Hydrocyanic Acid / Thiocyanate Inhibition of Thyroperoxidase: Rutin Mediation by Kinetic and Docking Studies

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ABSTRACT

The present study explores the effects of phenolics and flavonoids as potential therapeutic agents for management of raised serum levels of some cassava food toxicants, SCN / HCN as reported in our previous study. Michaelis-Menten kinetics, together with Lineweaver-Bulk plot are employed to determine the kinetic constants of porcine thyroid peroxidase (pTPO), a principal enzyme in thyroid hormone synthesis; while the Schrodinger Suite (2020) is used to dock phenolics and flavonoids test compounds against the human TPO (PDB ID: 1PRX), with ascorbic acid and methylimidazole serving as the control compounds. In the absence of inhibitory compounds, TPO exhibits typical Michaelis-Menten kinetics, with values for Vmax 0.014126Ms^{-1}, Km 0.001297M, and Kcat 5.389s^{-1}. Upon the addition of some individual phytochemicals, all the kinetic parameters altered, (much less so when the SCN was introduced), exhibiting an overall catalytic efficiency as noncompetitive or mixed-type inhibitors, thereby modulating enzyme function and catalysis. The kinetics and or docking studies suggest epicatechin, chlorogenic acid, quercetin, gallic acid and the toxicants SCN and HCN act as noncompetitive inhibitors and exhibited similar binding with a key amino acid residue, ALA-151, as that of the control inhibitor compound, ascorbic acid - an interaction deemed to affect enzyme's structural conformation and activity, contributing to the phytochemicals' potential as modulators / regulators of enzyme activities. Contrarily, rutin acts as an allosteric activator, increasing the rate of breakdown of ES complex to products and so a potential remedy for conditions of hypothyroidism. This kinetic behaviour also aligns with its binding interactions with amino acid residues different from those of its counterpart inhibitor components. The studied phytochemicals including rutin, show higher binding affinity (docking scores -6.52 to -5.06 Kcal/mol), displaying the most stable binding conformation and supporting their potential roles as enzyme modulators than those obtained from the inhibitor toxicants (-3.58 to -3.04 Kcal/mol) and the standard ascorbic acid ligand (4.27) Kcal/mol. The results from chlorogenic acid and epicatechin suggest both possess therapeutic relevance in hyperthyroidism and in TPO homeostasis respectively. More understanding into the complex interplay between enzyme, substrate, and regulatory / modulatory factors in thyroid hormone synthesis, function, advancement of diagnostics and therapeutic approaches for thyroid disorders and associated health conditions, has been provided. These findings have significant implications for drug design as activators / inhibitors / modulators of enzyme functions and in nutraceutics preparations.

Keywords: TPO, Phytochemicals, Thiocyanate, Kinetic, Docking, Rutin.

INTRODUCTION

Thyroid peroxidase (TPO) plays a pivotal role in thyroid hormone synthesis, making it a key target for research aimed at understanding thyroid disorders, drug design and developing diagnostic tools. Encoded by the TPO gene situated on chromosome *2p25* in humans (Marlena and Paul 2019), the enzyme structure consists of a large glycoprotein anchored to the membrane of thyroid follicular cells and contains a haem prosthetic group at its catalytic site to facilitate enzymatic oxidation of iodide ions (Williams et al, 2020). TPO's activity is facilitated by its interaction with hydrogen peroxide, which acts as a co-substrate; and

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catalyses two key reactions: the oxidation of iodide (I^-) to reactive iodine species and the iodination and coupling of tyrosine residues in thyroglobulin to form thyroxine (T_4) and triiodothyronine (T_3) (Kochman et al, 2021). Inhibition of TPO has been linked to disruptions in thyroid hormone production, which can lead to hypothyroidism and associated goiter, developmental abnormalities, and metabolic disorders (Joma et al, 2024; Shahid, Ashraf, and Sharma, 2021; Madukosiri, 2011).

The present study is informed by the incidence of goitre, in some areas in Nigeria including Bayelsa state, where iodine deficiency has been recorded in the midst of high iodine levels in both food and blood samples of subjects (Madukosiri, 2011; Madukosiri and Ikale, 2011), therefore it was logical that certain factor(s) (which in this case represent SCN / HCN inhibition of thyroperoxidase) could be obstructing the availability of iodine in the subjects, making the protracted efforts of UN's intervention for iodine supplementation in third world countries, partially ineffective. Given the critical function of TPO in endocrine physiology, understanding how various compounds interact with this enzyme is essential for assessing potential therapeutic approaches for thyroid disorders.

A wide range of synthetic and natural compounds such as phytochemicals have been reported to inhibit TPO activity, and they include phenolics, flavonoids, and certain toxicants. (Madukosiri et al, 2015; Makhuvele et al, 2020). Phenolic compounds such as gallic acid and chlorogenic acid are widely distributed in plant-based foods and are known for their antioxidative and enzyme-modulating properties (Dai & Mumper, 2010; Madukosiri et al, 2025 in press). Similarly, flavonoids such as catechin, quercetin, rutin, kaempferol, and epicatechin exhibit diverse biological activities, including interactions with key metabolic enzymes (Hao et al 2024). Some of these polyphenolic compounds have been linked to the modulatory effect on thyroid function through direct interactions with TPO, potentially influencing hormone synthesis (Madukosiri et al, 2015). Contrarily, toxicants such as hydrogen cyanide (HCN) and thiocyanate (SCN) had been implicated in TPO inhibition (Madukosiri et al, 2015) and reported as competitive inhibitors of TPO, reducing iodine organification and impairing thyroid hormone biosynthesis (Vargatu, 2016; Madukosiri, 2011). Given their dietary and environmental prevalence, these compounds may contribute to thyroid dysfunction, even in population with adequate or high iodine intake, how much less in iodine-deficient regions of sub-Saharan Africa. Phytochemicals had often been ascribed to as having dual and conflicting functions; although, it was then unclear how a chemical compound can mitigate both conditions of, for example, hypothyroidism and hyperthyroidism. The solution lies not only on insightful elucidation and understanding of the biochemical pathway of the compound in question but in the understanding of the structural conformations of the ligand that influence the nature of binding interactions with its enzyme.

The flavonoids are a group of polyphenols naturally occurring plant phytochemical substances with variable phenolic structures, having the general structure of a 15-carborn skeleton consisting of two phenyl rings (A and B) and a heterocyclic ring (C) (Mutha, Tatiya, and Surana, 2024). They are synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PALO), and consist of a large group of polyphenolic compound with a benzo-y—pyrone structure and a chemical nature exemplified by structural hydroxylation and conjugation. More than 500 naturally occurring flavonoids exist in plant kingdom and often classified into four major groups -the flavones (including apigenin, kaemferol, luteolin, rutin, and quercetin); the flavonones (hesperetin, naringin, naringenin); flavans (catechin, qallocatechin, catechin 3-gallate, gallocatechin 3-gallate, epicatechins); and anthocyanins (cyanidin, petunidin, peonidin, delphinidin, and malvidin), (Mokhtari and Asgarpanah, 2021; Pandey and Rizvi, 2009; Bolling and Bohn, 2018). Published food sources include, tea, apple fruit, red wine, berries, broccoli, fruit peels, grapes, lettuce, olives, onions parsley, celery, among others (Perez-Vizcaino and Duarte, 2010; USDA Database, 2018). Phenolic acids belong to the group of simple phenols (going by their chemical structure) having carboxylic acid functional group bonded to either the phenol ring with at least one hydroxyl group examples of which include gallic acid, chlorogenic acid, ellagic acid; and p-hydroxybenzoic acid, or to sinnamic acid skeleton and include caffeic acid, p-coumoric acid, ferulic acid and sinnapic acid, abundant in various plant-based foods, particularly in skins or peels of fruits, seeds, tea, coffees, cheries, apples, cereals, grains, berries and leaves of vegetables (Belščak-Cvitanović, 2018).

Phytochemical components of phenolic acids and flavonoids have been found useful in ameliorating the adverse effects of thiocyanate and consequent thyroid dysfunction in nutrition (Madukosiri et al, 2015). Their

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nutritional benefits are often based on their capacity as antioxidant compounds, (Wu et al, 2023). Several authors have shown that these compounds are capable of mitigating, treating or even preventing many of the effects of toxicants and of their free radicals in the body, (Dwivedi et al, 2021; Kunwar & Priyadarsini, 2011; Madukosiri, Opara and Victor, 2015a). Although, the mechanisms of their actions are yet to be fully understood, many scholars agree they include mediation by direct scavenging of free radicals, induction of the synthesis of antioxidant enzymes or by chelating redox-active metal ions and or the avoidance of the free radical generation – hydroperoxide conversions into oxyradicals (Tumilaar et al, 2024; Nguyen et al 2024; Kumar and Pandey, 2013; Anthony et al., 2012). Food processing with polyphenol-rich compounds had been found to significantly reduce the glycosides component of such foods (Madukosiri et al, 2023, in press). Literature reports also agree on the capacity of flavonoids together with phenolic to act as anti-tumour agents, although their mechanisms of action are yet to be fully clarified (Nguyen et al 2024; Kumar et al 2013). Apart from acting as powerful antioxidant, flavonoids and phenolics can also modulate some peroxidases activity (Tungmunnithum et al, 2018).

