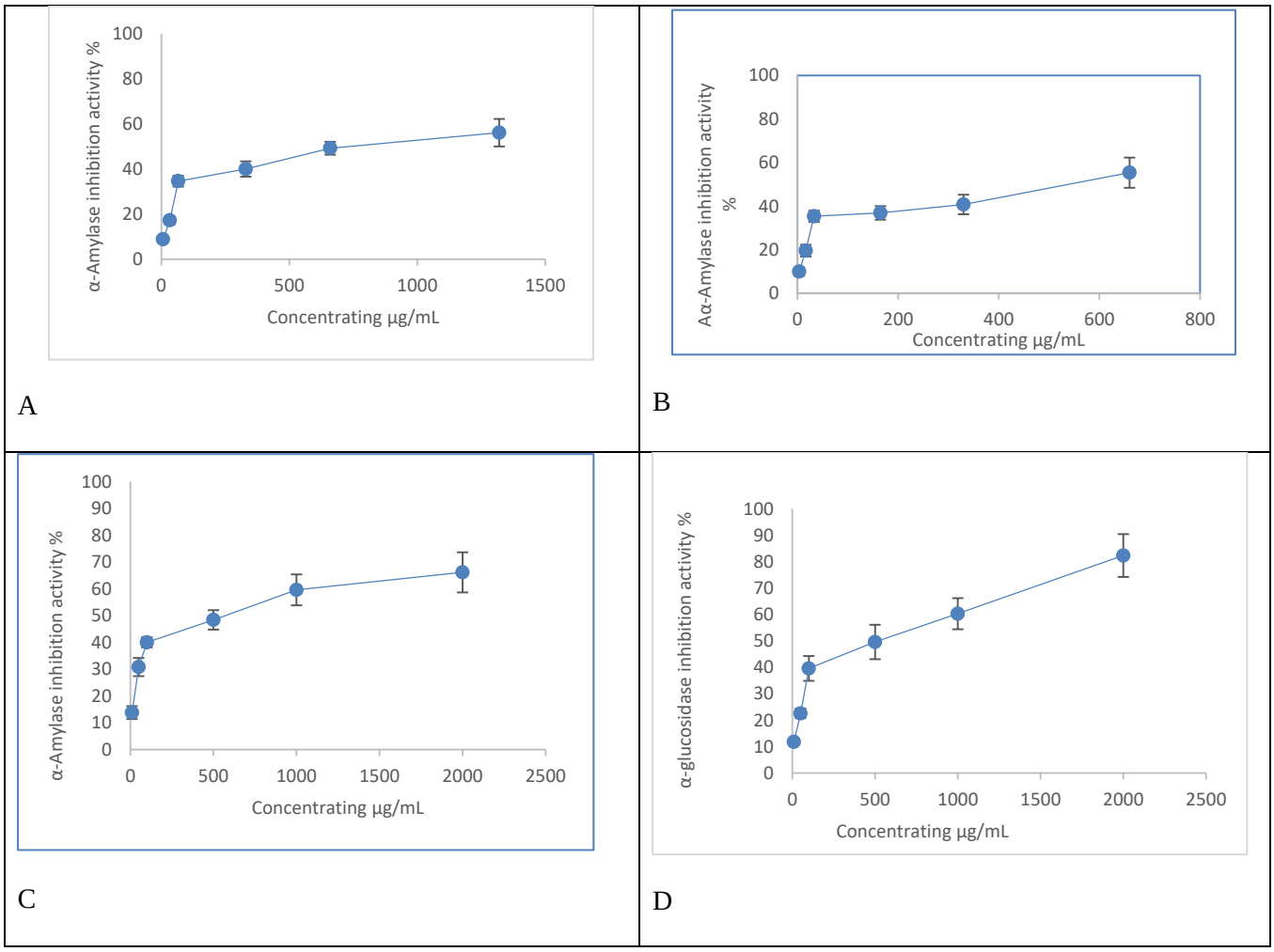


Figure 7. α-amylase inhibitory activities of (A) ZnO, (B) CGA (C) ZnO/CGA mixture, (D) ZnO/CGA NPs. Each point is the mean of three independent measurements (n=3).

