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Research Article

A detailed research on Antioxidant and therapeutic potential of Immuxanth implementing in silico and in vitro approach

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Abstract

Imuuxanth powder, a dietary supplement enriched with natural antioxidants, is explored for its nutritional properties and potential health benefits. The powder provides 5.26 Kcal energy, 1.90g protein, zero fat and added sugar, and 1.80g fiber per serving. Suitable for low-calorie diets, it supports protein intake, tissue repair, and muscle development. With no fat or added sugar, it aids weight management and blood sugar control. Amino acid analysis highlights its diverse essential and non-essential amino acids. GC-MS identifies flavonoids, xanthones, and carotenoids for antioxidant properties.

Antioxidant assays (DPPH, FRAP, ABTS) confirm Imuuxanth's potential to combat oxidative stress. Molecular docking studies predict interactions with proteins related to antioxidant effects. Top berries intensify its antioxidant activity due to active compounds. Imuuxanth powder presents promising nutritional properties, including low energy, moderate protein, and absence of fat and added sugar. Its diverse bioactive compounds contribute to potent antioxidant capabilities. In silico studies support its potential as a dietary supplement for overall health, warranting further research for specific benefits.

Keywords: Immuxanth powder, Antioxandant potential, GCMS Analysis, Nutritional Analysis, Insilico Analysis

Introduction:

Berries, recognized for their nutritional richness and potential health benefits, belong to the category of simple fruits with seeds and pulp originating from a single flower's ovary. Notable families such as Rosaceae and Ericaceae encompass diverse berries like blackberries, strawberries, and cranberries. These fruits are esteemed for their abundance of bioactive compounds, including phenolic compounds, flavonoids, tannins, and phenolic acids, known for their antioxidant, anti-inflammatory, and anti-proliferative properties.

Renatus Wellness offers Imuuxanth powder, a supplement derived from a blend of berries, including blackberries, blackcurrants, blueberries, cranberries, raspberries, and strawberries. This formulation aims to capitalize on the health-promoting potential of these berries, providing a natural immune system boost. However, a thorough nutritional analysis is crucial to ensuring its efficacy and safety.

To comprehend Imuuxanth's composition, including vitamins, minerals, and essential nutrients, a comprehensive nutritional analysis is imperative. This information is vital for assessing dietary benefits and potential risks, particularly for individuals with dietary restrictions or health conditions. Furthermore, understanding the bioactive compounds present in the powder is crucial for evaluating its potential health-promoting effects.

Various techniques, including gas chromatography and mass spectrometry (GC-MS), are employed to identify and quantify organic compounds in the powder. GC-MS enables precise identification of individual compounds, providing insights into the powder's chemical composition. Antioxidant evaluation is essential to determine Imuuxanth's ability to combat oxidative stress, crucial in various health conditions.

In silico activities, such as computer-based molecular modeling, offer insights into the bioactive compounds' interactions with biological targets. This predictive approach aids in understanding potential efficacy and mechanisms of action. Relevant studies Yuan et alunderline the significance of molecular simulations in drug design and target identification.

Conducting a comprehensive nutritional analysis, including GC-MS, antioxidant evaluation, and in silico activities, is vital to unravel the health-promoting potential of Imuuxanth powder. These analyses collectively support its efficacy as an antioxidant-rich and immune-boosting supplement, promoting overall well-being. References to studies involving similar analytical techniques in diverse contexts Jianu et al., further substantiate the importance of such investigations

Materials and Methods

Nutritional profiling of immuxanth powder

Proximate analysis

The proximate composition of the samples was analyzed following the methods outlined by the Association of Official Analytical Chemists (AOAC) [23]. The analyses were conducted using standard procedures available in India to assess the nutritional composition of the samples.

To determine the dry matter content, the samples were dried at 105 °C until reaching a constant weight using an Indian drying oven.

Crude protein (CP) content was assessed using the Kjeldahl method with a Büchi B-324 distillation unit manufactured in Switzerland. The nitrogen content was multiplied by 6.25, as per the standard AOAC method 945.18, to calculate the crude protein content.

Crude fiber (CF) content was determined as the residue left after sequential treatment with 1.25% H_2SO_4 and 1.25% NaOH using an ANKOM220 Fiber Analyser or an equivalent Indian fiber analyser available in the local market, following the AOAC method.

Total carbohydrates were calculated as follows: Nitrogen-free extract (NFE) (%) = 100 - % (moisture + crude protein + crude fat + crude ash + crude fiber).

By utilizing these standard Indian laboratory procedures and equipment, we obtained precise and accurate measurements of the proximate composition of the samples, enabling us to gain valuable insights into their nutritional content and potential health benefits.

Vitamin Content Profiling: Vitamin C (Ascorbic Acid) by AOAC Official Method 967.24. Vitamin A (Retinol): AOAC Official Method 970.64 Vitamin D (Calciferols): AOAC Official Method 994.10. Vitamin E (Tocopherols): AOAC Official Method 993.14 Vitamin B12 (Cyanocobalamin): AOAC Official Method 944.12 Folate (Folic Acid): AOAC Official Method 2005.06 ; Amino Acid Analysis by AOAC Official Method 982.30 Mineral Analysis Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg): AOAC Official Method 968.08 (Fe): AOAC Official Method 985.35.Zinc (Zn): AOAC Official Method 990.08 Copper (Cu): AOAC Official Method 985.35.Selenium (Se): AOAC Official Method 999.10 .

GCMS Profiling

GC-MS analysis was conducted on imuuxanth powder to identify its organic compounds. The analysis used an Agilent 7890-Jeol AccuTOF GCV system with Elite 1 column and helium gas as the carrier. The temperature was raised from 40 to 280°C during the 5-minute isothermal run. Compounds were identified based on retention time, MS fragment ions, and percentage from peak area. The compounds' MS spectrum patterns were compared to the NIST Mass Spectra Database. GC-MS revealed the complex chemical composition and potential bioactive components of imuuxanth powder.

Antioxidant Profiling of Immuxanth powder

The antioxidant capacity of Immuxanth powder was evaluated using several chemical assays, including the DPPH Radical Scavenging assay, ABTS radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, determination of total phenolic content, and determination of total flavonoid content. The experiments were performed following established methods with slight modifications. All assays were conducted in triplicate to ensure accuracy and reproducibility of the results.

1. DPPH Radical Scavenging Assay:

The DPPH radical scavenging activity of Immuxanth powder was determined based on a previously reported method. A stock solution of stable DPPH radical was prepared by dissolving 2.4 mg of DPPH in 100 ml of methanol. A test solution containing 5 µl of Immuxanth powder was added to 3.995 ml of methanolic DPPH radical solution. The mixture was vigorously shaken and kept at room temperature in the dark for 30 minutes. The absorbance of the reaction mixture was measured at 515 nm using a spectrophotometer. A blank without Immuxanth powder was also measured. The DPPH scavenged percentage was calculated using the equation.

DPPH Scavenged (%) = ((AB - AA) / AB) × 100

Where AB is the absorbance of the blank at t=0 min, and AA is the absorbance of the antioxidant at t=30 min. A calibration curve was constructed using the % DPPH scavenged versus the concentration of the standard antioxidant (Trolox).

2. ABTS Radical Scavenging Assay:

The ABTS radical scavenging activity of Immuxanth powder was determined using the ABTS cation decolorization assay . ABTS radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in water. The solution was stored in the dark at room temperature for 12-16 hours before use. The ABTS cation solution was diluted with methanol to achieve an absorbance of 0.700 at 734 nm. A test solution containing 5 µl of Immuxanth powder was added to 3.995 ml of diluted ABTS cation solution, and the absorbance was measured after 30 minutes of initial mixing. A solvent blank was run in each assay. The ABTS scavenging effect was calculated as follows:

ABTS•+ Scavenging Effect (%) = ((AB - AA) / AB) × 100

Where AB is the absorbance of ABTS radical + methanol, and AA is the absorbance of ABTS radical + Immuxanth powder extract/standard (Trolox).

