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Estimation of the Kinetic Parameters of the Inhibition of Tyrosinase by an Extract of *S. Mombin* (Root Bark) and the Investigation of Likely Interactions of Composite Phytochemicals Using Molecular Docking Calculations

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Abstract The GCMS analysis of the ethyl acetate fraction of crude aqeous methanol extract of the root bark of *Spondias mombin* revealed the composition of 18 compounds of which two methyl esters of long chain carboxylic acids (methyl palmitate and (*E*)-9-octadecenoic acid methyl ester) account for 52% of the entire extract both having % peak area of 25.6% and 26.4% respectively. The ethyl acetate fraction of the *S. mombin* aqeous methanolic extract inhibited tyrosinase from *Agaricus bisporus* (mushroom) with an IC_{50} of 1.016 ± 0.003 mg/ml which was 25 fold higher than that of kojic acid which was used as a standard inhibitor of tyrosinase in a control experiment with an IC_{50} of 0.04 ± 0.006 mg/ml. The interaction between the EtOAc fraction of *S. mombin* and tyrosinase was investigated through fluorescence quenching studies. The fluorescence emission spectra of tyrosinase were recorded in the range of 300 – 500 nm with the excitation and emission wavelengths of tyrosinase at 290 nm and 345 nm respectively. The Intrinsic fluorescence quenching indicated that the test fraction interacted and quenched the fluorescence intensity of tyrosinase in a concentration dependent manner. Kinetic studies with the extract showed that the test fraction elicited a competitive mode of inhibition for the tyrosinase (from *A. bisporus*). The 3D structures of the 18 compounds detected as constituents of the fraction from GCMS analysis were generated and prepared for docking using a combination of software packages (ChemDraw Ultra 12.0 and MGL tools v1.5.4) and docked (using autodock vina v.1.1.2) with the 3D, X-ray crystallographic structure of the protein (obtained from the protein databank, rcsb.org, pdb code 2Y9X) in order to estimate their binding affinity and interactions with the protein. The docking calculations revealed that five compounds out of the eighteen had higher binding energy (-5.8 kcal/mol to -7.5 kcal/mol) relative to that of the standard, kojic acid (-5.6 kcal/mol). The compound identified to have the highest binding affinity for the tyrosinase is (E)-4-((4-(2-hydroxybenzamido)phenyl)amino)-4-oxobut-2-enoic acid with a binding energy of -7.5 kcal/mol.

Keywords: *tyrosinase, fluorescence quenching, molecular docking, kinetic studies*

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1. Introduction

Tyrosinase is a metalloenzyme, which is ubiquitous in nature. It is the critical and rate-limiting enzyme in the biosynthesis of melanin, it catalyzes the hydroxylation and subsequent oxidation of tyrosine. Melanin is the major biological compound that protects the skin from the deleterious effects of UV radiation in humans, however over production results in a number of skin pigmentation disorders such as age spots, freckles, melisma [1,2,3,4] as well as cancer [5] and post inflammatory melanoderma [6]. Tyrosinase is also linked to Parkinson's and other neurodegenerative diseases [7], oxidizing excess dopamine to produce DOPA quinones, highly reactive compounds which induce neuronal damage and cell death [8]. Several tyrosinase inhibitors such as hydroquinone, arbutin, kojic acid, azelaic acid, *L*-ascorbic acid, ellagic acid, and tranexamic acid have been used as skin-whitening agents and all have certain drawbacks [9].

Plants being rich sources of bioactive chemicals, which are mostly free from harmful side-effects, have become the focus of researchers interested in developing new potent antityrosinase inhibitors with little or no side effects. It is expected that lead compounds from plants, as