Table 1. IC50 of the tested compounds against DPPH oxidation, NO production, amylase and glucosidase activity and FRAP-EC50

	IC50 (µg/mL)				EC50 (µg/mL)
	DPPH	NO	Amylase	Glucosidase	FRPA
ZnO	0 ± 0	0± 0	721.684 ± 0.15	519.1 ± 0.15	0 ± 0
CGA	27.6 ± 0.15	833.35 ± 0.15	581.58 ± 0.15	740.11 ± 0.15	464.39 ± 0.15
ZnO/CGA Mixture	221.87 ± 0.15	3146.98 ±0.15	381.56 ± 0.15	744.96 ± 0.15	4437.96 ± 0.15
ZnO/CGA-NPs	71.26 ± 0.15	1722.52 ±0.15	132.08 ± 0.15	402.73 ± 0.15	480.55 ± 0.15

The results are mean ± SD of three independent measurements (n=3).

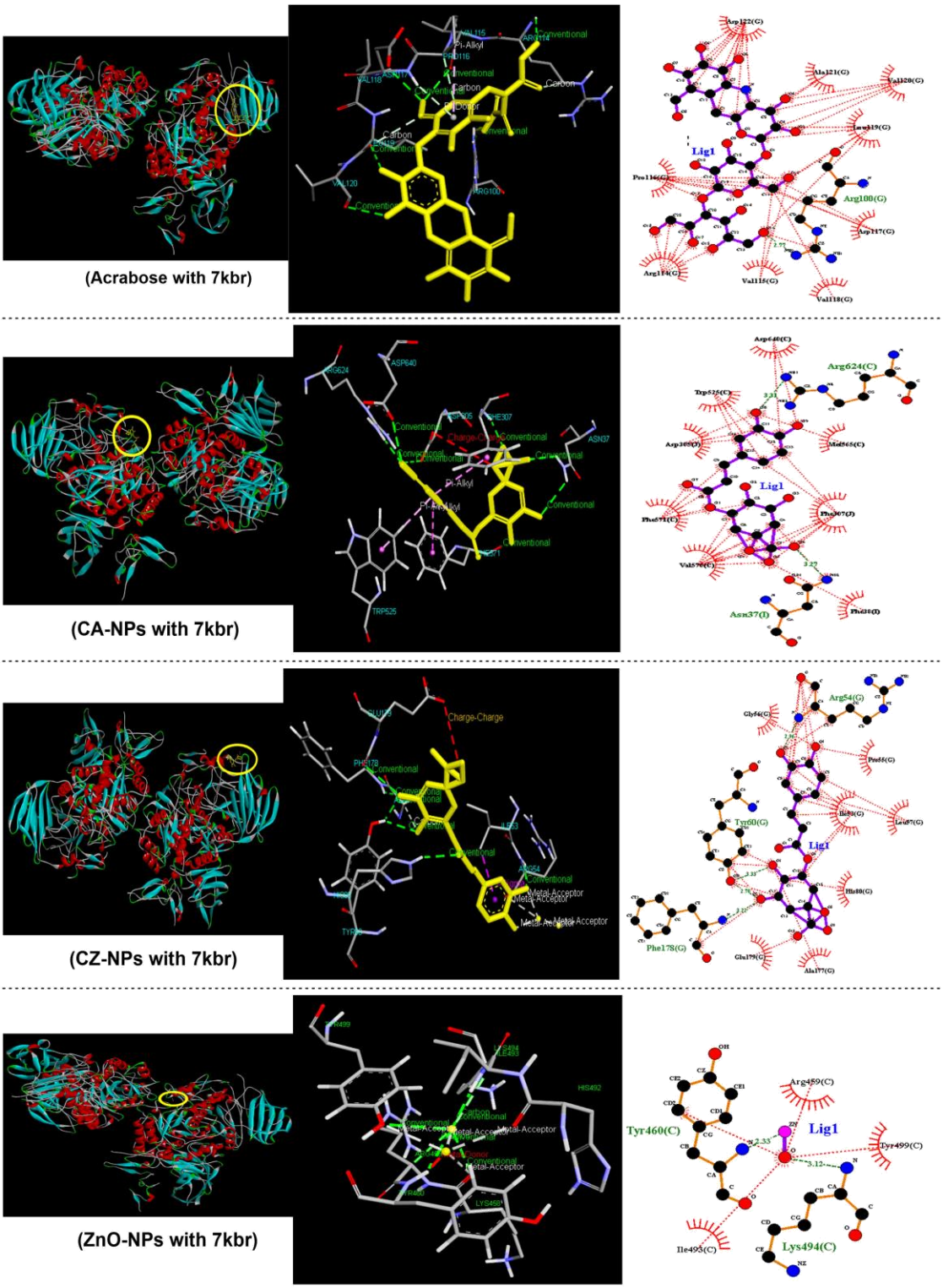


Figure 8. docked poses of the best conformations and ligplus analysis of Acarbose, CGA (CA)-NPs, ZnO/CGA (CZ_-NPs, and ZnO-NPs with 7KBR receptor.

Table 2. docking results of the tested ligands with 7KBR and 8OR6 receptors.

Parameters	acarbose	ZnO/CGA-NPs	CGA-NPs	ZnO-NPs
7KBR				
H-bonding	LIG O-ARG100(G) N LIG O-ARG114(G) N, 3.55 A° LIG O-ARG114(G) N, 2.91 A° LIG O-VAL115(G) O LIG O-VAL118(G) N LIG O-VAL120(G) O LIG O-LEU119(G) O, 3.03 A° LIG O-LEU119(G) O, 3.51 A° LIG O-ASP117(G) N LIG O-PRO116(G) N	LIG O-PHE178(G) N LIG O-TYR60(G) O, 2.70A° LIG O-TYR60(G) O, 3.33A° LIG