3. Ferric Reducing Antioxidant Power (FRAP) Assay:

The FRAP assay was performed according to the method described by Benzie and Strain [6]. The FRAP reagent, containing 300 mM acetate buffer, 10 ml of TPTZ in 40 mM HCl, and 20 mM FeCl3.6H2O in the proportion of 10:1:1, was prepared and incubated at 37°C. Freshly prepared working FRAP reagent (3.995 ml) was mixed with 5 µl of appropriately diluted Immuxanth powder, and the mixture was incubated at 37°C for 30 minutes. The absorbance of the intense blue color complex formed by the reduction of Fe3+ TPTZ to Fe2+ was measured at 593 nm against a reagent blank (3.995 ml FRAP reagent + 5 µl distilled water). The FRAP values were determined by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe3+ and expressed as mg of Trolox equivalent per gram of Immuxanth powder.

3.1 In Silico Prediction of Bioactivity and Molecular Docking Studies

Molecular docking simulations were carried out using the antioxidant compounds (ligands) of different berries used in the production of Imuuxanth (Table 1) against the target proteins. The 3D structures of the proteins were retrieved from Research Collaboratory for Structural Bioinformatics - Protein Data Bank (RCSB PDB) (Berman *et al*., 2000) database in pdb format and structure of antioxidant compounds were retrieved from the PubChem database (*https://pubchem.ncbi.nlm.nih.gov/*) in sdf format. However, for further analysis the required format of files is pdb so, these were then converted from sdf to pdb format using Open babel file converter software 2.4.1 (O'Boyle *et al*., 2011). Molecular

docking was carried out using AUTODOCK v1.5.6 (Hetenyi and Spoel, 2002) and Windows PowerShell. The docking grid box coordinates and size were chosen on the basis of active site prediction by BIOVIA Discovery studio 2021 (Rizvi *et al.,* 2013). Docking scores were generated as ΔG binding energy values (kcal/mol). Best run coordinates of the ligands and target proteins were analyzed and visualized through PyMOL v2 and docked complex structures were then prepared (Delano, 2002). BIOVIA Discovery studio 2021 was also used to analyze protein-ligand binding interactions (Rizvi *et al.,* 2013).

The number of berries employed Gooseberry, Acai berry, Xanthone, Cranberry, Strawberry, Gojiberry, Cherry, Raspberry Bilberry Black Currant, Blueberry, Redberry, Elderberry, Huckle Berry, Mulberry, Stone berry, Sea Buckthorn, Sea Buckthorn.

Table 2. Protein targets used in the antioxidant analysis of berries

S. No.	Protein	PDB ID
1	Lipoxygenase	1N8Q
2	NADPH-oxidase	2CDU
3	Xanthine oxidase	3NRZ

Result and discussion

Nutritional properties of Imuuxanth powder

Energy(Kcal)	05.26
Protein	1.90
Fat	0.00
Carbohydrate	01.25
Added sugar	0.00
Fibre	01.80

The data provided represents the nutritional composition of "imuuxanth powder." Each value is measured in units of kilocalories (Kcal) or grams (g) per serving. The powder contains 5.26 Kcal of energy, 1.90g of protein, 0.00g of fat, 1.25g of carbohydrates, 0.00g of added sugar, and 1.80g of fiber.

Energy Content: Imuuxanth powder has a relatively low energy content, which suggests that it may be suitable for individuals looking to manage their calorie intake or as part of a low-calorie diet.

Protein Content: The powder contains 1.90g of protein, which might be considered moderate. Protein is essential for various bodily functions, including tissue repair and muscle development. However, the protein content in this powder might not be sufficient as a sole protein source, but it can still contribute to the overall daily protein intake when combined with other foods.

Zero Fat and Added Sugar: Imuuxanth powder has no fat and added sugar. This aspect could be advantageous for those aiming to reduce fat and sugar intake for health reasons, such as weight management or controlling blood sugar levels.

Carbohydrate and Fiber: The powder contains 1.25g of carbohydrates and 1.80g of fiber. While the carbohydrate content is relatively low, the presence of dietary fiber is beneficial. Fiber aids in digestion, helps maintain bowel regularity, and can contribute to a feeling of fullness, which may assist in controlling appetite.

Amino Acid Analysis

Immuxanth powder appears to have a diverse range of amino acids, which is beneficial as it suggests a good variety of protein sources. Amino acids are essential for tissue repair, enzyme function, and overall health.

Essential amino acids are those that the body cannot synthesize on its own and must be obtained from the diet. Immuxanth powder contains a total of 79.9 mg/g of essential amino acids, which is an important aspect of its nutritional profile. These amino acids are crucial for supporting various bodily functions, including muscle growth and repair.

Non-essential amino acids can be synthesized by the body, and they are also important for various physiological processes. Immuxanth powder contains a total of 141 mg/g of non-essential amino acids.

It's important to note the relative proportions of specific amino acids in immuxanth powder. For instance, glutamic acid appears to be present in relatively high amounts (41.9 mg/g), and it is a non-essential amino acid involved in neurotransmitter function and protein synthesis. Additionally, histidine (28.6 mg/g) is an essential amino acid that is important for certain metabolic pathways.

The amino acid profile of a protein source is an essential factor in determining its overall quality and biological value. To fully assess the protein quality of immuxanth powder, its amino acid profile should be compared to established protein quality standards and the recommended daily intake of essential amino acids.

GCMS Analysis of Immuxanth powder

During the GC-MS (Gas Chromatography-Mass Spectrometry) analysis of imuuxanth powder, several bioactive compounds were identified, each contributing to the powder's chemical composition. Among the compounds detected were Emblicanin A and Emblicanin B, which belong to the xanthone class. Vitamin C (Ascorbic Acid), a well-known water-soluble antioxidant and essential nutrient, was also present. Flavonoids such as Cyanidin-3-glucoside, Cyanidin-3-rutinoside, Delphinidin 3-glucoside, Delphinidin-3-rutinoside, Cyanidin-3-sophoroside, Delphinidin-3-galactoside, Cyanidin-3-sambubioside, and Cyanidin 3-galactoside were identified. These flavonoids are known for their potential health benefits and antioxidant properties. Additionally, Proanthocyanidin A2 and Malvidin-3-glucoside were among the identified compounds. The presence of Zeaxanthin, a carotenoid with antioxidant properties, was also noted. The diverse array of bioactive compounds in imuuxanth powder suggests it may have various potential health benefits. Flavonoids, xanthones, and carotenoids are known for their roles in supporting health and well-being. However, it's essential to consider the specific concentrations of each compound and their potential synergistic effects within the powder. As with any dietary supplement or product, it is important to consult with a healthcare professional or qualified expert before using imuuxanth powder to ensure it is safe and appropriate for individual needs and health conditions.

Antioxidant evaluation

In the realm of modern nutrition research, the quest for natural compounds with potent antioxidant properties has been fervently pursued. As a response to this growing interest, the present investigation delves into the evaluation of the antioxidant activity of Immuxanth powder, a carefully

formulated supplement enriched with a selection of natural sources known for their antioxidant and immune-enhancing properties. To gauge the potential health benefits and efficacy of this innovative product, a battery of commonly accepted assays, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, and ABTS radical scavenging assay, were employed. These assays have been widely recognized for their ability to assess the free radical scavenging capacity and overall antioxidant potential of various compounds, making them indispensable tools in the investigation of dietary antioxidants. By subjecting Immuxanth powder to rigorous testing using these established methodologies, we aim to shed light on its antioxidant capabilities, contributing to the understanding of its potential role in supporting overall health and well-being.

Antioxidant Assay of Immuxanth powder

Sample	FRAP	ABTS	DPPH
Immuxanth powder	14.23±0.09	14.32±0.06	14.69+0.32

The antioxidant activity of Immuxanth powder was evaluated using three commonly accepted assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, and ABTS radical scavenging assay. The results, as presented in Table 4, demonstrate the potential antioxidant capabilities of Immuxanth powder.

In the DPPH assay, the total antioxidant activity of Immuxanth powder ranged from 14.32 to 14.90 mg trolox equivalent per gram of dry weight (mg TEAC/g dw). This assay measures the ability of antioxidants to scavenge the stable DPPH radical and reduce it to a non-radical form. The results show that all the extracts from the plants comprising Immuxanth powder exhibited varying degrees of radical scavenging capacity, highlighting the presence of active antioxidant compounds in the formulation.

