Original Research Article

UNVEILING THE POTENTIAL OF ANTIOXIDANT PROTEINS WITH THE INTEGRATION OF LITTLE MILLET PHYTOCHEMICALS FROM GC-MS STUDIES THORUGH *in silico* APPROACH

ABSTRACT

Aim: Millet extracts contain bioactive compounds that have antioxidant properties, anti-diabetic, anti-inflammatory, and other health-promoting properties. Little millet contains more protein, minerals, vitamins, and carbohydrates than rice and wheat. The dynamics of soil organic matter may be significantly impacted by the presence of antioxidants compounds in the soil. Antioxidants and protein-modifying substances are dietary components that change numerous characteristics and, in some cases, reverse ageing. Thus, exploring the phytochemicals in little millet is very much essential in understanding its biological functional implications.

Methodology: We have carried out the GCMS analysis for the little millet (seed). Further, we have performed the molecular docking and molecular dynamics simulation for the shortlisted phytochemicals.

Results: We screened the metabolites using GCMS analysis due to the unexplored phytochemicals of little millet. Docking against the little millet phytochemicals was done with a focus on key antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase. Acetin compound displayed strong binding with superoxide dismutase and glutathione peroxidase, while hexadecenoic acid exhibited best affinity with catalase. Through molecular dynamics simulations, we found the glutathione peroxidase complex to be the most stable. This stability implies enhanced antioxidant activity, crucial in counteracting oxidative stress.

Conclusion: This study uncovers the untapped potential of little millet's phytochemicals. By elucidating their interaction with vital antioxidant proteins, it opens avenues for innovative anti-aging strategies, health interventions and helps in enhancing the plant defence mechanism.

Keywords: acetin, antioxidant, catalase, GCMS, glutathione peroxidase, little millet, superoxide dismutase.

Abbreviations:

GC-MS – Gas chromatography Mass Spectrometry

HPLC – High Performance Liquid Chromatography

SOD – Superoxide dismutase

GPx – Glutathione peroxidase

CAT – Catalase

MSTFA – N- Methyl – N –(trimethylsilyl)trifluoroacetamide

1. INTRODUCTION

Millets are considered as Nutri-cereals and have been linked to potential health advantages, therefore minor millets have become the curiosity among experts in recent years [1]. Generally minor millets include foxtail millet (*Setaria italica*), kodo millet (*Paspalum scrobiculatum*), little millet (*Panicum sumatrense*), barnyard millet (*Echinochloa frumentacea*), proso millet (*Panicum miliaceum*) whereas major millets include sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), and finger millet (*Eleusine coracana*). Millets include phytochemicals, which contribute to grain nutritional content and this allows the researchers to investigate potential health benefits and minor millet phytochemicals have health promoting properties that include antioxidant activity, anti-diabetic activity, anti-microbial activity, anti-proliferative activity and anti-inflammatory activity [2]. Little millet can withstand both drought and water logging. Insufficient phosphorus may lead to a decrease in crop yield, according to [3], but historically little millet is grown in low-fertile soils with minimal or no fertiliser application and also, they claimed that by using experimental methods. When compared to rice and wheat, little millet contains more protein, fat, carbohydrates, crude fibres, minerals, and vitamins [4]. The phytochemicals found in little millet have not been thoroughly studied; however, phenols, flavonoids, tannins, and phytate have been listed in a few studies and fractions of phenolic compounds found in methanol extracts of little millet exhibit potential phytochemicals [5], [4].