O-ARG54(G) N LIG O-ALA177(G) C LIG O-HIS80(G) N LIG O-GLU179(G) O	LIG O-ASN37(I) N, 3.05 A LIG O-ASN37(I) N, 2.54 A LIG O-ARG624(C) N LIG O-ASP640(C) O, 2.94 A LIG O-ASP640(C) O, 3.31 A LIG O-PHE571(C) O LIG O-PHE307(J) N LIG O-ASP305(J) O	LIG Zn-TYR460(C) N LIG Zn-ILE493(C) N LIG O-LYS494(C) N LIG O-TYR499(C) O LIG O-LYS458(C) O
Binding energy Kcal/mol	-6.66	-6.90	-5.36	-2.78
Inhibition constant Ki µM	13.19	8.69	117.42	9.21
8OR6				
H-bonding	LIG O-ARG90(B) N, 2.63 A° LIG O-ARG90(B) N, 2.46 A° LIG O-HIS210(B) O LIG O-SER8(B) N, 2.19 A° LIG O-SER8(B) O, 3.15A° LIG O-ARG10(B) N, 2.06 A° LIG O-ARG10(B) N, 2.62 A° LIG O-ALA86(B) O LIG O-LYS82(B) O, 2.91 A° LIG O-LYS82(B) C, 3.10 A° LIG O-LYS82(B) C, 3.60 A°	LIG O-GLN61(A) N LIG O-TRP56(A) N LIG O-ARG291(A) C LIG O-VAL152(A) O LIG O-THR335(A) C, 3.42 A° LIG O-THR335(A) O, 2.81 A° LIG O-TRP57(A) O, 2.78 A° LIG O-TRP57(A) O, 2.91 A° LIG O-TRP57(A) C, 3.09 A° LIG O-TRP57(A) N, 3.59 A°	LIG O-GIN61(B) N LIG O-LYS51(B) N	LIG Zn-ARG291(A) N, 2.26A° LIG Zn-ARG291(A) N, 2.90A° LIG O-PHE330(A) N LIG O-SER329(A) N
Binding energy Kcal/mol	-6.12	-7.27	-5.13	-2.76
Inhibition constant Ki µM	32.84	4.71	174.44	9.53