The ABTS assay provided further insights into the relative antioxidant ability of Immuxanth powder to scavenge the ABTS radical cation compared to the standard Trolox. The ABTS TEAC values ranged from 14.01 to 14.62 mg TEAC/g dw. Notably, the TEAC values obtained by the ABTS assay were higher than those obtained by the DPPH assay, indicating a potentially stronger antioxidant capacity against the ABTS radical in the Immuxanth powder.

The FRAP assay, which measures the reducing potential of an antioxidant by reacting with a ferric tripyridyltriazine (Fe3+-TPTZ) complex, yielded FRAP values for the studied plants in the range of 14.12 mg to 14.5 mg Trolox equivalent per gram of dry weight (mg TEAC/g dw). The observed variation in FRAP values indicates the diverse antioxidant potential of the individual components present in Immuxanth powder.

Comparing the antioxidant results with existing literature, we find the findings align with previous studies, thus lending support to the reliability and reproducibility of the conducted assays.

The combination of DPPH, ABTS, and FRAP assays provides a comprehensive assessment of the antioxidant activity of Immuxanth powder. The varied results obtained from these assays reflect the presence of multiple antioxidant compounds in the formulation, contributing to the overall free radical scavenging capacity and reducing potential. Such a synergistic effect can potentially enhance the effectiveness of Immuxanth powder in combating oxidative stress and promoting overall health and well-being.

In conclusion, the antioxidant assays demonstrate the promising antioxidant activity of Immuxanth powder. The results indicate that the formulation possesses a diverse range of antioxidants, with the potential to scavenge free radicals and protect against oxidative damage. These findings further support the significance of Immuxanth powder as a potential dietary supplement to bolster the body's natural defenses and support overall health. Nonetheless, further studies are warranted to explore the specific antioxidant compounds present in Immuxanth powder and to assess its biological activity and potential health benefits in vivo.

Comparison to market formulation

The comparison of antioxidant capacities between Imuuxanth™ powder and Market Formulation reveals notable differences in their Oxygen Radical Absorbance Capacity (ORAC) values. Imuuxanth™ powder demonstrates a significantly higher ORAC value of 122.69 µmol Trolox/g FW, whereas Market Formulation exhibits a lower ORAC value of 39.09 µmol Trolox/g FW. This substantial contrast underscores the superior antioxidant capacity of Imuuxanth™ over Market Formulation, suggesting Imuuxanth™'s greater potential in neutralizing free radicals and mitigating oxidative stress. The implications of these findings are significant: Imuuxanth™'s robust antioxidant capacity offers enhanced protection against oxidative damage, potentially safeguarding cellular health more effectively. Conversely, while Market Formulation does possess antioxidant properties, its lower ORAC value implies a diminished ability to combat oxidative stress. Therefore, consumers evaluating these options should consider the discernible disparity in antioxidant efficacy, with Imuuxanth™ emerging as a compelling choice for those prioritizing optimal cellular health and seeking potent antioxidant support

4.1 In silico Prediction of antioxidant activity by Molecular Docking Studies

Computational methods are now an important tool for reducing the time required to unravel the action mechanisms of pharmacologically active substances. Molecular docking is a computational technique that allows the user to dock candidate molecules into the active site of a biological target and then classify the compound set based on their binding affinity, which is calculated using a scoring function (Cheng *et al*., 2012; Jianu *et al*., 2022). By using molecular docking simulations antioxidant activity of the Imuuxanth's ingredients are deduced against potential targets that can be related to a protein-targeted antioxidant effect.

4.1.1 Protein Selection

The targets consist of proteins that play critical roles in the metabolic generation of reactive oxygen species as byproducts and whose inhibition can reduce metabolic oxidative stress. This includes lipoxygenase, NADPH-oxidase, and Xanthine oxidase (Jianu *et al*., 2022). The crystal structures with lowest resolution of these proteins were downloaded from RCSB PDB database with PDB IDs 1N8Q, 2CDU and 3NRZ respectively. The lowest resolution benefits in the resolving quality of the

protein structure and these structures were prepared using X-ray diffraction (Huang, 2007).

Lipoxygenases are enzymes that catalyze the oxygenation of polyunsaturated fatty acids that can form lipid metabolites involved in several biological functions, including oxidative stress (Conteh *et al*., 2019; Ríos *et al*., 2006). Oxidative stress is a stressed state of the body in response to environmental stimuli, reflects an imbalance between the oxidative and antioxidative systems, and mainly presents as excessive production of reactive oxygen species (ROS) and/or the depletion of antioxidant enzymes and antioxidants (Huang *et al*., 2019). This also plays a vital role in the pathophysiological mechanisms of human diseases, including cancer, cardiovascular disease, diabetes, rheumatoid arthritis, Alzheimer disease, and Parkinson disease (Sosa *et al*., 2013). Furthermore, it was proven in previous studies that the inhibition of lipoxygenases can reverse the issue of the oxidative stress (Huang *et al*., 2019).

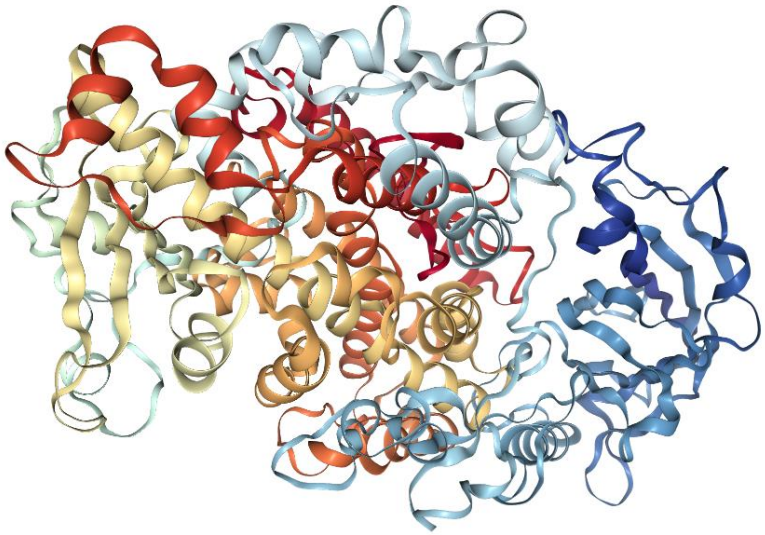

Figure 1: Structure of Lipoxygenase (1N8Q) obtained from RCSB PDB

NADPH oxidases are a family of enzymes that generate reactive oxygen species (ROS). The NOX1 (NADPH oxidase 1) and NOX2 oxidases are the major sources of ROS in the artery wall in conditions such as hypertension, hypercholesterolaemia, diabetes and ageing, and so they are important contributors to the oxidative stress, endothelial dysfunction and vascular inflammation that underlies arterial remodeling and atherogenesis (Drummond *et al*., 2011). These enzymes are widely expressed in vascular and non-vascular cells, and also there is mounting evidence that NOX are an important source of ROS and oxidative stress in glaucoma and other retinal diseases (Gaskin *et al*., 2021).