The present study focuses on the *in vitro* TPO enzyme inhibition assay to assess the potential effects of select phenolic acid, flavonoids, and toxicant on the enzyme activity. For more understanding into the action of flavonoids and phenolics on TPO enzyme kinetics and potential development of therapeutics, this study include molecular docking analysis together with radical scavenging activities to examine the binding interactions between these phytochemical components and the amino acid residues of the enzyme hTPO focusing on key molecular forces that govern enzyme inhibition / activation. By integrating experimental and computational approaches to characterize the enzyme-ligand binding information and mechanics, this research aims to contribute to a better understanding of TPO inhibition mechanisms and their broader implications for healthy thyroid function and endocrine health in general. These computational approaches provide insights into key binding interactions, such as hydrogen bonding, van der Waals forces, polar, salt bridge and Pi-cation formation, which are essential for stabilizing enzyme-ligand complexes (Xue et al., 2022). Molecular interactions of this sort are helpful in determining the binding efficiency of ligands which explain their potential impact on enzyme function. The implications of these interactions extend beyond basic enzyme kinetics, as they can influence toxicological risk assessment, dietary recommendations related to thyroid health and ultimately drug design. A deeper understanding of how naturally occurring compounds and environmental toxicants interacting with TPO could aid in the identification of potential thyroid-disrupting chemicals and inform regulatory guidelines, is provided. These findings provide better understanding into the therapeutic potential of certain polyphenols as activators / inhibitors / modulators of thyroid function for individuals having critical to high levels of thiocyanate (obtained from certain food crops such as cassava - *Manihort esculenta* crantz) with consequent health challenges, (Madukosiri, Opara and Unuaru, 2015; Madukosiri, 2011). The effects of certain phenolics and flavonoids as potential therapeutic agents for management of thyroid dysfunction related to increased serum levels of food, water and environmental toxicants, HCN or SCN, are presented.

In this study, porcine thyroid peroxidase (pTPO) was employed in place of human thyroid peroxidase (hTPO) due to its demonstrated structural and functional similarity to hTPO. Previous studies have shown that pTPO closely mimics the enzymatic behavior of hTPO, making it a suitable model for investigating thyroid hormone synthesis. Notably, pTPO may exhibit variations in substrate affinity compared to hTPO, which could influence the efficiency of thyroid hormone production (Smith et al., 2018; Jones & Brown, 2019). These characteristics rendered pTPO a practical and relevant choice for the experimental objectives of this research.

MATERIALS AND METHOD

Materials

Major Equipment / Apparatus

Major equipment used include constant temperature water bath, model: HH.W21.Cr 42II, Q/STYS008-2001), uv-vis spectrophotometer (Searchtech Instrument England, Code: 7252204083), electronic weighing balance (Multitech Analytical, 12051, New Delhi), centrifuge (BL- 110 Biomedical Engineering INC. USA), Smart Digital pH tester (Model no: PHS-3C, HS, code: 9025800000, China), and others such as freezer (-20^{0}C), micropipettes, cuvettes, Latex and gloves.

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Chemicals / Reagent

Sucrose, (Pharmachem and Co; Kolkata), Tris (hydroxymethyl) aminomethane (Spectrum chemical select science, 26158938,Gardena), potassium chloride (Sisco Research Laboratories PVT, LTD.), sodium chloride (Spectrum Reagents and Chemicals PVT.LTD., Edayar, Cochin), magnesium chloride ($MgCl_2$, CAS 7786-30-3, Luchschen), sodium hydroxide (CAS.No:1310-73-2 cpmplany place), guaiacol (2-Methoxyphenol, Spectrum chemical), hydrogen peroxide (Service Pharmaceutical Co Ltd.21, Benin city.), Biuret reagent (R1), thiocyanate (Xilongchemical XL, China), catechin (Central Drug House; New Delhi, India; Product code: 205315; batch no. 040823),quercetin (Central drug house; new Delhi, India; Product code: 05622100005; batch no. 030917), methyl imidazole (MOLYCHEM, Mumbai India; Product code: 16283; batch no. MCR-22500-01), methanol (M; Cat No.:351696T; Index No: 612-032-00-2, Hohenbrunn, Germany), gallic acid (MOLYCHEM, Mumbai 400002, India; Product code: 14500; batch no. MCR-18404), rutin (LOBA CHEMIE PVT. LTD; Product code: 05621; batch no. BC437872306.

Sample collection

The study animal was purchased from GSM Farm along AIT/Elebele Road by Chief Preye Street (Calabar Church Road) Kpansia II, Yenagoa. Porcine thyroid gland was harvested at a local abattoir in Efekeama, Amassoma (where the animal was sacrificed), and was immediately placed in a cooling flask containing ice block for onward transfer for preservation and storage at -20^0C in the laboratory until required for use within 30 hours.

The morphology of the animal specimen include: Species, Sus scrofa domesticus (domestic pig); Subspecies, Duroc breed; sex: female (Sow). Data was collection by the animal Taxonomist, Department of Animal Science, Faculty of Agriculture, Niger Delta University, Wilberforce Island Bayelsa State).

Physical characteristics of animal include, size, medium-sized; black head and pink upper body and a black lower body; floppy ears; long snout; legs were restrained for easier transportation and handling. Age, 10 months old; weight, 150 kg and length, 1.5M. The specimen exhibited good health with no visible injuries or abnormalities noted during initial assessment. The handling procedures were in accordance with the ethical conditions of the Faculty of Agriculture, Niger Delta University and with the Guidelines for the Ethical Treatment of Nonhuman Animals (Behavioral Research and Teaching, Ethical Committee, 2023). Apart from the leg restraints during transportation, the specimen was gently handled using a padded sling to minimize stress and ensure safety.

Sample preparation

The assay was conducted as explained in Habza-Kowalska et al, 2019. The thyroid gland The frozen thyroid gland was cut into slices. Using an electronic weighing balance the total thyroid gland was weighed in grams which gave 51.89g. Afterwards,1.0g of each sliced thyroid gland was weighed into a plastic crucible mortar and homogenized in a 9.0 mL buffer, (to make 10% - w/v homogenate), containing 0.25mM sucrose, 2mM Tris-HCl,100mM KCl, 40mM NaCl, and 10mM $MgCl_2$ (pH 7.4) was added to each plastic crucible mortar containing the 1g thyroid gland after homogenization in a refrigerated centrifuge. The homogenized thyroid gland was then centrifuged two times at 4000 RPM per 15min at a temperature 4ºC. This step was performed to remove cellular debris and nuclei content. The resulting supernatant was stored at -20ºC in a freezer until required for enzyme activity which was carried out within 48 hours.

The Radical Scavenging Activity of Polyphynols - Phenolic and Flavonoid Components:

The radical scavenging assay was performed using 2,2'-diphenyl-1-picryilhydrazyl (DPPH) according to the method described by Brand-Williams, et al., (1995) and applied by Guchu, et al, (2020). Different concentrations of test compounds were prepared in methanol. The standard antioxidant used was L-ascorbate while the negative control was DPPH solution. The absorbance was read off a spectrophotometer at 517nm. Determinations were performed in triplicate, while the % radical scavenging activity (RSA) at various concentrations was estimated by the ratio: [difference in absorbance (abs of control - abs of sample) / abs of control) x 100. The half maximal inhibitory concentration (IC50) of the components was found by either

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extrapolating from a plot of % RSA versus concentration (mg/mL) or by calculation using the straight line equation from the graph of IC50, y = mx + b.

Total protein determination

The total amount of protein was spectrophotometrically determined in line with the manual for the Randox biochemical kit's method (Agappe, 2023). In alkaline solution, the peptide bonds and cupric ions react to give a colored complex, the absorbance of which is measurable at 546nm in a uv-vis spectrophotometer. The total protein concentration was determined by multiplying the ratio of OD (optical density) of sample / OD standard, by the standard concentration.

Guaiacol peroxidation assay for measuring TPO activity.

The activity of thyroperoxidase was determined using the Guaiacol assay method as explained in Habza-Kowalska et al, (2019). The freshly prepared solutions of 33mM guaiacol, 0.29mM H_20_2, and 33mM sucrose buffer were pre-incubated at 37ºC before the procedure. Into a cuvette were added 180µL of the buffer, 25µL guaiacol and 40µL TPO and mixed. The reaction was started by the addition of 25µL of H_2O_2 and absorbance read every minute for 40mins in a uv-vis spectrophotometer set at 470nm. In this reaction, guaiacol is oxidized to tetraguaiacol (a colored product) by TPO in the presence of H_2O_2. The molar extinction coefficient (ε) at 470 nm = 26,600 M^{-1} cm^{-1} (for tetraguaiacol). Assay setup:

TPO + H_2O_2 + Guaiacol → Tetraguaiacol (monitored at 470 nm and faster than the iodide oxidation assay).