Diabetes mellitus may cause changes in the endogenous free radical scavenging defence systems, leading in insufficient scavenging of reactive oxygen species, resulting in oxidative damage and tissue loss [6]. Millet extracts contain bioactive substances with antioxidant activity, anti-diabetic, anti-inflammatory, and other health-promoting qualities[7]. Antioxidants are a diverse collection of compounds that are difficult to categorize based on common structural features[8]. Antioxidants and protein-modifying chemicals are the dietary compounds which changes several features and, in some cases, it overcomes aging [9]. Phenolic acids have become recognized antioxidants because they provide hydrogen or electrons[4]. Polyphenols such as tocopherols and tocotrienols are a diverse group of plant-derived natural compounds and they are the important components of biological membranes, serving as both antioxidants and non-antioxidants[8]. The researchers discovered that 95% of total dietary flavonoids came from two components, 63% from quercetin and 32% from kaempferol[10].The antioxidant power of phenolics is best explained thing in terms of free radicals created by cell metabolism[11]. Oxidative stress is caused by an imbalance between the formation of reactive oxygen species (ROS), mostly free radicals, and the protective effects including free radical inhibition, direct free radical scavenging, or detoxification[12]. The free radicals cause lipid peroxidation of the membrane lipids, which causes the red blood cells to burst and this can be treated or avoided by taking antioxidant supplements [13].Reactive oxygen species (ROS), which are formed either directly or indirectly as a result of environmental stresses, are believed to be responsible for a large portion of the harm that plants experience [14]and they stated that copper and zinc deficiency influenced SOD activities. Superoxide dismutase[15], catalase[16], and glutathione peroxidase [17] were the assays analysed and tested for the antioxidants. Plant cells contain an antioxidant system to defend them from such harm, which consists of: (1) low-molecular-weight antioxidants such ascorbate, -tocopherol, glutathione, and carotenoids; and (2) protective enzymes that work as follows: Superoxide

dismutase (SOD) neutralises superoxide radicals, and the ascorbate-glutathione cycle, which contains the enzymes ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase, can detoxify the reaction's end product which is H_2O_2 [18]. Present study focused on finding the interaction and stability between the antioxidant target proteins namely superoxide dismutase, catalase, glutathione peroxidase and the bioactive compounds obtained from little millet (*Panicum sumatrense*) seeds through GC-MS analysis.

2. MATERIALS AND METHODS:

2.1 COLLECTION OF PLANT MATERIALS

The seeds of little millet (*Panicum sumatrense*) (ATL 1) variety were taken as the sample for GC-MS analysis and it was obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore. It is located at a height of 411.98 m and between 11°N latitude and 77°E longitude. The collected seed samples were kept at -20°C for an extended period of time.

2.2 SAMPLE PREPARATION AND EXTRACTION

The bioactive compounds were identified in little millet (ATL 1) seeds, through the metabolomic study GC-MS analysis. The protocol by [19] was used for the compound extraction with slight modifications. The seeds (200mg) were homogenised for roughly 2 minutes at 50hz in a mixer mill. The enzyme was deactivated with the addition of 1.4 ml of 100% HPLC-graded methanol. The mixture was shaken for 15 minutes in a water bath at 65 °C and centrifuged for 10 minutes at 11,000 g. After transferring the supernatant to a new 2ml Eppendorf tube, 750 µL of chloroform was added. Then 1.4ml of Milli-Q water was added and vortexed for 50 seconds. The mixture was centrifuged again for 10 minutes at 10,000 rpm, and the aqueous layer was filtered through 0.45µm and transferred to a fresh tube. In a vacuum concentrator for 45 minutes, the extract was totally dried. The derivatization reagent MSTFA (70ml) was added and incubated at 37^0 c for 30 mins.

2.3 GCMS ANALYSIS

Shimadzu triple quadrupole GC-MS-TQ8040 NX Gas Chromatograph-Mass Spectrometer (GC-MS) was used to analyse the derivatized samples. 1 µL of aliquot was injected into the GC column at a split ratio of 1:20 at an injection temperature of 280°C. The temperature of the column (SH-Rxi-5Sil MS column) was set to start at 70°C for 1 minute, then increased to 320°C for 10 minutes. The mass spectrometer's programming is as follows: The mass range was 50 m/z to 650 m/z, with the ion source at 230 °C, the interface at 280 °C, and the solvent cut at 5.0 min.

2.4 PREPARATION OF PROTEIN AND LIGAND

The antioxidant target proteins namely superoxide dismutase (SOD) catalase (CAT), glutathione peroxidase (GPx) and the crystallographic three-dimensional structure of these enzymes were taken from the Protein Data Bank (PDB) [20]. The phytochemical compounds were taken from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Both target proteins and ligands were prepared for the docking analysis using the BIOVIA Discovery Studio Visualizer (DS4.5, Accelrys, Inc., San Diego, CA, USA)[21]. Macromolecule module was used to create the receptor protein, which involved energy minimization and the creation of protonation sites to ensure structural stability. The CHARMm force field was used to minimise protein energy for retaining the root mean square deviation (RMSD) at 0.25. Binding pocket residues were predicted. Using the software's small molecule methodology, the ligands were minimised and prepared for docking. To construct a 3D conformation of a metabolite process such as ionisation, the tautomer and isomers are set to their default values, allowing for accurate and efficient docking simulations.