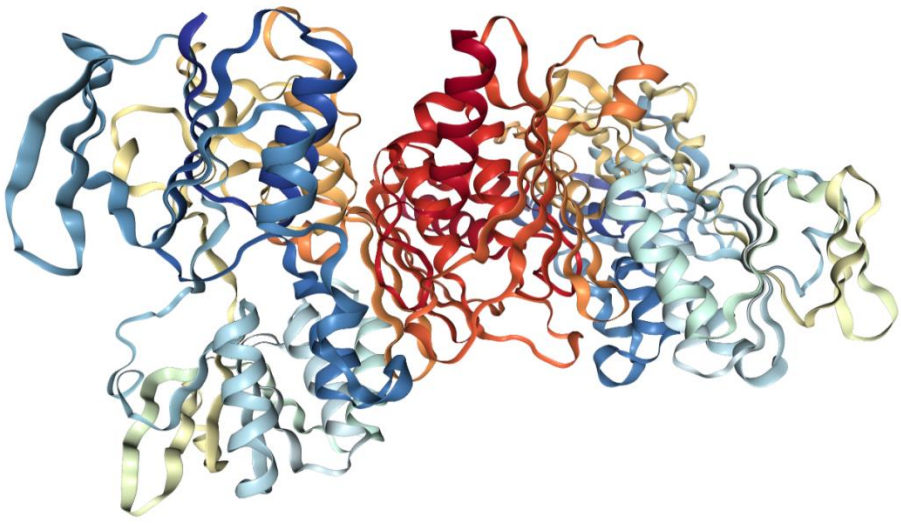

Figure 2: Structure of NADPH-oxidase (2CDU) obtained from RCSB PDB

Xanthine oxidase (XO) is an important enzyme catalyzing the hydroxylation of hypoxanthine to xanthine and xanthine to uric acid which is excreted by kidneys. Excessive production and/or inadequate excretion of uric acid results in hyperuricemia (Kostić *et al*., 2015). Uric acid is the end product of purine metabolism in humans. Hyperuricemia is a metabolic disease caused by the increased formation or reduced excretion of serum uric acid (SUA). Alterations in SUA homeostasis have been linked to a number of diseases, and hyperuricemia is the major etiologic factor of gout and has been correlated with metabolic syndrome, cardiovascular disease, diabetes, hypertension, and renal disease. Studies have demonstrated that hyperuricemia is closely related to the generation of reactive oxygen species (ROS). Therefore, XO is considered a drug target for the treatment of hyperuricemia and gout (Liu *et al*., 2021).

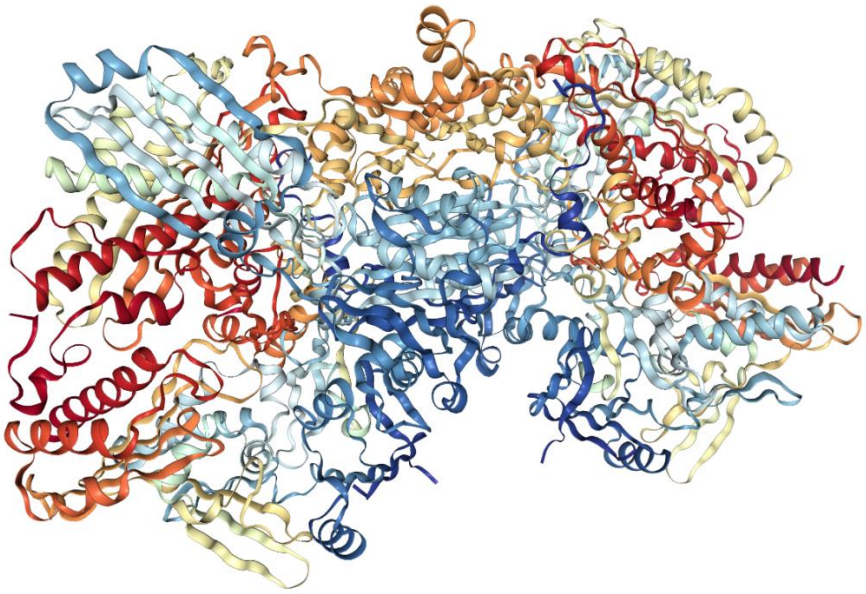

Figure 3: Structure of Xanthine Oxidase (3NRZ) obtained from RCSB PDB

4.1.2 Ligands Selection

A number of ingredients were used in the production of Imuuxanth, out of which eighteen exhibit antioxidant activity including different berries and Xanthone. GC-MS analyses identified several components in each berries and molecular docking was performed using the components that are highly active and present in abundance in all the selected berries. According to the analysis and previous findings majorly active compound which is responsible for the higher antioxidant activity is anthocyanin, these include cyanidin, delphinidin, peonidin and malvidin (Munoz-Espada *et al*., 2004; Liang *et al*., 2012; Moze *et al*., 2011). The list of selected compounds (ligands) and their 2D structures obtained by PubChem are shown in Table 3 and Figure 4 respectively.

Table 3. List of selected compounds responsible for the antioxidant activity of the berries

S. No.	Ligands
1	Emblicanin A
2	Emblicanin B
3	Vitamin C
4	Cyanidin-3-glucoside
5	Cyanidin-3-rutinoside
6	Xanthone
7	Proanthocyanidin A2
8	Zeaxanthin
9	Cyanidin-3-sophoroside
10	Delphinidin 3-glucoside
11	Delphinidin-3-rutinoside
12	Delphinidin-3-galactoside
13	Cyanidin-3-sambubioside
14	Cyanidin 3-galactoside
15	Malvidin-3-glucoside

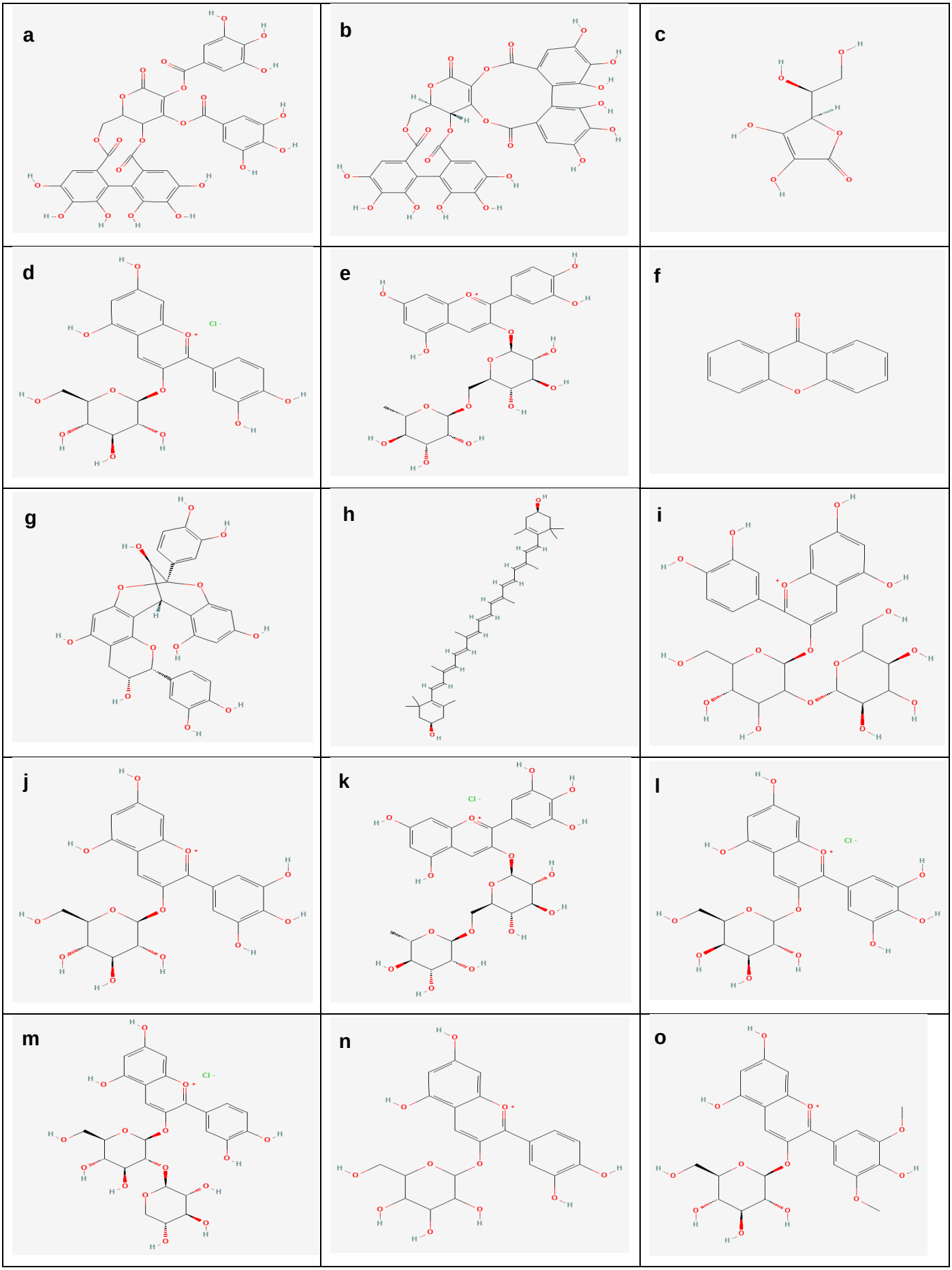

Figure 4. (a) Emblicanin A; (b) Emblicanin B; (c) Vitamin C; (d) Cyanidin-3-glucoside; (e) Cyanidin-3-rutinoside; (f) Xanthone; (g) Proanthocyanidin A2; (h) Zeaxanthin; (i) Cyanidin-3-sophoroside; (j) Delphinidin 3-glucoside; (k) Delphinidin-3-rutinoside; (l) Delphinidin-3-galactoside; (m) Cyanidin-3-sambubioside; (n) Cyanidin 3-galactoside; (o) Malvidin-3-glucoside