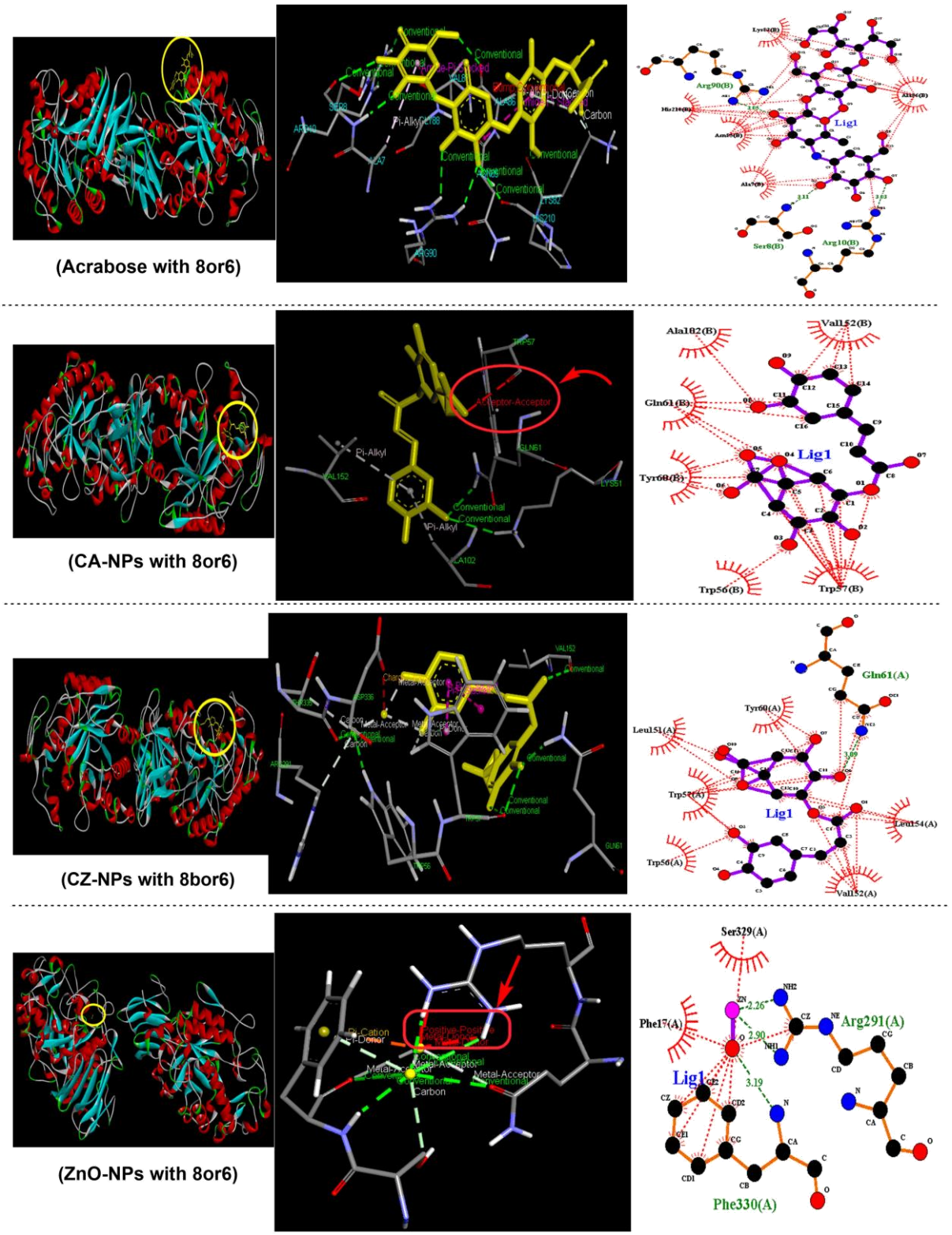


Figure 9. docked poses of the best conformations and ligplus analysis of acarbose, CGA (CA)-NPs, ZnO/CGA (CZ)-NPs, and ZnO-NPs and 8OR6 receptor.