With the enzyme and buffer volumes remaining constant, H_2O_2 and guaiacol volumes were subsequently increased gradually from 25µL up to 1000µL and assayed in same manner. A plot of the enzyme TPO absorbance (470nm) versus Time (per 60 seconds) was made, followed by subsequent calculation of initial Velocity (V_0), Michaelis-Mentes plot of Enzyme Velocity (Vo) versus Substrate Concentration(M), Line-weaver Burk Plot, 1/Vo versus 1/[S] (M/Sec), and finally the determination of Vmax, Km and Kcat for the non-inhibited reaction.

The thyroperoxidase inhibition assay was prepared and assayed the same way as that of the uninhibited enzyme reaction with the exception of the presence of an inhibitor compound - SCN, HCN, flavonoid and phenolic component at each assay run, as the case may be. The kinetic constant of the inhibited reactions were calculated accordingly.

Molecular Docking Protocol

In order to elucidate the structural and biochemical basis of the phytochemicals (phenolics and flavonoids), HCN and thiocyanate binding to the enzyme's active site, the present study has employed molecular docking tools for more insight into these binding interactions and their implications for thyroid function and health. The Schrodinger Suite (Maestro, Schrödinger LLC, 2020) method was used to dock phenolic and flavonoid test compounds against the enzyme, with ascorbic acid serving as the control. The crystal structure of the human peroxidase enzyme (PDB ID: 1PRX) was retrieved from the RCSB Protein Data Bank (Choi et al., 1998). Ligands, including phenolic and flavonoid compounds, were obtained from the PubChem database.

Preparation of Ligands and Protein

The protein structure was prepared by removing water molecules and adding hydrogen atoms, the ligands optimized using the LigPrep tool in Schrödinger, while the docking process was carried out using the Glide module, utilizing extra-precision (XP) docking.

Evaluation Criteria

The binding affinity was assessed using docking scores (kcal/mol), while the protein-ligand interactions were examined through hydrogen bonds, hydrophobic, polar interactions, and salt bridges. Docking Score (kcal/mol) represents the binding affinity between the ligand and the target enzyme (thyroperoxidase, TPO). A more

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negative value indicates a stronger binding interaction. Ligand Efficiency (LE) measures the binding efficiency relative to the size of the ligand. A more negative LE suggests better interaction per atom. Glide E model is a scoring function that combines binding energy and geometric considerations. Lower (more negative) values suggest stronger and more stable interactions.

RESULTS AND DISCUSSION

Demonstrated in the present study are the roles of select phenolic and flavonoid compounds in modulating TPO enzyme activity, their various binding interactions with the protein-enzyme amino acids residues deduced from molecular docking and their radical scavenging activities compared to the standard antioxidant, L-ascorbate and the negative control DPPH. The findings reinforce the pharmacological significance of these phytochemicals in modulating the hydrocyanic acid (HCN) or its metabolic product, thiocyanate (SCN) inhibition of the enzyme, thyroperoxidase (TPO).

Enzyme Assay: Effect of the Phytochemical Components on pTPO Activity

This study evaluates the effects of various polyphenols (gallic acid, quercetin, rutin and catechin), the toxicant, thiocyanate, and the standard, methylimidazole (MIM) on the kinetics of porcine thyroid peroxidase (pTPO). The kinetic parameters are analyzed in terms of maximum velocity (Vmax), substrate affinity (Km), catalytic efficiency (Kcat) as represented in table 1, and are further visualized and explained using Lineweaver-Burk (double reciprocal) plot (fig. 1).

The introduction of gallic acid decreases Vmax ($0.0088M^{-s}$) and is represented as a significant increase on LWB plot. The slope (Km/Vmax) is also increased, but calculations of Km from the fits shows the value remain nearly unchanged or slightly lower compared to the control. If gallic acid acts as an inhibitor under those conditions, then the data support it behaves as a noncompetitive inhibitor (fig.1). Quercetin shows no significant inhibition while both the control, MIM, and SCN test toxicant, depit a mixed-type inhibition with a noncompetitive nature - inorder words, there's a decrease in Vmax (represented by an increase in Y-intercept), while X -intercept shifts suggesting an effect on Km. Though the substrate binding affinity is higher (low Km), Kcat remains decreased (figs 1a & 1b). (Reactions for a non-competitive inhibitor is the same as with a single substrate, the present work adopts one substrate reaction instead of two, and so the later is adopted in the present study). Compounds which fall under noncompetitive inhibitors are more useful as drugs since they inhibit the enzyme independent of the [S].

Contrarily, rutin (fig.1f), shows a significant increase in Vmax, with possibly reduced enzyme-substrate affinity but with increased Kcat, suggestive of allosteric activation, increasing the rate of breakdown of ES complex to products and so could offer a remedy for conditions of hypothyroidism. Catechin introduction brings about little or no increase in Vmax (0.014138) with a slight reduction in Km (0.00128). Since Vmax and Km are both nearly unchanged, catechin does not exhibit classical competitive, uncompetitive, or non-competitive inhibition , rather acts as a slight activator ligand.

Table 1: Kinetic Parameters of pTPO with Inhibitor / activation Compounds.

Compound (& pTPO)	**Vmax (Ms^{-1})**	**Km (M)**	**Kcat (s^{-1})**	***Overall catalytic efficiency**
(+ve Control) gaiacol	0.014126	0.001297	5.389	Normal uninhibited enzyme activity
Catechin	0.014134	0.00128	3.998×10^{-5}	Weak enzyme activation
Quercetin	0.005998	0.00148	2.266	No significant inhibition or activation
Rutin	0.057647	0.007130	21.79	AA
Gallic Acid	0.008569	0.001392	2.267×10^{-5}	NI or mixed-type inhibition
(-ve control) methyl Imidazole	0.003815	0.000984	1.009×10^{-5}	Haem coordination inhibition, NI
Thiocyanate (SCN)	0.003525	9.648×10^{-5}	9.33×10^{-6}	NI, mixed-type inhibition

**NI = Noncompetitive Inhibition; AA, Allosteric Activation. MIM (Methyl Imidazole) is a negative control while SCN* (thiocyanate) *is a test food / environmental toxicant.*

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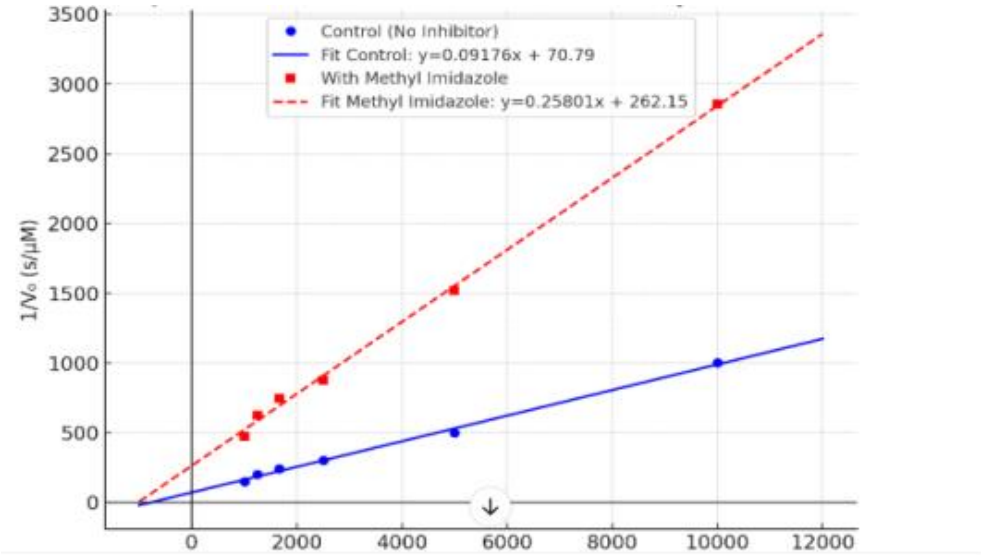

Fig. 1a: MIM: mixed-type inhibition with a Noncompetitive nature.