4.1.3 Active sites Prediction

The prediction of active site is a very helpful step in the docking simulations, that can exclude target proteins or binding sites that have weak or no binding ability to ligands. In addition, identifying binding sites is not only beneficial for the functional characterization of proteins but also provides the knowledge about them to guide the design of inhibitors and antagonists (Capra *et al*., 2009; Liao *et al*., 2022). This stage is important considering that up to 60% of failures in the clinical trial phase may be attributed to non-druggable targets (Mohs and Greig, 2009; Schuhmacher *et al*., 2016). The significant criteria for all the 3 target proteins-ligand interactions are depended on whether the ligand is properly oriented and conformed or not on the active site of the proteins. The predicting procedure for active site generated the sites on which the ligand was binding, out of which the grid box measurements of the active sites were 21.954744 x 0.918539 x 19.525457 A° for lipoxygenase, 2.382925 x 2.239538 x -1.191920 A° for NADPH-oxidase and 39.305740 x 7.966833 x 15.289809 A° for Xanthine Oxidase (Figure 5-7).

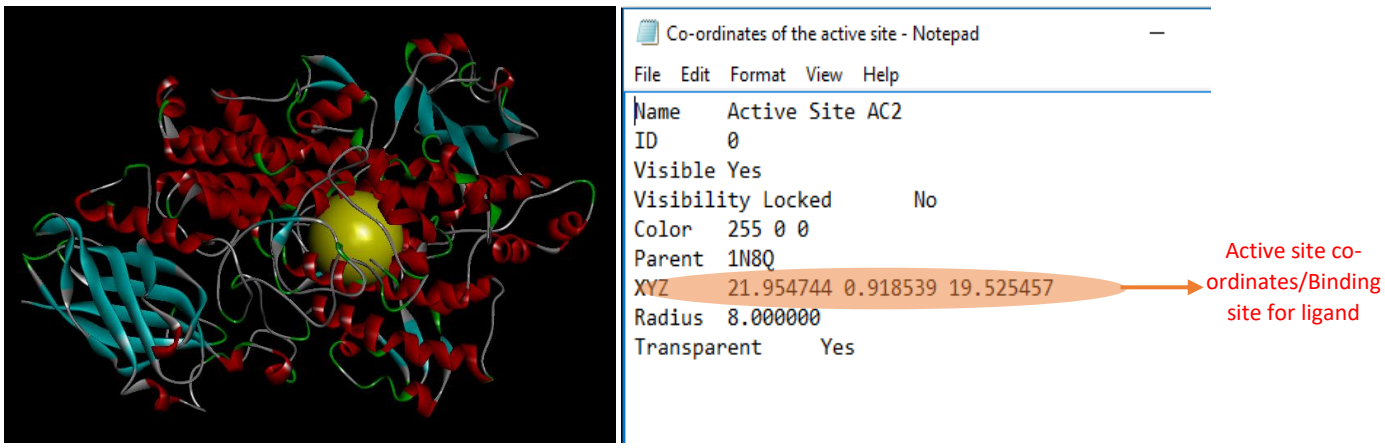

Figure 5. Binding site in the Lipoxygenase represented by sphere in BIOVIA Discovery Studio (a) represent binding site sphere; (b) acting residues in the active site of protein.

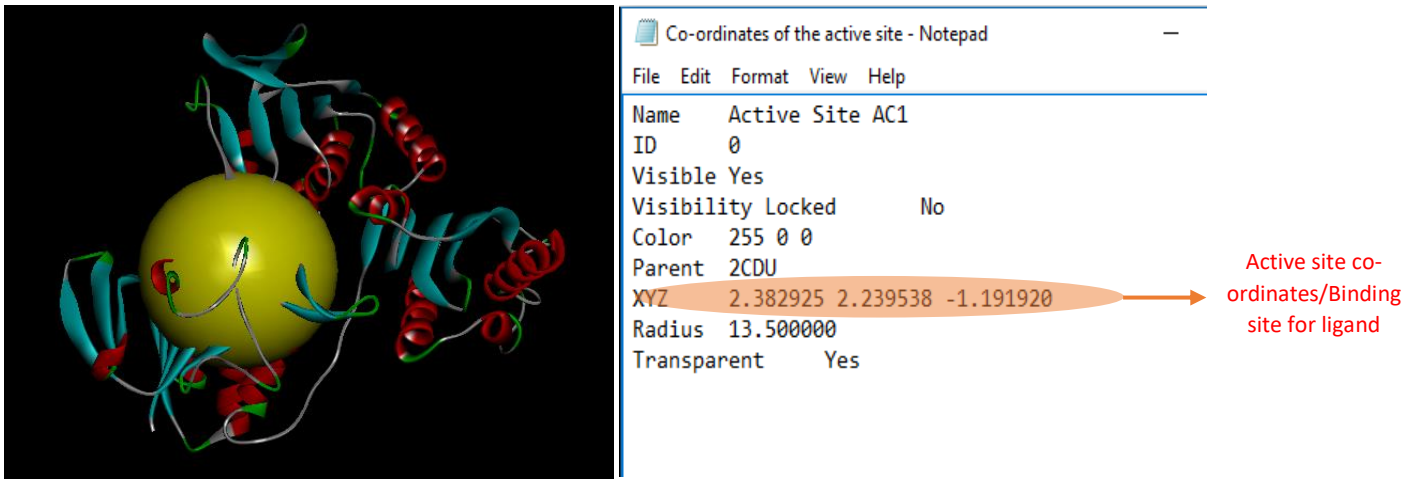

Figure 6. Binding site in the NADPH-oxidase represented by sphere in BIOVIA Discovery Studio (a) represent binding site sphere; (b) acting residues in the active site of protein.

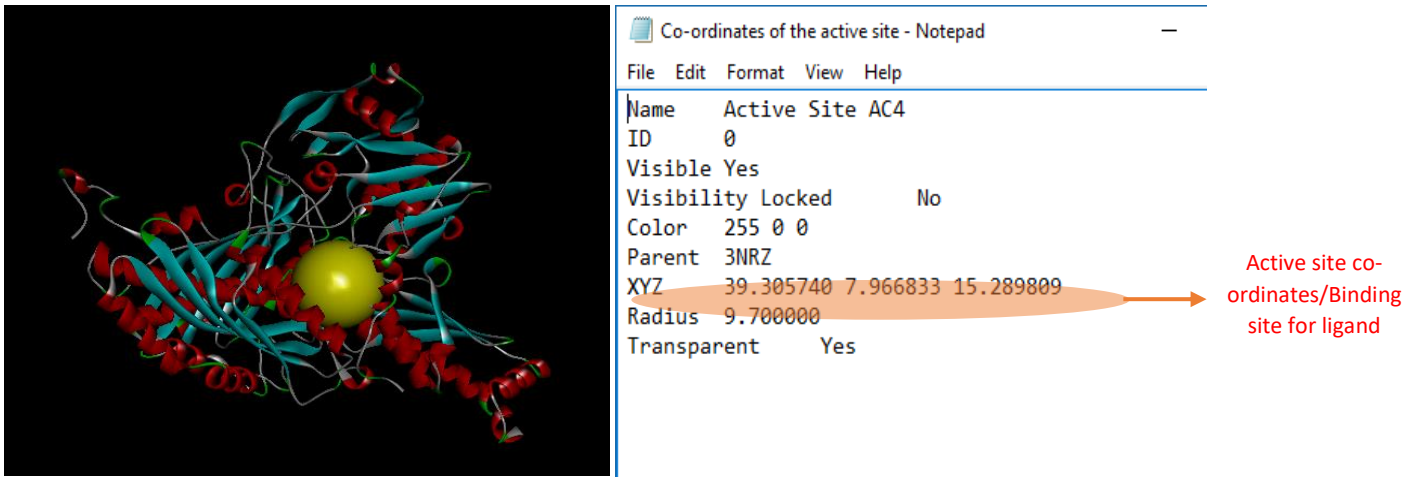

Figure 7. Binding site in the Xanthine Oxidase represented by sphere in BIOVIA Discovery Studio (a) represent binding site sphere; (b) acting residues in the active site of protein.