tyrosinase inhibitors may be obtained which will then be optimized by synthetic modifications. *Spondias mombin* Linn belongs to the family *Anacardiacae*. It grows in the rain forest and in the coastal areas. Extracts from various parts of the plant are commonly used in folk medicine to produce a wide variety of remedies. *S. mombin* has been scientifically proved to exhibit antimicrobial, antibacterial, antifungal, and the antiviral properties [10]. The plant extract has been demonstrated to contain mainly phenolic derivatives which have been shown to possess antioxidant and anti-inflammatory properties [11]. In the study by Elufioye e*t al.*, 2009 [12], it was observed that the crude methanolic extract from the leaves of *S. mombin* exhibited both anti-cholinesterase and anti-butyrylcholinesterase activities. In a similar study, by the same authors, involving the leaf extract of *S. mombin*, the anxiolytic effect, sedative, antiepileptic and antipsychotic effects of the leaves extract in mice and rats were also established [13]. The aim of this study is to investigate the potential antityrosinase properties of the ethylacetate fraction of the root bark of *S. mombin* and study the intrinsic fluorescence quenching of the extract, estimate the kinetic parameters and establish the mode of binding of the extract to the protein. The components of the fraction studied will be identified using GC-MS and docked with the tyrosinase to estimate the binding affinity of these components with the protein in a bid to ascertain the effect of each component on the protein and identify potential clinically useful tyrosinase inhibitors.

2. Materials and Methods

2.1. Chemicals and Reagents

Lyophilized Mushroom Tyrosinase (M.W. 128 kDa), L-3, 4 dihydroxyphenylalanine (L-DOPA) and kojic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All reagents used in the *in-vitro* experiment were obtained from commercial sources and were of analytical grade.

2.2. Preparation of Plant Material

The root bark of *Spondias mombin* was obtained from the botanical garden, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria. The plant material was authenticated at the IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile Ife. Dried samples of the plant material (root bark of *Spondias mombin*) were ground to a coarse powder and extracted with 70% methanol by maceration. The hydroalcoholic extract obtained was concentrated in *vacuo* at 40°C and the concentrate partitioned using 100 ml (3×) of the following solvent, n-hexane, dichlorommethane, ethylacetate (EtOAc) and n-butanol (n-BuOH) consecutively in a separating funnel. The ethylacetate solvent fraction was further concentrated and freeze-dried to obtain an ethylacetate fraction of the crude extract with a yield of 15.32 %

2.3. Tyrosinase Inhibitory Activity

Anti-tyrosinase activity of the EtOAc fraction of *S. mombin* was performed as described by Ashraf *et al.*, 2014 [14] with slight modification. The reaction mixture containing 140 mL of phosphate buffer (0.1M, pH 6.8), 20 mL of mushroom tyrosinase (40 U/ml) and 60 ml of varying sample concentrations were added to a 96-well microtitre plate and incubated for 10 min at room temperature. After incubation, 40 mL of L-DOPA (3, 4-dihydroxyphenylalanine) (3 mM) was added and incubated at room temperature for 20 min. Subsequently the absorbance of dopachrome was measured at 475 nm using a micro plate reader. Kojic acid was used as a reference inhibitor and for negative tyrosinase inhibitor, phosphate buffer was used. The % inhibition of the tyrosinase activity was estimated using the formula shown below.

$$\%\ \text{inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}.$$

The IC_{50} value of the EtOAc fraction of *S. mombin* was calculated by linear fitting.

2.4. Estimation of Kinetic Parameters

The estimation of kinetic parameters (K_m, V_{max}), were carried out using varying concentration of the substrate at a fixed concentration of the sample (inhibitor). The assay was carried out at three different sample concentrations and the kinetic parameters were obtained from the non-linear regression fit of the Michaelis-Menten plot and Lineweaver-Burk plot using Graph Pad Prism 5.0 The assay mixture contained 20 µl of 40 U/ml of mushroom tyrosinase, 100 µl assay buffer (0.1M phosphate buffer, pH 6.8) and 10, 30 and 60 µl of extract solution to give a final concentration range of 0.16, 0.50 and 1.0 mg/ml respectively in the reaction mixture. The concentrations of the substrate ranged from 0.6-3 mM of L-DOPA. Formation of DOPAchrome was continuously monitored at 475 nm for 5 min at a 30 s interval in the microplate reader after addition of enzyme. The inhibition type was determined by Lineweaver Burk plots of the inverse of velocity (1/V) versus the inverse of substrate concentration 1/[S] mM.