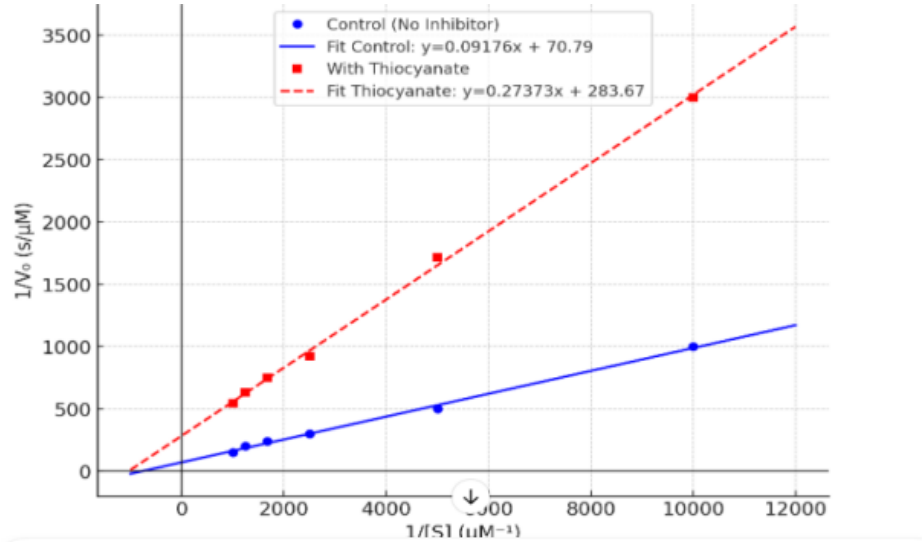

Fig 1b: SCN - Noncompetitive inhibition, mixed-type

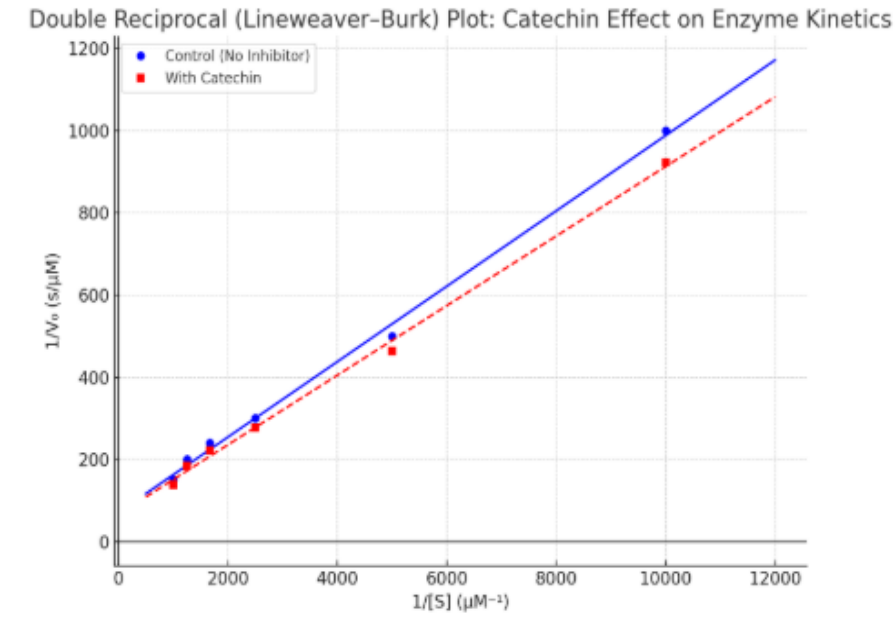

Fig 1c: Catechin - slight activator

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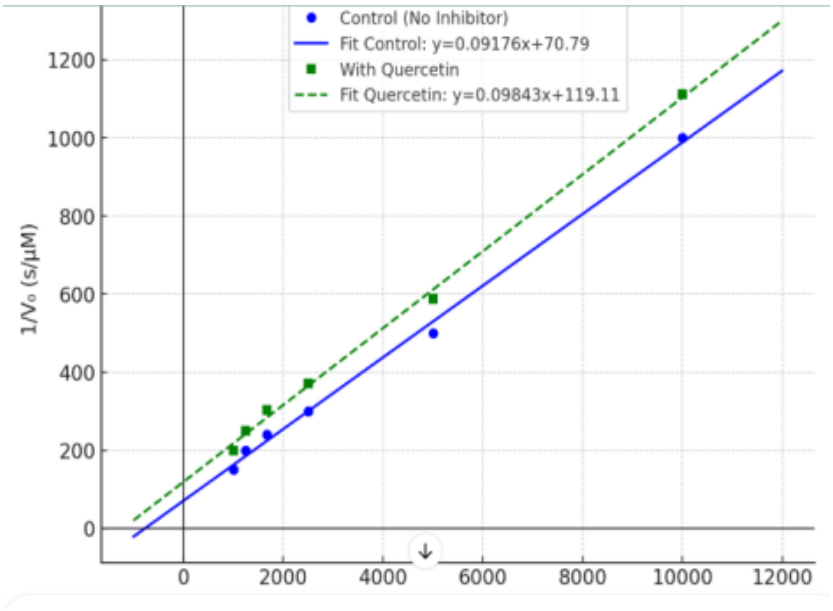

Fig. 1d: Quercetin - No significant inhibition.

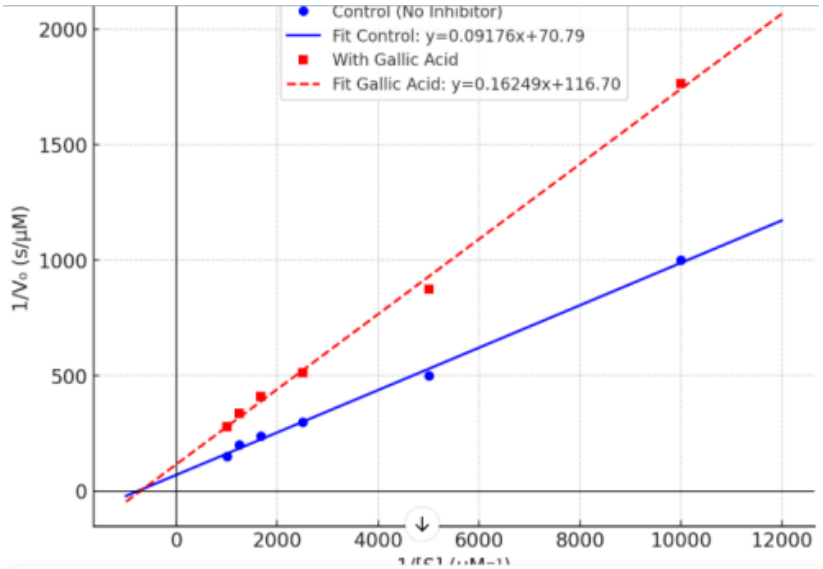

Fig. 1e: Gallic acid - Noncompetitive inhibition.

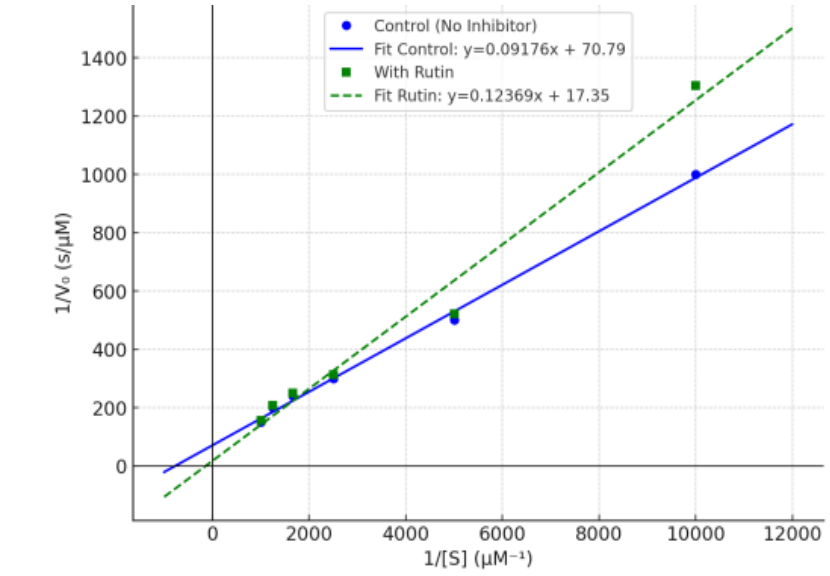

Fig 1f: Rutin - Allosteric activation.

Double Reciprocal (Lineweaver-Burk) Plot for the Inhibition /Activation of pTPO Enzyme by Phytochemical Components; fig 1a (methyl- imidazole - negative control), 1b (thiocyante - test toxicant), 1c (catechin), 1d (quercetin), 1e (gallic acid), 1f (rutin).