Visualization of docked complex

The docking was conducted using AutoDock and Windows PowerShell in order to determine the potential virulent spike proteins that were responsible to destabilize the ligand molecule. The docking conducted via these generated several results based on the Binding Free Energy ΔG (kcal/mol) of the interaction, out of which first was selected because of the best free energy score, i.e., lower the binding free energy, higher will be the stability of protein-ligand binding interaction (Chu *et al*., 2018). Table 3 displays the docking scores obtained for the 15 docked compounds.

Table 3. Docking scores for compounds 1–15 (binding energy, ΔG kcal/mol); compounds with better docking scores are highlighted in bold.

Target PDB ID0		1N8Q	2CDU	3NRZ
S. No.	**Ligands**	**Binding Free Energy ΔG (kcal/mol)**		
1	**Emblicanin A**	-9.6	**-10.0**	-9.2
2	**Emblicanin B**	-9.3	**-10.2**	-9.8
3	Vitamin C	-5.6	-6.6	-6.2
4	**Cyanidin-3-glucoside**	**-9.0**	**-9.0**	-8.0
5	**Cyanidin-3-rutinoside**	-10.0	**-10.8**	-9.7
6	Xanthone	-7.2	-7.6	-7.5
7	**Proanthocyanidin A2**	**-10.3**	-9.2	-9.8
8	Zeaxanthin	-6.6	-10.8	-8.6
9	**Cyanidin-3-sophoroside**	**-9.2**	-9.1	-8.4
10	**Delphinidin 3-glucoside**	-8.7	**-9.2**	-8.2
11	**Delphinidin-3-rutinoside**	-9.8	**-10.9**	-9.1
12	Delphinidin-3-galactoside	-8.7	-8.9	-7.6
13	**Cyanidin-3-sambubioside**	-9.3	**-9.5**	-8.7
14	Cyanidin 3-galactoside	-9.2	-9.2	-7.8
15	Malvidin-3-glucoside	-8.1	-9.2	-7.8

According to the Docking scores all the compounds were significantly effective against the target proteins and majorly against NADPH-oxidase and after that lipoxygenase. The further analysis was then performed on the basis of the best docking scores of the compounds that covers top 10 berries. The compounds with best docking scores includes, Emblicanin A, Emblicanin B, cyanidin-3-glucoside, cyanidin-3-rutinoside, Proanthocyanidin A2, cyanidin-3-sophoroside, delphinidin 3-glucoside, delphinidin-3-rutinoside and cyanidin-3-sambubioside showed the highest antioxidant activity. And top 10 berries that contains above mentioned compounds in abundance are Gooseberry, Acai Berry, Cranberry, Cherry, Raspberry, Bilberry, Black Currant, Blueberry, Elderberry and Mulberry (Table 5). These berries are majorly responsible for the higher antioxidant activity of the Imuuxanth.

The docked complex structures were then prepared by using PyMOL v2 and were further saved in .pdb format. PyMOL is an open-source molecular visualization tool and has been extensively used for visualizing the macromolecules in 3D and it becomes one of the most popular tools for preparing hi-res (high-resolution) images of macromolecules for publications (Yuan *et al*., 2017). Figure 8 & 9 showing the complex structures prepared by PyMOL, in which blue surface representation of structure is the target protein and middle stick structure represents ligand.

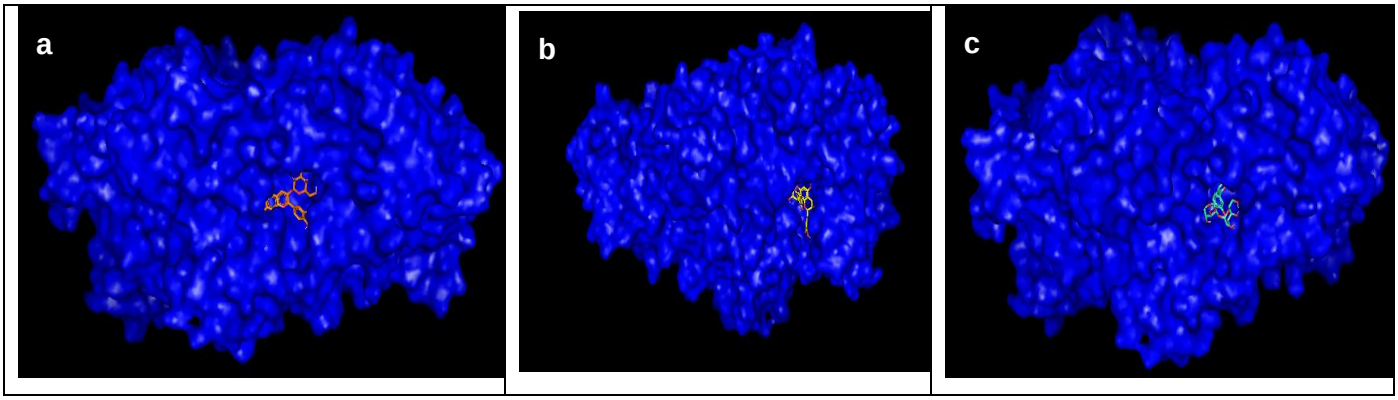

Figure 8: Surface representation of Lipoxygenase in complex with docked compounds. (a) Cyanidin-3-glucoside; (b) Proanthocyanidin A2; (c) Cyanidin-3-sophoroside.