2.5. Intrinsic Fluorescence Studies

Fluorescence quenching studies were performed as described by Kim *et al.*, 2006 [15] with slight modification. The fluorescence intensities were recorded using a Hitachi F-450 spectrofluorophotometer with an excitation wavelength of 280 nm. The total reaction mixture (2ml) consists of a solution of 0.1 M phosphate buffer (pH 6.8), containing a constant concentration of 0.25 ml of 1.08 µM mushroom tyrosinase with different concentrations of the EtOAc fraction of *S. mombin* (0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 mg/ml). The change in the fluorescence emission intensity was measured following the addition of *S. mombin* to tyrosinase, and fluorescence quenching was estimated by the Stern–Volmer equation;

$$\frac{F_0}{F} = 1 + K_q \sigma_0 [Q] = 1 + K_{SV} [Q]$$

Where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively, K_q is the

bimolecular quenching constant σ_0 is the lifetime of the fluorophore in the absence of the quencher, [Q] is the concentration of the quencher, and K_{SV} is the Stern–Volmer quenching constant. Hence, the above equation is applied to determine the K_{SV} using linear regression of a plot of F_0/F against [Q].

2.6. Gas Chromatography and Mass Spectrometry (GC-MS) Analysis

The analysis of the EtOAc fraction of *S. mombin* obtained was performed using Agilent GC-7890A series Gas Chromatograph with a column of specification, DB-1 fused silica capillary column (30 x 0.25 mm i.d, film thickness 0.25 µm). The initial oven temperature was held at 80 °C for 5 min, and increased at the rate of 15 °C/min to 250 °C. Helium was used as the carrier gas at a flow rate of 1 ml/min, and the sample size was 0.1 µl, split ratio, 50:1. The percentage composition of the EtOAc fraction was determined with a Class-GC computer programme and the relative percentages of the chemical constituents were expressed as percentages by peak area normalization. For the GC-MS detection a tandem Mass Spectrophotometer (Model 5975C VLMSD), using the injector (Model 7683B Agilent Technology) with an electron ionization system with ionization energy of 70 eV was used. The samples were diluted at a ratio 1:100, v/v in dichloromethane and 1.0 µl were injected manually in the split-less mode. Identification of volatiles components was based on GC retention indexes calculated by using n-hydrocarbons and mass spectra by computerized matching of compounds with the National Institute of Standards and Technology (NIST) and by comparison of the fragmentation patterns of the mass spectra with those reported in literature.

2.7. Molecular Docking Studies

The compounds identified from the GC-MS analysis as being components of the EtOAc fraction of *S. mombin* were docked with a tyrosinase protein from *Agaricus bisporus* in a bid to investigate the interaction of the chemical constituents of the EtOAc fraction of *S. mombin* and estimating their binding energy with the protein using AutoDock Vina (1.1.2). The 3D crystal structure of *Agaricus bisporus* tyrosinase (PDB ID 2y9x) was obtained from the protein databank (www.rcsb.org). The compounds identified from the GC-MS analysis of the EtOAc fraction of *S. mombin* were converted to 3D structures and energy minimized using the Chemdraw Ultra 12.0 and Chem3D Pro 12.0 program. For the docking of ligand molecules to the tyrosinase protein structure, search space coordinates were provided to the AutoDock Vina using AutoDock Tools [15,16]. The dimensions of the grid box were set in a manner to ensure that the ligand could bind to all the potential binding sites of the protein and, hence, provide the best binding conformation. Default values provided by the program were retained for the rest of the parameters. The number of grid points in xyz was set to the spacing value equivalent to 0.375 Å and the grid center to -9.780, -24.50 and -39.814. The output was obtained in the form of binding energies (Kcal/mol) and the best binding conformations. The binding conformations with the lowest energy coefficients were selected and visualized in the PyMOL molecular graphics interface. Spatial (3D) and linear (2D) interaction maps were studied in order to determine the amino acids involved in the ligand-protein interactions. Kojic acid as prepared for docking in the same manner as the other compounds and docked with the protein as reference.