Both Copeland (2013) and Lehninger, (2021) agree on the four major classification of enzyme inhibitors: competitive, non-competitive, uncompetitive, and mixed-type inhibitors. Non-competitive inhibitors bind to a site, an allosteric site, distinct from the catalytic or active site, causing a decrease in Vmax while leaving Km unaffected. Non-competitive inhibitors are known to be particularly valuable in drug design because they can maintain efficacy even when substrate concentrations rises - facts which could be found beneficial in fluctuating physiological conditions (Copeland, 2013; Lehninger, 2021). Another advantage of non-competitive inhibitors are their applications in situations where full inhibition of enzyme activity is not the focus or goal, as they do not affect substrate binding but reduce the enzyme's catalytic efficiency, making them relevant in regulating of metabolic pathways or in hyperthyroidism, (Lehninger, 2021). However, both scholars are in agreement regarding the challenges posed by the non-competitive inhibitors where Vmax is reduced, thus potentially limiting the enzyme's maximum catalytic output - a reduction which could lead to unintended physiological and cellular processes. However, this obstacle can be overcome by optimizing the inhibitor's binding affinity to secure selective partial inhibition, while maintaining sufficient enzyme activity and forestalling significant metabolic disruption. In Addition, the authors posit the development of more specific inhibitors to selectively target disease-specific enzyme isoforms or conformations could minimize those setbacks and enhance therapeutic outcomes.

According to them, Mixed-type inhibitors, offer a more generalized inhibition, binding to both the free enzyme and the enzyme-substrate complex, though with different affinities. The effects of mixed-type inhibitors seem more complex since both Km and Vmax are altered depending on the relative binding preferences of the inhibitor for each state of the enzyme. Since mixed-type inhibitors affect both substrate binding and catalysis resulting in the disadvantage of unpredictable effects on enzyme kinetics and complicating their application in therapeutics, Copeland, (2013) proposed the application of the inhibitor's specific binding affinity for both the free enzyme and the enzyme-substrate complex, in other to overcome the disadvantage. Alternatively, the application of advanced structure-based drug design methods, such as the use of computational tools, to optimize inhibitors' binding preferences and preferentially target either the free enzyme or the enzyme-substrate complex, could reduce the unpreditable, off-target or the side effects while improving specificity and therapeutic efficacy (Copeland, 2013; Lehninger, 2021).

Among the investigated ligands, rutin exhibited an allosteric activation effect, significantly enhancing enzyme activity - result which aligns with our docking study and also with the findings by Tang et al. (2024) and Mahfoudi et al (2017), who reported that glycosylated flavonoids could induce conformational changes in enzymes, increasing their catalytic efficiency rather than inhibiting activity, effect which maybe linked to their antioxidant capacity. This supports the slight activation effect of catechin, though with an antioxidant potency slightly lower than that of rutin. The report from the present study also lends weight to the ameliorative effect of the rutin-rich phytochemical plant agents administered to laboratory animals that were induced with cassava-cyanide toxicity, (Madukosiri et al, 2015). Activators in drug design are linked with positive modulators at a site different from the active site. In the present study, this activation site or positive modulator site aligns with THR 192 residue of hTPO enzyme as obtained from the docking studies.

The present study supports the ability of both flavonoids and phenolic acids to act as either inhibitors or activators of enzymes depending on their structural conformation and binding interactions - effects which are demonstrated in the present study and aligned with previous literature documentations, (Safe et al, 2021; Panche, Diwan and Chandra, 2016). The current study also posits that substrate stabilization, enhanced binding specificity, affinity and facilitated catalysis could be achieved depending on the structure of the ligand or the polyphenol compound **(**typically the content of aromatic rings), which in turn influences the binding interactions and final catalysis. This understanding has thrown more light into the often deemed dual roles played by natural supplements. Comparisons with a standard inhibitor such as methylimidazole or the test toxicant, thiocyanate, reveal that while phytochemicals such as gallic acid, exhibit inhibitory properties, they are less potent than the standard or test compounds, aligning with the above modulatory rather than complete inhibitory role for the phytochemicals, (and confirmed by comparing the binding activities of the respective

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ligands). This is consistent with the report by Dai & Mumper (2010), who suggests that plant-derived phenolics and flavonoids function as mild enzyme regulators rather than direct inhibitors, which possibly contributes to their beneficial effects in maintaining enzyme homeostasis in biological systems. This could explain why the phytochemical-rich compounds should seem beneficial (depending on the body concentration of the relevant component) in either hyperthyroidism or, for the present, hypothyroidism state of malfunctioning thyroid gland, (Madukosiri, 2013; Madukosiri et al, 2015). An example is the present case where thiocynate, a potent TPO inhibitor as described in the present experiment, could result in low generation of iodine radicals, compromised organification, coupling reactions, low production of thyroid hormone and possibly, goiters development. Our result indicates that rutin, the activator compound, could find application in the management of TPO inhibition by thiocyanate - one underlining condition in goitre development among cassava-cyanide consuming populations, as found in several populations across countries where consumption of inadequately processed cassava products has been reported, (Mwadzombo et al, 2019; Madukosiri, 2013).

Radical Scavenging Activity of Phytochemical Compounds

The results of radical scavenging activity (RSA) of the pure polyphenol compounds were gallic acid (0.473mg/mL), Rutin (0.484mg/mL), catechin (0.513mg/mL) and quercetin (1.251mg/mL) (Fig 2a) of which were ranked (relative to % L-ascorbic acid standard) in the following order of potency, gallic acid (51%) > rutin (50%) > catechin (47%) > quercetin (19%), (Fig 2b), a trend that aligns with previous studies on phytochemical antioxidants (Mahfoudi et al., 2017; Kumar et al., 2021). Many phytochemicals are potent antioxidants as their molecular structures allow them to donate electrons or hydrogen atoms to neutralize reactive spec-ies. The superior radical-scavenging ability of gallic acid is attributed to its three hydroxyl groups, which enhance electron donation and metal chelation, a feature extensively reported in antioxidant research (Molski, 2023). Natural activators or inhibitors of enzyme activity are ligands found to possess potent antioxidant capacity as well as the ability to bind to the relevant effector site on enzyme - positive effector site or negative modulator site respectively. Literature report on enzyme inhibition involving mixed-type and noncompetitive inhibition by flavonoids and phenolics against peroxidases and oxidases include those from Duda-Chodak and Tarko, (2023). Rutin exhibited almost same radical scavenging activity as gallic acid, and its potent enzyme-modulating effects (Table 1) suggest a complementary role in oxidative stress reduction through both direct antioxidant activity and enzymatic regulation (Table 1). Plant-rich phytochemical agents had been used as food additives to mitigate the toxicity of residual SCN / HCN in cassava-*Manihort esculenta* food products, in a bid to improve the health of consumers (Madukosiri et al 2015 a & b).

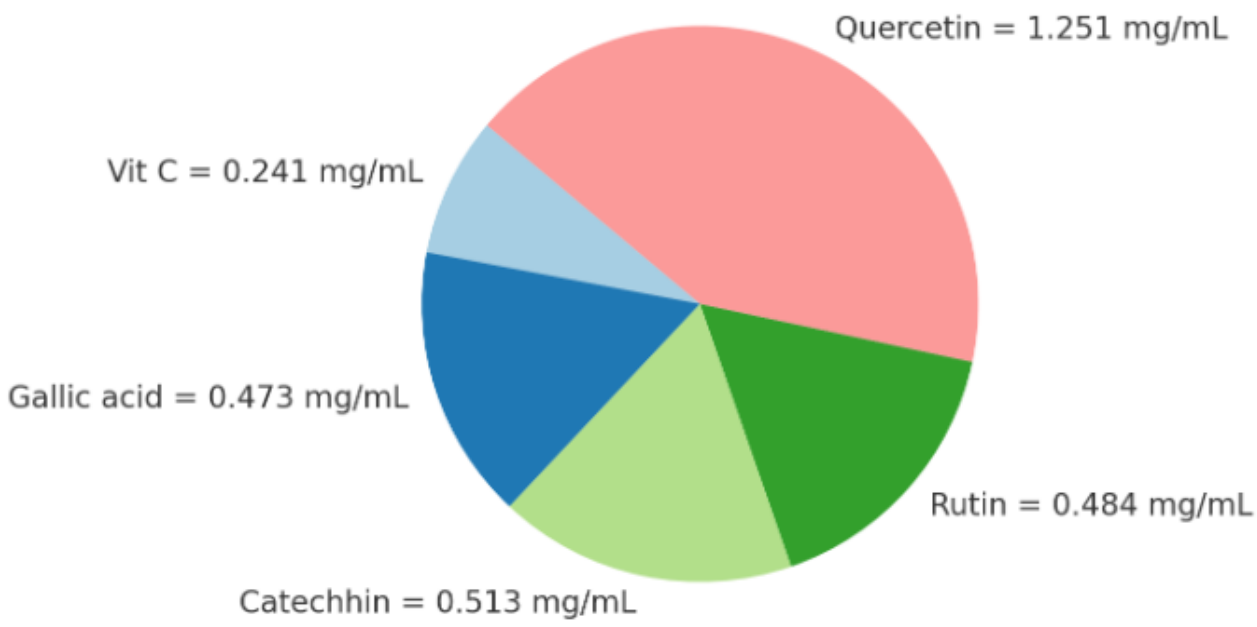

Fig 2a: Pie Chart of RSA of Phytochemical Components.