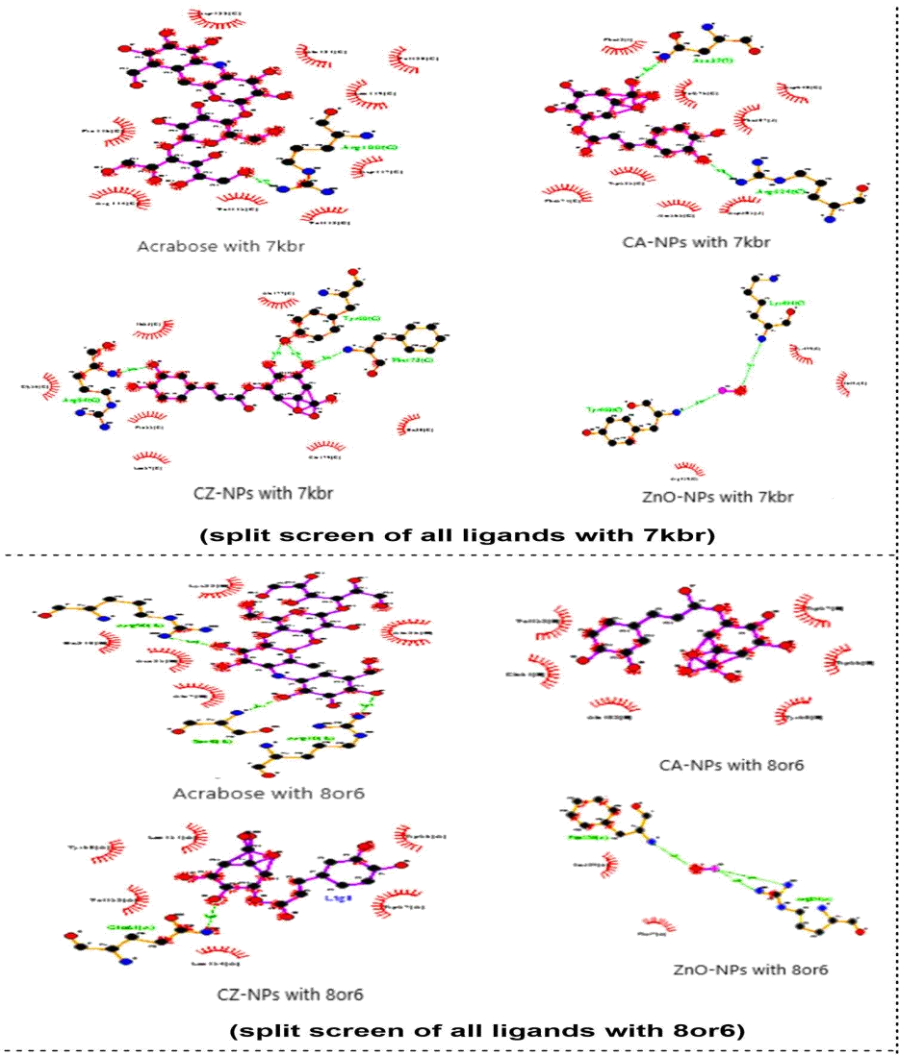


Figure 10. Orientation and interaction of each compound with 7KBR and 8OR6 amino acids.

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