3. Results and Discussion

3.1. Tyrosinase Inhibition Assay

In the tyrosinase inhibition assay, the EtOAc fraction of *S. mombin* obtained showed a dose dependent inhibitory effect against mushroom tyrosinase activity. Moreover, the inhibition efficiency of the fraction (IC_{50} value of 1.016 ± 0.003mg/ml) was compared to the standard, kojic acid (IC_{50} of 0.04 ± 0.006 mg/ml), and found to be 25 fold higher. The IC_{50} value of the fraction shows that the kojic acid is still a better inhibitor of the tyrosinase relative to the fraction. This result serves as a preliminary screening for potential antityrosinase agents present as a chemical constituent of the EtOAc fraction of *S. mombin*.

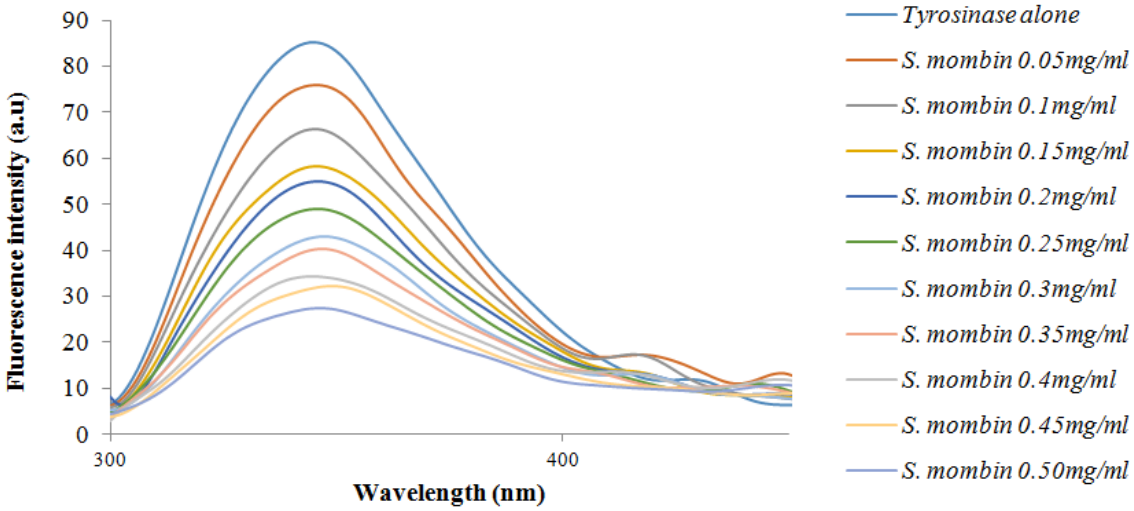

Figure 1. Chart showing the gradual reduction of the fluorescence intensity of *A. Bisporus* tyrosinase as the concentration of the EtOAc fraction of *S. mombin* increased