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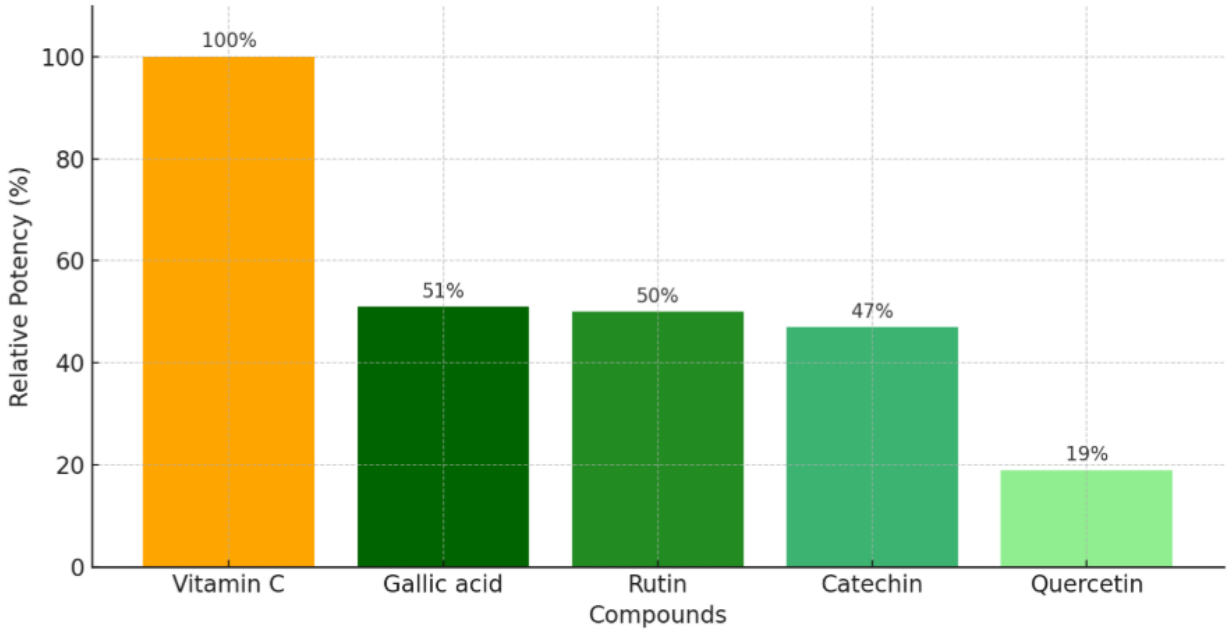

Fig 2b: Bar Chart of %RSA of Phytochemical Components Relative to Standard Ascorbic Acid

As potent antioxidants, many polyphenols and flavonoids, can influence the free radical production associated with hTPO through two main mechanisms: by modulating the enzyme's activity and by directly scavenging the free radicals once they are formed. Binding near an oxidatively active region of hTPO could influence the enzyme's redox behavior or reduce free radical formation, (Halliwell, and Gutteridge, 2015). Hydroxyl radicals (OH•), produced during the breakdown of H_2O_2 by hTPO, are among the most reactive and damaging species. Phytochemicals can intercept these radicals thereby modulating hTPO catalytic activity, before damage is done to cellular components, (Halliwell, and Gutteridge, 2015; Braverman, and Cooper, 2021). Computational docking studies show mitigative role could be achieved through, allosteric binding of phytochemicals to sites on hTPO that are distinct from the classic catalytic haem pocket affecting the conformation of the enzyme and efficiency of electron transfer during the catalytic cycle, interfering with substrate accessibility, and positioning thereby reducing the rate at which H_2O_2 reacts to generate radical species. According to Habza-Kowalska et al., (2019), this reduction could disrupt the normal redox cycling of the haem group, which is critical for both iodide oxidation and the unintended formation of free radicals like hydroxyl (OH•) and iodine (I•) radicals. Going by the present radical scavenging assay, the potency for anti-radical effect resides more with gallic acid (a potent inhibitor ligand), followed by rutin (a potent activator), while quercetin (with the least antioxidant potency) shows little or no effect on pTPO activity, (Fig 2b). This trend is supported by their binding studies (table 4).

Docking Scores

The results of docking scores indicate that the control compounds namely, ascorbic acid and ABTS exhibited moderate binding affinity (Table 2), possibly as stabilizers, compared to the test compounds (Table 2 & 3). ABTS is similar to ascorbic acid in binding scores, but has a lower ligand efficiency. However when judged as a function of binding energy and geometry, ABTS scores higher according to the order: ABTS > ascorbi acid > SCN > HCN and likely interacts differently with the enzyme, possibly stabilizing or altering enzyme function. On the other hand, ascorbic acid has a better binding affinity than the rest (Table 2). SCN shows a comparatively stronger binding affinity than HCN, indicating it may have a more pronounced inhibition of TPO activity than the later, and inturn, lower than the other phytochemical test components (Table 3), throwing more light on the ability of the later (most of which acted as noncompetitve inhibitors in present study) to mitigate the toxicity effects of the metabolic products of cyanogenic glycosides from cassava foods. Of note are the stronger binding interaction / affinity or docking scores of some select phytochemical compounds (Table 3) including epicatechin (-6.52 kcal/mol), chlorogenic acid (-5.57 kcal/mol), rutin (-5.53 kcal/mol), and gallic acid (-5.06 kcal/mol), (Table 3), facts which informed their ability as enzyme inhibitors and activator compounds. These findings confirm the binding efficiency of flavonoid and phenolic compounds as potential noncompetitive inhibitors / activators of 1PRX (Table 4)

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Table 2. Docking Scores of standard and control samples with hTPO

Ligand ID Controls	PubChem ID	Docking Score kcal / mol	LE	Glide Emodel
AA(std)	54670067	-4.27	-0.36	-26.5
ABTS	35688	-4.17	-0.13	-50.38
HCN	768	-3.04	-1.52	-7.17
SCN	9322	-3.58	-1.2	-10.15

STD – Standard; ABTS - 2, 2'azinobis-(3-ethylbenzothiaoline-6-sulfonic acid, Negative controls. LE – ligand efficiency. SCN / HCN, test toxicants.

Table 3. Docking Scores of Phenolics and Flavonoids with hTPO

Phenolics / Flavonoids	PubChem ID	Docking Score (kcal/mol)	LE	Glide Emodel
Gallic acid	370	-5.06*	-0.42	-28.15
Caffeic acid	689043	-4.9	-0.38	-29.69
p-CA	637542	-4.83	-0.4	-26.6
p-0H-BA	135	-5.52*	-0.55	-25.47
Chlorogenic acid	1794427	-5.57*	-0.22	-45.87
Ellagic acid	5281855	-5.22*	-0.24	-38.36
Catechin	9064	-5.27*	-0.25	-37.48
Quercetin	5280343	-5.13*	-0.23	-39.48
Rutin	5280805	-5.53*	-0.13	-67.22
Kaempferol	5280863	-4.98	-0.24	-37
Epicatechin	72276	-6.52*	-0.31	-42.85
Isoquercetin	5280804	-4.19	-0.13	-46.21

*Enzyme's PDB ID: 1PRX; hTPO: human TPO; p-CA: p-coumaric acid; p-0H-BA: p-hydroxybenzoic acid; STD – Standard; ABTS - 2,2'azinobis-(3-ethylbenzothiaoline-6-sulfonic acid, LE – ligand efficiency. *Test compounds with better protein-ligand affinity than control compound – ascorbic acid.*

Enzyme protein-ligand interactions

Protein-ligand interaction analysis particularly revealed hydrogen bonding, hydrophobic, and polar interactions, (Table 4, Figs 5-8), contributing to the stability of these complexes.