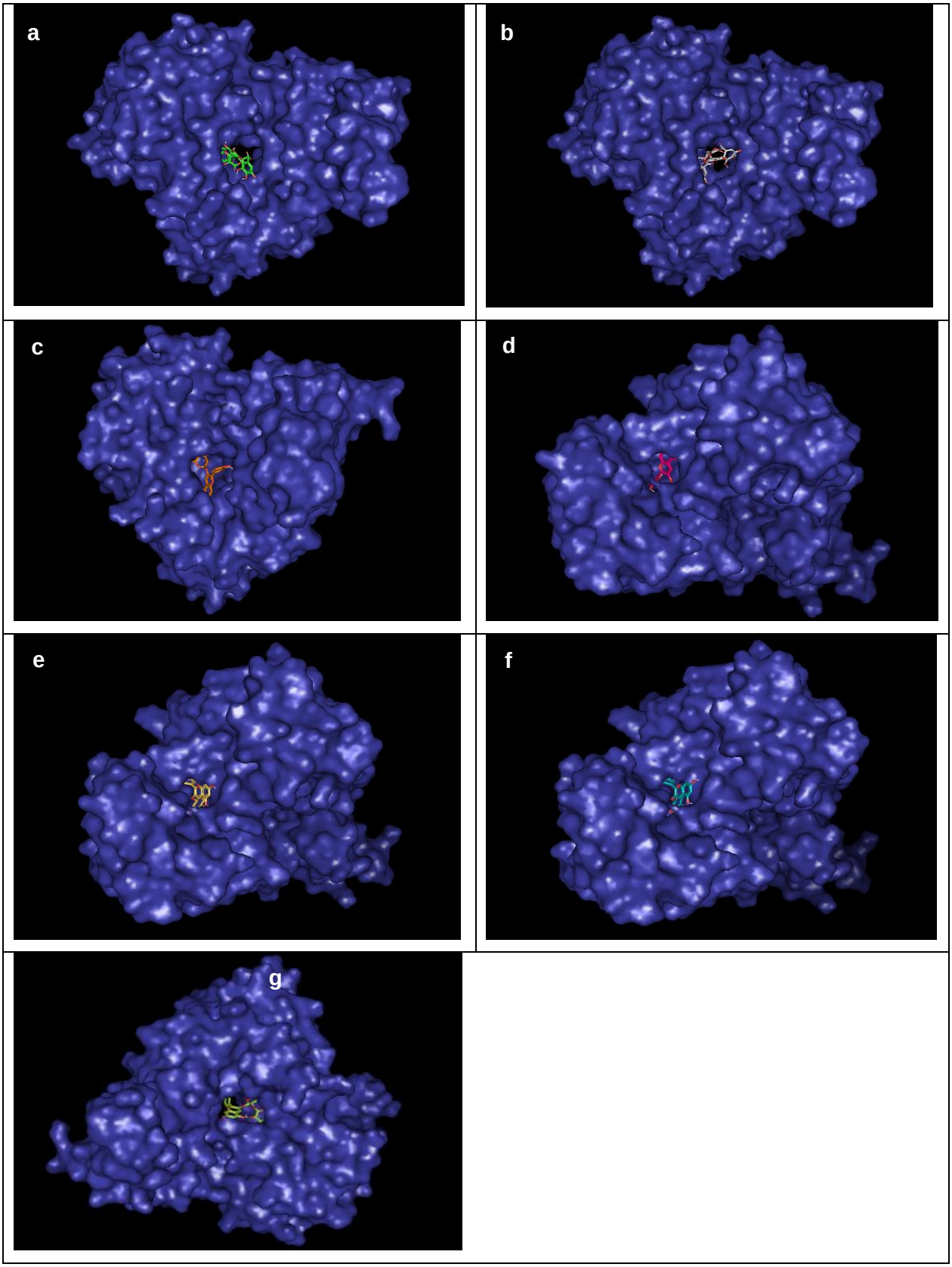

Figure 9: Surface representation of NADPH-oxidase in complex with docked compounds. (a) Emblicanin A; (b) Emblicanin B; (c) Cyanidin-3-glucoside; (d) Cyanidin-3-rutinoside; (e) Delphinidin 3-glucoside; (f) Delphinidin-3-rutinoside; and (g) Cyanidin-3-sambubioside.

The docked complexes were then visualized by using BIOVIA Discovery Studio (Figure 10 & 11), which was able to show all the hydrophobic as well as the hydrogen bonds between protein ligand interactions along with the protein amino acid residues involved (Table 4). Apart from hydrophobic and hydrogen bonds, some docked complexes had the interactions that are electrostatic in nature were also depicted in Discovery Studio but they don't contribute much neither in affinity nor in the interactions that are made with the significant group having charges, so they were hidden for the clearer picture of the important interactions (Clackson and Welis 1995). Interactions of the docked compounds, acting amino acid residues, type of bonds formed between protein and ligands are shown in series of picture below (Figure 10 a-c and Figure 11 a-g).

Table 4. Interacting amino acid residues in the docked complex structures.

S. No.	Proteins	Ligands	Hydrogen bonds	Hydrophobic interactions
1	Lipoxygenase	Cyanidin-3-glucoside	LYS A:278, TYR A:150, ARG A:260, 2 LEU A:258	LEU A:560, ALA A:263
2	Lipoxygenase	Proanthocyanidin A2	2 ASP A:592, ASP A:431, GLN A:598	PRO A:432
3	Lipoxygenase	Cyanidin-3-sophoroside	2 ASN A:556, VAL A:256, PHE A:264, LEU A:258, LYS A:278, ASP A:255	PHE A:272, ALA A:263
4	NADPH-oxidase	Emblicanin A	LYS A:134, ASP A:282, PRO A:298, LEU A:258, ALA A:300, HIS A:10	PHE A:245, ALA A:303, LEU A:40
5	NADPH-oxidase	Emblicanin B	LYS A:134, ASP A:282, ALA A:300	ILE A:160
6	NADPH-oxidase	Cyanidin-3-glucoside	HIS A:10, LYS A:134, ASP A:282, GLY A:329	ILE A:160, 2 ILE A:44
7	NADPH-oxidase	Cyanidin-3-rutinoside	2 ALA A:11, THR A:112, ASN A:34, ASN A:248, LYS A:134, SER A:41	MET A:33, ALA A:300
8	NADPH-oxidase	Delphinidin 3-glucoside	2 THR A:112, 2 THR A:9, PHE A:39, ASN A:36, ASN A:135, SER A:115, ASN A:248	LYS A:134
9	NADPH-oxidase	Delphinidin-3-rutinoside	2 LYS A:134, 2 ASN A:36, THR A:9, ASN A:135, ASN A:34, ASN A:248, PHE A:39, SER A:115, ASP A:282	PRO A:117, ALA A:300, LYS A:134
10	NADPH-oxidase	Cyanidin-3-sambubioside	SER A:41, LYS A:134, HIS A:10, ASP A:282, ALA A:300, PRO A:298, 1LE A:160, TYR A:159, TYR A:188, GLY A:329	2 LEU A:299

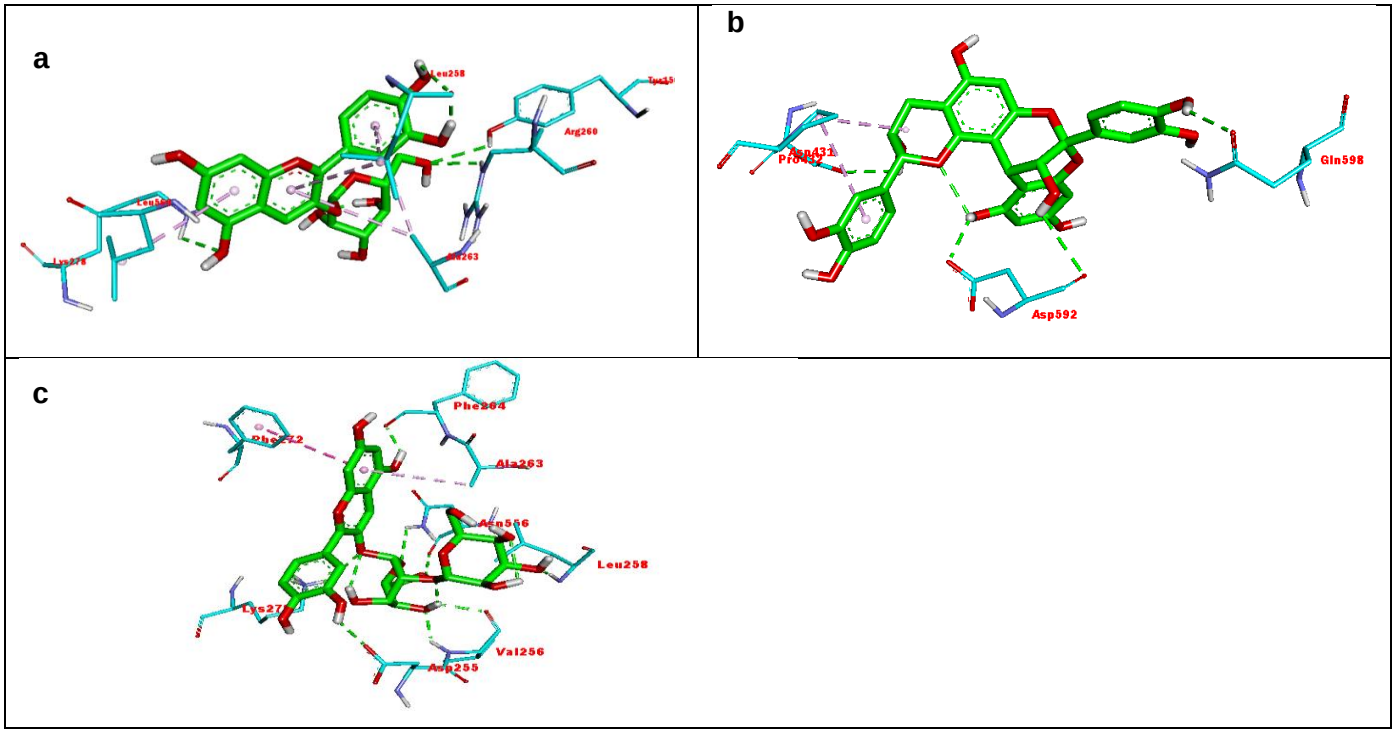

Figure 10: Docked structure interaction visualization of Lipoxygenase in complex with docked compounds (3D). (a) Cyanidin-3-glucoside; (b) Proanthocyanidin A2; (c) Cyanidin-3-sophoroside.