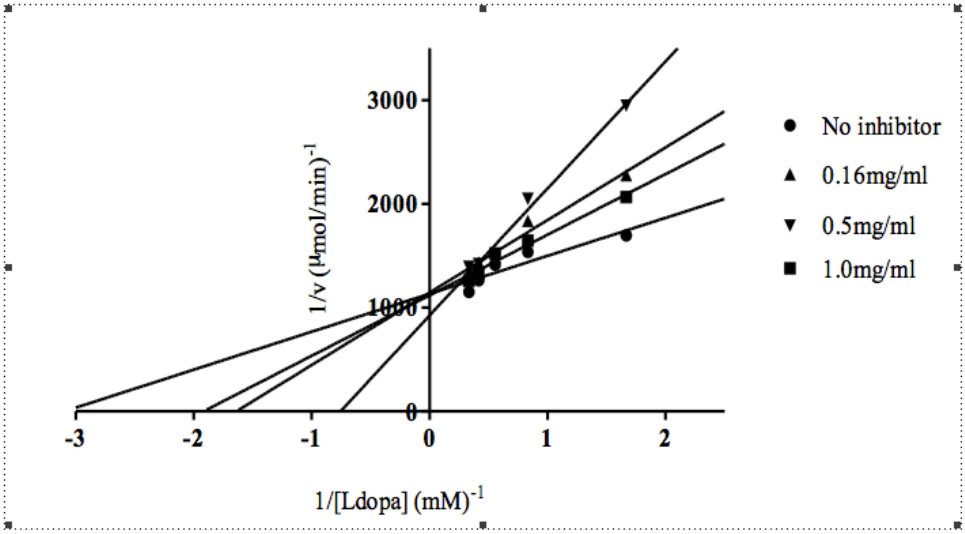

Figure 2. Lineweaver-Burk Plot of Inhibition on *A. Bisporus* tyrosinase by EtOAc fraction of *S. mombin*

3.2. Kinetic Analysis

The inhibition kinetic behavior of the tyrosinase by the EtOAc fraction of *S. mombin* at different substrate concentration was evaluated through Lineweaver Burk plot (Figure 2). The change in both K_m and V_{max} values was observed in lineweaver burk plot, which tends towards a competitive type inhibition as evidenced by the marked increase in slope and intercept on the coordinate (1/[1/S]) axis at all concentrations of inhibitor used for the assay. This ultimately brought about a change in the K_m values at different concentrations of the inhibitor used (Table 1)

Table 1. Kinetic Constants of Mushroom tyrosinase by EtOAc fraction of *S. mombin*

	No inhibitor	0.16 mg/ml	0.5 mg/ml	1.0 mg/ml
V_{max}	0.001232	0.001427	0.001262	0.001139
K_m	0.7307	1.555	1.591	1.735

3.3. Intrinsic Fluorescence Quenching Study

The interaction between the EtOAc fraction of *S. mombin* and tyrosinase was investigated through fluorescence quenching studies. The fluorescence emission spectra of tyrosinase were recorded in the range of 300-500 nm with the excitation and emission wavelengths of tyrosinase at 290 nm and 345 nm respectively. Figure 1 shows that increasing the concentration of the fraction caused a decrease in the intrinsic fluorescence intensity of tyrosinase in a dose dependent manner. A linear Stern–Volmer's plot (Figure 3) was obtained and the constant (K_{Sv}) was estimated to be 3.708 mg/ml which indicates the interaction of the EtOAc fraction of *S. mombin* with tyrosinase; the binding affinity towards the tertiary structure of the tyrosinase enzyme was thus determined by intrinsic fluorescence study.

Table 2. Interactions between selected compounds from the EtOAc fraction of *S. mombin,* kojic acid (standard) and the active site of *A. bisporus* tyrosinase

Compound	Binding energy (kcal/mol)	Binding Site	Interactions	
			Polar	Key residues
1	5.5	Cu-Cu domain	-	HIS 61, HIS 259, ASN 260, HIS 263, PHE 264, MET 280, GLY 281, SER 282, VAL 283, ALA 286, CU 400, CU 401
3	5.8	Cu-Cu domain	GLY 281, VAL 283	HIS 244, VAL 248, ASN 260, HIS 263, PHE 264, MET 280, GLY 281, SER 282, VAL 283
6	6.4	-	-	GLN 307, THR 308, TYR311, GLU356, ASP 357, TRP 358, GLU 359, PHE 368, LYS 376, LYS 379
7	5.8	Cu-Cu domain	-	HIS 61, HIS 85, HIS 244, VAL 248, GLU 256, HIS 259, ASN 260, HIS 263, PHE 264, GLY 281, SER 282, VAL 283, ALA 286, CU 401
9	5.8	Cu-Cu domain	-	HIS 61, HIS 85, HIS 244, VAL 248, GLU 256, HIS 259, ASN 260, HIS 263, PHE 264, GLY 281, VAL 283, ALA 286, CU 401
16	7.5	Cu-Cu domain	-	HIS 61, HIS 85, HIS 244, VAL 248, GLU 256, HIS 259, ASN 260, HIS 263, PHE 264, VAL 283, ALA 286, CU 401
Kojic acid (standard)	5.6	Cu-Cu domain	MET 280 CU 400 CU 401	HIS 61, HIS 85, HIS 259, ASN 260, HIS 263, PHE 264, MET 280, GLY 281, SER 282, VAL 283, ALA 286, PHE 292, HIS 296, CU 400, CU 401
Tyrosinase active site				HIS 61, CYS 83, HIS 85, HIS 94, HIS 244, HIS 259, ASN 260, HIS 263, RESIDUES 279 – 282, ALA 286 HIS 296 , CU 400, CU 401