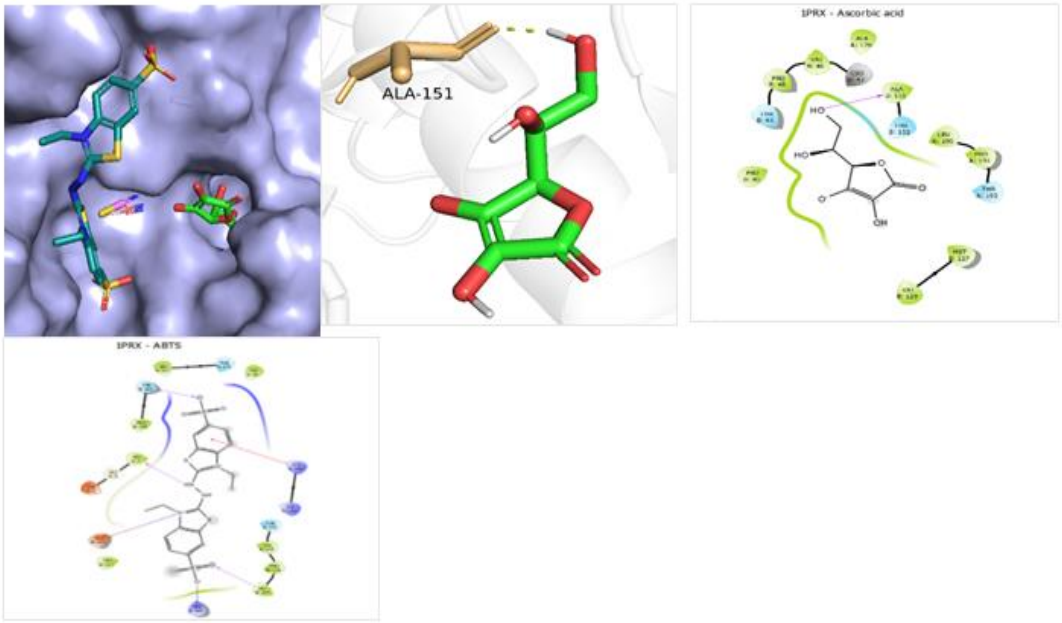

Figure 5. 3D and 2D Interaction of std Ascorbic acid & control ligand with 1PRX

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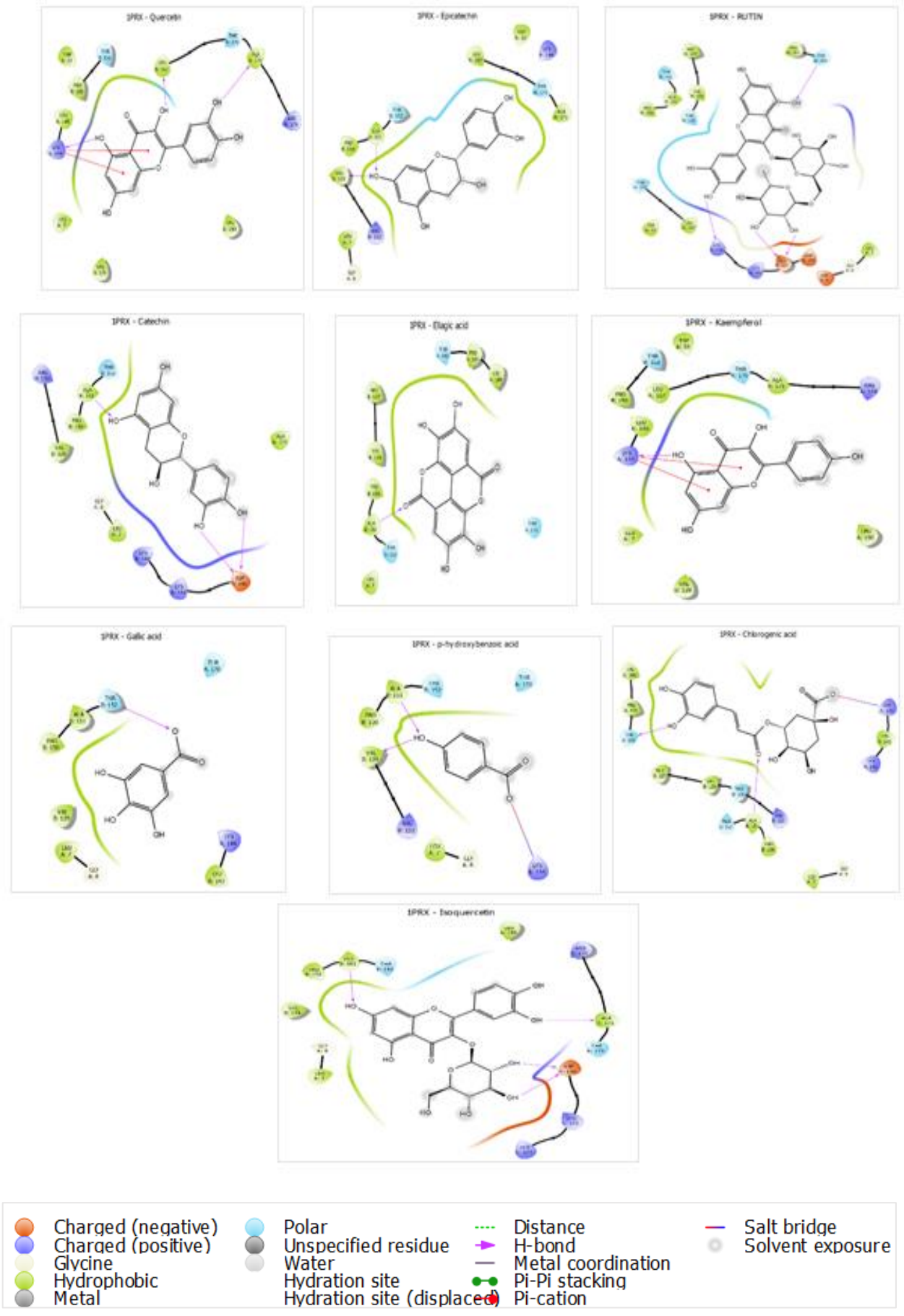

Figure 6. 2D interaction of Flavonoids and Phenolic acids with hTPO (1PRX). *The compounds with the same or similar binding pose with control show potential competitive inhibition, while others show allosteric binding to the target. Also, compounds binding to the same protein (amino acid) residues depict potential cmpetitive inhibition.*

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Table 4. Protein-ligand Interactions: amino acid residues of hTPO (1PRX) vs Ligands

Ligand	H-bonding	Hydrophobic	Polar	Pi-cation	Salt bridge
Ascorbic	ALA-151	PRO-40, PRO-45, VAL-46, ALA-176, LEU-190, PRO-191, MET-127, VAL-129	THR-44, THR-152, THR-192		
acid					
ABTS	THR-152, LUE	LEU-167, LEU-7, LEU-117, TRP-33, MET-127, PRO-126, VAL-129	THR-152, THR-130, THR-170,	LYS-144	GLU-121
	-7, MET-127				
Gallic	THR-152	LEU-7, VAL-129, ALA-151, PRO-150, LEU-143	THR-152,		
acid			THR-170		
Caffeic	ALA-151, LEU-167	TRP-33, LEU-167, ALA-171, PRO-150, LEU-7, VAL-129	THR-152,	LYS-144	
acid			THR-170		
p-ouma	VAL-46,	PRO-45, MET-127, PRO-191, LEU-190, ALA-151, ALA176	THR-44, THR-152, THR-192		
ric acid	THR-192				
pOHben zoic acid	ALA-151, VAL-129	PRO-150, LEU-7, VAL-129	THR-152,	LYS-144	
			THR-170		
Chlorogenic acid	ALA-151, THR-192	LEU-7, LEU-143. PRO-150, VAL-129, MET-127, PRO-191, LEU-190	THR-130, THR-152, THR-192	LYS-144	
Ellagic	ALA-151	PRO-191, LEU-190, PRO-150, VAL-129, MET-127	THR-192, THR-152, THR-170		
acid					
Catechin	ASP-140	ALA-151, PRO-150, VAL-129, LEU-7	THR-152		
Quercetin	ALA-171, LYS-144, LEU-167	TRP-33, PRO-150, LEU-145, LEU-7, VAL-129, LEU-190	THR-152, THR-170	LYS-144	
Rutin	THR-192, LYS-144, GLU-121	PRO-191, MET-127, VAL-129, ALA-151, PRO-150, LEU-167, TRP-33, LEU-7	THR-152, THR-170, THR-130, THR-192		
Kaemp	LYS-144	ALA-171, TRP-33, LEU-167, PRO-150, LEU-145, LEU-190, LEU-7	THR-152, THR-170	LYS-144	
-ferol					
Epicate-chin	ALA-151, VAL-129	LEU-7, VAL-129, PRO-150, LEU-167, TRP-33, ALA-171	THR-152, THR-170		
Iso-quer	ALA-151, ALA -171, ASP-140	LEU-190, PRO-150, VAL-120, LEU-7, ALA-151, ALA-171	THR-152, THR-170		
cetin					

hTPO active site amino acids residues: His494, Arg491, Glu399, His239, Asp238 (Baker, et al, 2023).

Hydrogen bonds play a crucial role in substrate recognition and enzyme specificity, enhancing binding affinity and influencing inhibitory potency (Madushanka et al, 2023). Van der Waals interactions contribute to non-specific stabilization within the enzyme's active site, while Pi–cation interactions are vital because they not only boost the overall binding affinity but also contribute to the selectivity and proper orientation of ligands which is essential for efficient enzyme activity. Salt bridge formed between oppositely charged residues can strengthen enzyme-inhibitor complexes and influence conformational changes (Dougherty, 2025).