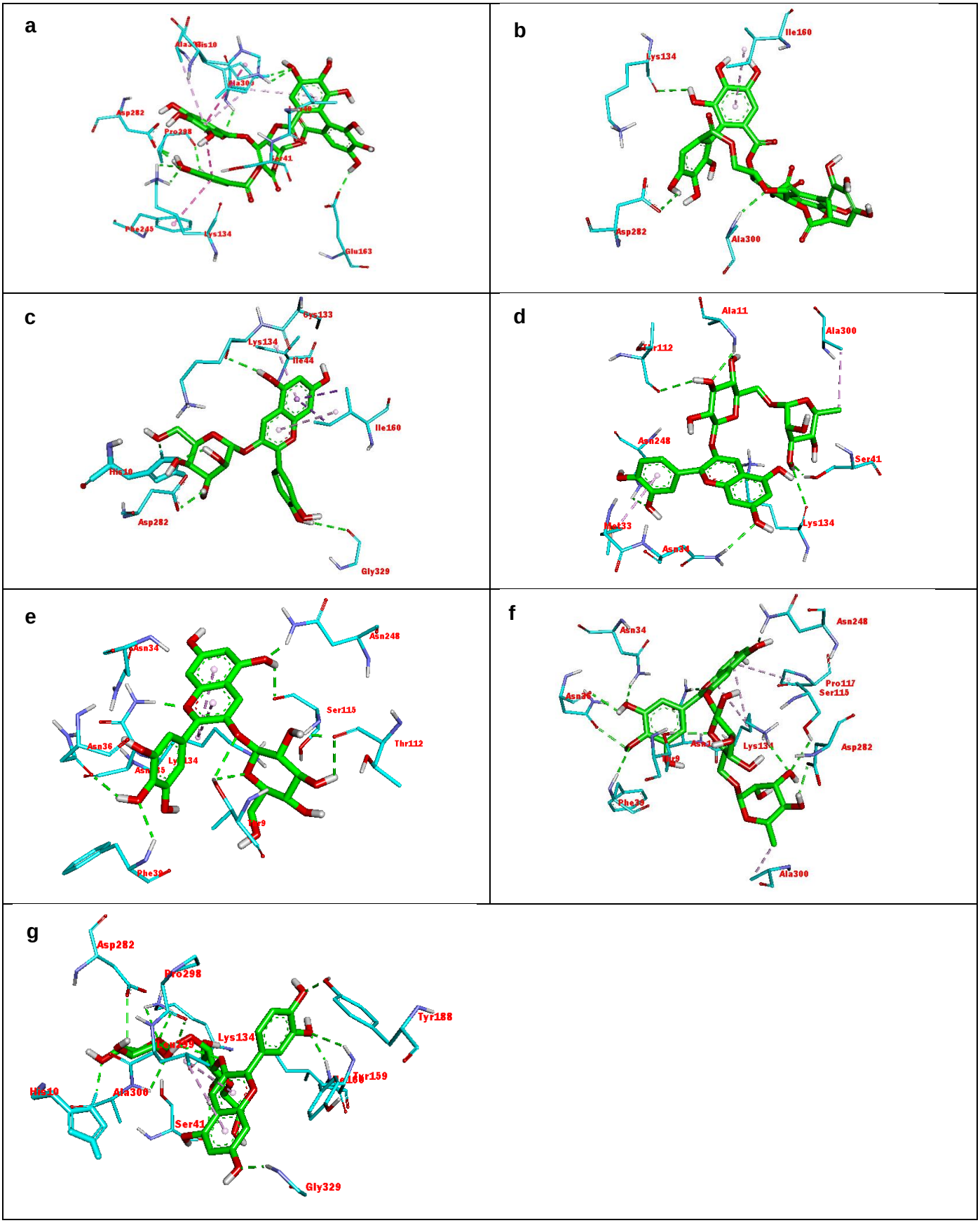

Figure 11: Docked structure interaction visualization of NADPH-oxidase in complex with docked compounds (3D). (a) Emblicanin A; (b) Emblicanin B; (c) Cyanidin-3-glucoside; (d) Cyanidin-3-rutinoside; (e) Delphinidin 3-glucoside; (f) Delphinidin-3-rutinoside; and (g) Cyanidin-3-sambubioside. HB interactions are depicted as green dotted lines and hydrophobic interactions as purple dotted lines.

The research investigated the nutritional properties and antioxidant evaluation of Imuuxanth powder, a dietary supplement formulated with a selection of natural sources known for their antioxidant and immune-enhancing properties. The nutritional analysis revealed that Imuuxanth powder contains 5.26 Kcal of energy, 1.90g of protein, 0.00g of fat, 1.25g of carbohydrates, 0.00g of added sugar, and 1.80g of fiber per serving. While the energy content is relatively low, the powder's protein and fiber content could contribute to overall nutritional intake.

Amino acid analysis demonstrated that Imuuxanth powder has a diverse range of amino acids, including essential and non-essential ones. The presence of essential amino acids is crucial for various bodily functions, such as tissue repair and muscle growth.

GC-MS analysis identified several bioactive compounds in Imuuxanth powder, including xanthones, flavonoids, carotenoids, and vitamin C. These compounds are known for their potential health benefits and antioxidant properties.Antioxidant evaluation was conducted using three assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Ferric Reducing Antioxidant Power (FRAP) assay, and ABTS radical scavenging assay. The results demonstrated the promising antioxidant activity of Imuuxanth powder, with potential benefits in scavenging free radicals and protecting against oxidative damage.Furthermore, in silico molecular docking studies were performed to assess the antioxidant activity of selected compounds in Imuuxanth powder against critical enzymes involved in metabolic oxidative stress. The compounds, such as Emblicanin A, Emblicanin B, cyanidin-3-glucoside, and others, showed effective docking scores against the target proteins, suggesting their potential role in reducing oxidative stress. The research highlights the nutritional and antioxidant properties of Imuuxanth powder, indicating its potential as a dietary supplement to support overall health and well-being. The diverse array of bioactive compounds, along with the promising antioxidant activity, suggests that Imuuxanth may contribute to combating oxidative stress and promoting better health. However, further studies, including in vivo research, are necessary to fully understand the health benefits and safety of Imuuxanth powder.

Conclusion:

The research findings indicate that Imuuxanth powder is a nutritionally valuable product, with moderate protein content and no added sugar or fat. It is a potential supplement for individuals seeking to manage calorie intake and control fat and sugar consumption.The amino acid analysis demonstrates the presence of essential and non-essential amino acids, contributing to the overall protein quality of Imuuxanth powder. The diverse range of amino acids enhances its nutritional profile.

GC-MS analysis revealed the presence of various bioactive compounds in Imuuxanth powder, including xanthones, flavonoids, carotenoids, and vitamin C. These compounds offer potential health benefits and antioxidant properties.

The antioxidant evaluation highlights the significant antioxidant activity of Imuuxanth powder, indicating its potential in scavenging free radicals and protecting against oxidative stress.

Molecular docking studies suggest that selected compounds in Imuuxanth powder have effective interactions with critical enzymes involved in metabolic oxidative stress. This suggests the potential of Imuuxanth in reducing oxidative stress and supporting overall health.

Overall, the research provides valuable insights into the nutritional and antioxidant properties of Imuuxanth powder, making it a promising dietary supplement with potential health benefits. However, further research, including in vivo studies, is needed to fully explore its effects and safety in a practical setting. Individuals interested in using Imuuxanth powder as a dietary supplement should consult healthcare professionals to ensure its suitability for their individual needs and health conditions.

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