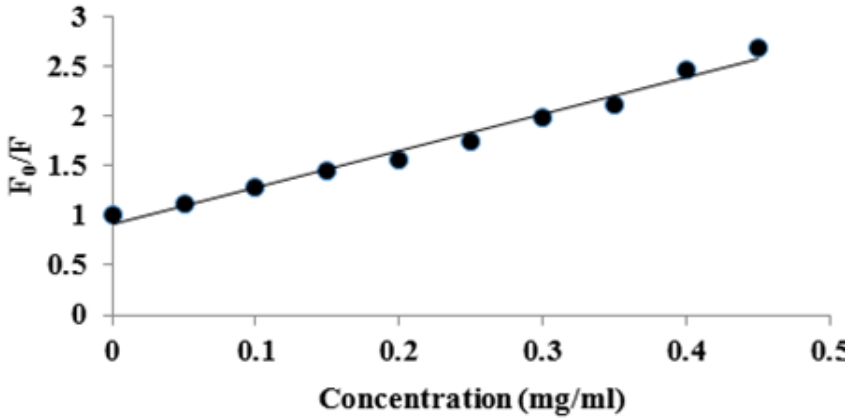

Figure 3. A linear Stern-Volmer's plot including the interaction of the EtOAc fraction of *S. mombin*

3.4. Gas Chromatography Mass Spectrum (GCMS) Analysis of the Ethyl Acetate Fraction of *Spondias mombin*

The GC-MS analysis revealed the presence of 18 compounds present in the EtOAc fraction of *S. mombin* using GC-MS analytical methods and literature comparison. The major constituents are (E)-9-octadecenoic acid methyl ester (%PA: 26.4) and methyl palmitate (%PA: 25.64) along with other minor constituents also present (Table 3). All the chemical constituents were subjected to molecular docking study.

Table 3. Chemical constituents of the EtOAc fraction of *S. mombin*

SN	Compounds	% Peak area	Retention Time
1	p-Xylene	1.72	7.428
2	D-limonene	0.82	14.356
3	2, 4-bis (1, 1-dimethylethyl) Phenol	0.81	31.316
4	(E)-2-tetradecene	2.59	33.617
5	Dodecane	2.42	33.83
6	(1-methyldecyl)-benzene	1.23	36.783
7	(1-propylnonyl)-benzene	0.92	37.545
8	5-octadecene (E)-	7.03	38.166
9	(1-pentyloctyl)-benzene	1.22	38.244
10	9-heptadecanone	1.27	38.975
11	Methyl palmitate	25.64	39.415
12	1-nonadecene	11.21	39.886
13	10,13-octadecadienoic acid methyl ester	8.47	40.522
14	(E)-9-octadecenoic acid methyl ester	26.4	40.562
15	1-heptacosanol	3.15	42.337
16	4-[4-[(2-hydroxybenzoyl)amino]anilino]-4-oxobut-2-enoic acid	1.12	43.138
17	Phthalic acid di(2-propylpentyl) ester	2.7	43.735
18	Octacosyl acetate	1.29	44.159

3.5. Molecular Docking Study

The docking results showed binding energies (between -5.8 and -7.50 kcal/mol) of five compounds, higher than that of the standard, kojic acid (-5.6 kcal/mol), with (4-[4-[(2-hydroxybenzoyl)amino]anilino]-4-oxobut-2-enoic acid) having the highest binding energy with a value of -7.5 kcal/mol. Among the five compounds identified to have higher binding energy relative to kojic acid in the docking experiments, only one of them does not dock and interact at the protein's Cu-Cu domain (Table 2) at its active site which also implies that these compounds may have the capacity to disrupt the enzymatic activity of the protein. The observed binding energies for these five compounds and their preferred binding modes as well as polar interactions indicates that the fraction contains compounds that might be potent inhibitors of tyrosinase.