Molecular docking analysis revealed strong binding interactions between the test compounds and human thyroid peroxidase enzyme, (hTPO), (1PRX) . The binding affinities of the epicatechin, rutin, and chlorogenic

acid (Table 4) are consistent with earlier studies on flavonoid interactions with peroxidase enzymes, (Tang et al., 2024; Sharma., 2023). Epicatechin was shown as a potent inhibitor compound, facilitated by hydrogen-binding to the key residue, ALA 151. From the Glide Emodel scores (Table 3), which shows rutin in hydrogen-bonding to THR 192 (with a docking score of 67.22kcal/mol), displays the most stable binding conformation among the studied ligands, reinforces our kinetic results and supporting its role as a positive modulator. Chlorogenic acid possibly exhibits enhanced role in enzyme homeostasis due to its hydrogen bonding to both ALA 151 and THR192 (both of which seem to function as the negative and positive effector sites respectively). The strong affinity of rutin (a potent activator), and gallic acid (an inhibitor ligand) aligns with both the present kinetic study and previous reports identifying these compounds as modulators of oxidative enzymes through their hydroxyl-rich structures, which enhance free radical stabilization, scavenging or metal chelation (Nguyen et al, 2024).

Specific Enzyme Amino Acid Residues Interacting with different Phytochemical Components - Chlorogenic acid and epicatechin

The following compares the two sets of docking results—one involving phenolic compounds (Fig 7) (represented by chlorogenic acid) and the other involving flavonoid compounds (Fig 8) (represented by epicatechin)—along with common reference ligands (ascorbic acid, ABTS, HCN, and SCN). Both studies target human thyroid peroxidase (hTPO) but highlight slightly different binding modes and residues.

Since several compounds (chlorogenic acid, epicatechin, ascorbic acid, HCN, and SCN) target ALA-151, that which binds more comprehensively to this site would likely out-compete the others in occupying the site (Figs 7 & 8). Because multiple compounds target this same region, it strongly suggests a mixed-type inhibition mechanism in the present context, where these ligands either block the normal substrate and bind to E or ES complex. Compared to other control ligands, ABTS binds to a different set of residues, indicating a distinct binding mode that might not directly compete at the primary ALA-151 key spot. Although, ascorbic acid binds ALA-151, it does so in an allosteric manner (at an alternative site), which may affect the enzyme differently rather than simply blocking substrate binding. HCN and SCN are comparatively smaller molecules and only interact with ALA-151, suggesting their binding might be less dominant compared to a ligand engaging multiple critical interactions. Chlorogenic acid appears to have a strong competitive edge by way of dual interactions - hydrogen-bonding with both ALA-151 and THR-192 which could represent a regulatory / modulatry role in thyroid function (Fig.7). The prominent activator ligand, rutin also interacts with ALA-151 but by hydrophobic bonding possibly enhancing its role as a positive effector. Other amino acid residues which are key to the interaction of rutin include THR-192, THR 152, THR170, THR-130 and LYS-144. THR-192 residue could be an important allosteric site for hTPO activation which aligns with its role as an activator ligand. The present study therefore suggests that rutin, epicatechin and chorogenic acid could find application in future drug design for management of hypothyroidism, hyperthyroidism and in enzyme homeostasis, respectively. The trio ligands interact with hydrogen bonding to key residues THR 192, ALA 151 and to both, in that order with the later structurally suggesting a disposition towards enzyme homeostesis during conditions of fluctuating thyroid hormone production. The fact that those reported active site amino acids of hTPO - His494, Arg491, Glu399, His239, Asp238 (Baker, et al, 2023), of which are highly conserved among peroxidases, were not involved in the bonding interactions (Table 4) with the studied phytochemical compounds, strengthens our reports and confirming the ligands' functions as potential allosteric activator (for rutin), non-competitive inhibitor (gallic acid) and modulator (chlorogenic acid) of enzyme activity.

The phytochemical components, in this case, might have played a regulatory or an activating role depending on the prevailing conditions of TPO activity. This event (if inhibitory) could culminate in under production of thyroid hormone and consequent goitre development (Madukosiri, 2015). As shown from the present study, SCN, acted as a noncompetitive (or mixed-type) inhibitor of thyroperoxidase with resultant decrease in thyroid hormone production and development of some form of metabolic malfunctions. Although the inhibitor increases substrate affinity, the Vmax and the overall catalytic activity are reduced.

CONCLUSION

This study investigates the inhibitory, activation and modulatory effects of select phenolic acids, flavonoids, and environmental toxicants on human thyroid peroxidase (hTPO) using *in vitro* enzyme assays and molecular

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docking analysis. The findings have enhance understanding of how these compounds influence TPO activity with implications for thyroid health and therapeutic development. The dual roles of these phytochemicals as modulators of enzyme function and as antioxidants are highlighted.

Both kinetic and docking findings show that gallic acid, epicatechin, chlorogenic acid, quercetin, rutin and catechin, exhibit varying degrees of TPO inhibition or activation. Molecular docking reveals that these compounds interact with key amino acid residues such as ALA-151 and THR-192 which are distinct from classical catalytic or active site residues, supporting their effects on kinetics of TPO as noncompetitive / mixed-type inhibitors or allosteric activator respectively. Docking studies coupled with *in vitro* kinetic assays show rutin binds strongly to THR-192, supporting its role in kinetic studies as an allosteric activator molecule. On the other hand, epicatechin and or gallic acid demonstrates strong noncompetitive inhibition with binding to a key residue ALA151 - a role which aligns with its effect on TPO as an inhibitor ligand. Chlorogenic acid binds strongly to both ALA-151 and THR-192 and seem to support enzyme homeostasis role. Radical scavenging assays correlate with the above data, ranking antioxidant potency in the order: gallic acid > rutin > catechin > quercetin. These antioxidant properties reinforce their potential for regulating oxidative stress and supporting thyroid function. Toxicants such as SCN and HCN show lower binding energies with the former exhibiting greater potential to inhibit TPO in a mixed-type / noncompetitive fashion - a function which aligns with its binding to a key inhibitory site, ALA151. Reference compounds, including ABTS and ascorbic acid, display moderate binding and seem to act as noncompetitive inhibitors. These interactions suggest that environmental or food toxicants such as SCN and HCN (from improperly processed cassava food stuff) could disrupt TPO function and lower thyroid hormone production. This research enhances the current understanding of phytochemical-TPO interactions and supports the therapeutic potentials of these natural compounds as modulators of the enzyme functions and in managing disorders such as hypothyroidism and hyperthyroidism of malfunctioning thyroid gland. Continued *in vivo* and clinical investigations are necessary to confirm these findings and for subsequent application in drug development and nutraceutical formulations.

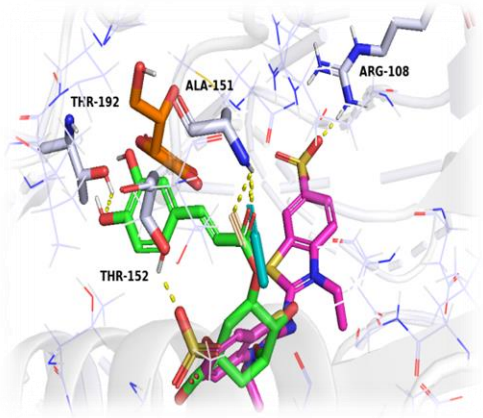

Fig 7: Phenolics.

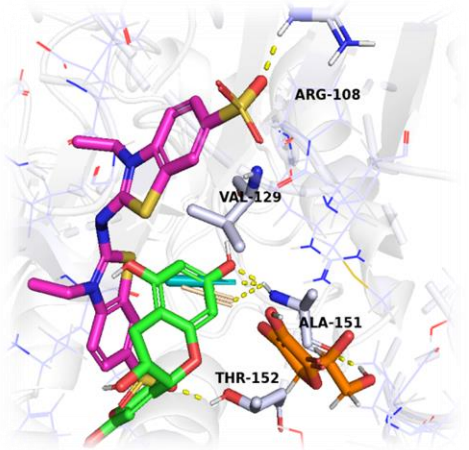

Fig 8: Flavonoids

Figure 7: Phenolic: Chlorogenic acid (green), ascorbic acid (orange) ABTS (magenta), HCN (wheat), SCN (teal). Chlorogenic acid interacted (formed H-bonds) with ALA-151 and THR192 residues respectively. ABTS interacted (formed H-bonds) with ARG-108 and THR-152. Chlorogenic acid, ascorbic acid, HCN, and SCN interacted (formed H-bonds) with ALA-151 residue (depicting competitive inhibition). The ascorbic acid showed allosteric inhibition by binding another site of the ALA-151 residue different from chlorogenic acid and the other control compounds. Protein presented in wire representation with grey carbon atoms, yellow dash lines represent hydrogen bonds.

Figure 8: Flavonoid: Epicatechin (green), ascorbic acid (orange) ABTS (magenta), HCN (wheat), SCN (teal). Epicatechin interacted with ALA-151 residue only. ABTS interacted with ARG-108 and THR-152. Epicatechin, ascorbic acid, HCN, and SCN interacted with ALA-151 residue (depicting competitive inhibition). The ascorbic acid showed allosteric inhibition by binding another site of the ALA-151 residue different from the other control compounds and chlorogenic acid.

Authors declare no conflict of interest between them.

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