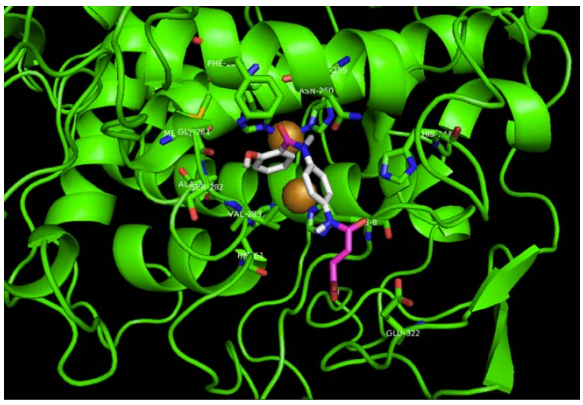

Figure 4. Cartoon rendering of the tyrosinase from A. bisporus showing the compound (4-[4-[(2-hydroxybenzoyl)amino]anilino]-4-oxobut-2-enoic acid) in the Cu-Cu domain of the protein active site

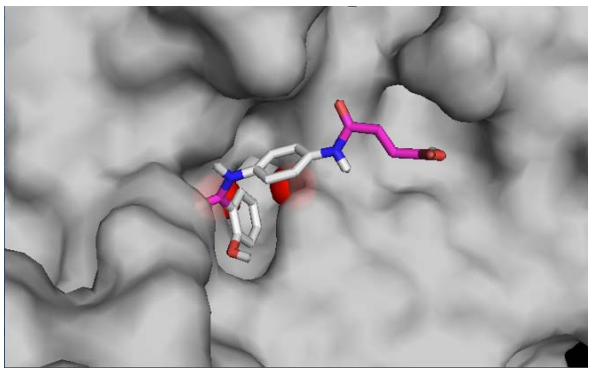

Figure 5. Surface rendering of the tyrosinase from A. bisporus showing the compound (4-[4-[(2-hydroxybenzoyl)amino]anilino]-4-oxobut-2-enoic acid) in the Cu-Cu domain of the protein active site

However, these five compounds happen to be among the compounds with the lowest % composition (a total of 5.3 % for all five compounds, with the compound having the highest binding energy being 1.12 %) in the fraction which means that their contribution to the inhibition of the

tyrosinase in the *in-vitro* experiment might not be significant at the test concentrations. The interactions at the Cu-Cu domain (Figure 4 & Figure 5) of most of the components in the fraction may also be responsible for the imperfect competitive-interaction profile observed in the kinetic analysis of the inhibition of the tyrosinase as shown in the Lineweaver-Burk Plot (Figure 2).

4. Conclusion

In conclusion, the results of this study suggest that some of the minor chemical constituents of the root bark of *S. mombin* can act as potential tyrosinase inhibitors in the food, cosmetics and pharmaceutical industry. A follow up study will be embarked upon, in which the five compounds with higher binding energy relative to that of kojic acid will be studied independently, *in-vitro*, in order to assess their tyrosinase inhibitory potential. Furthermore, the combination of GCMS profiling of the plant extract and molecular docking was vital in establishing the reasons for the low inhibition of the extract, the change in both K_m and V_{max} values which were observed in the lineweaver burk plot, tending towards a competitive type inhibition, and revealing that the compounds with the lowest % composition are those predicted to have the highest potential for inhibiting tyrosinase, prompting a follow-up study.

It is worthy to note that the use of a combination of *in-vitro* and *in-silico* models in investigating potential plant derived compounds with biological activity that could ultimately be applied in *in-vivo* studies is a veritabe approach in the field of medicinal chemistry for the overall goal of exploiting and promoting compounds with health benefits.

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