2.5 ADMET SCREENING

Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties was checked using SwissADME web-server [22] for all the ligands. SMILES (Simplified Molecular Input Line Entry System) notation of all the ligands was used throughout the process. The physio-chemical descriptors pharmacokinetic properties were computed. Lipinski rule of five includes number of hydrogen bond donors, number of hydrogen bond acceptors, molecular mass, partition coefficient was taken into consideration for the drug-likeness property of the compound.

2.6 VIRTUAL SCREENING OF THE COMPOUNDS AS ANTIOXIDANT INHIBITORS

Grid-based CDOCKER components of the Discovery Studio program were utilised for simulated screening of inhibitors against the antioxidant targets. The binding affinity of each chemical in the molecular complex was estimated using CDOCKER energies. The best-docked position for each ligand was chosen based on the CDOCKER energy and CDOCKER interaction energy.

2.7 NETWORK PHARMACOLOGY

The identified phytochemicals from the little millet seeds using GC-MS analysis and the top binding energy resulted ligands with the target proteins were taken for the construction of biomolecular interaction networks using Cytoscape v3.9.1[23].

2.8 MOLECULAR DYNAMICS SIMULATION

BIOVIA Discovery Studio and a solvation phase with specific water molecules and periodic boundary conditions were used to run MD simulations of the highest-ranking docking

conformations. The simulation time was set to 20,000ps (20 ns), and the system went through minimization and equilibration phases, followed by 20ps heating, equilibration, and production phases.

3. RESULTS

3.1 METABOLITE PROFILING

Derivatized metabolites from the seeds of little millet (ATL 1) were subjected to GC-MS analysis. The presence of 92 bioactive phytoconstituents were identified in seed extracts of little millet (*Panicum sumatrense*) using GC-MS (Table 1). The mass spectra of each plant metabolite at varying retention times were compared to the National Institute of Standards and Technology (NIST-14) mass spectra database. In GC-MS analysis, the retention time in minutes (RT), peak area (%, computed by dividing each compound's peak area by the total of all compounds' peak areas within the sample), and compound name were identified (Table 1). The resulting ion chromatogram of the little millet was shown in (Fig. 1).

Table 1 Compounds identified in little millet (ATL 1) by GC-MS analysis. CID-Compound ID, RT time – Retention Time

CID	Compound Name	RT Time (mins)	Area%
14273	2-Pyridinecarboxaldehyde	5.06	1.5
7362	2-Furancarboxaldehyde	5.14	1.9
8091	Methyl caprylate	5.267	0.25
673	Dimethylglycine	5.3	0.22
753	Glycerin	6.423	1.47
6287	Valine	6.593	0.77
8998	1,2,3,4-Butanetetrol	6.903	0.64
8468	Vanillic acid	6.903	0.64
612	Lactic Acid	6.955	0.05
5319706	Methyl linolenate	7.058	2.27
7824	Methyl caproate	7.257	0.16
23105108	1-[(Trimethylsilyl)oxy] propan-2-ol	7.281	0.41
757	Glycolic acid	7.363	0.03
439368	Pantolactone	7.403	0.38
135	4-Hydroxybenzoic acid	8.843	0.18
6112	Lipoic acid	8.903	0.07
135398638	Hypoxanthine	8.969	0.3
787	Hydroxylamine	9.194	0.12
91696644	Acetin, bis-1,3-trimethylsilyl ether	9.873	0.33
241	Benzene	9.993	0.28
5280863	Kaempferol	10.148	0.4
9231	Azulene	10.34	1.23
264	Butanoic acid	10.51	1.28
5288725	L-Alanine, N-methyl-	10.623	1.34
120047	Benzaldehyde, 4-propyl-	12.641	4.72
258796	2-Pyrazinecarbohydrazide	12.815	0.49
332	2-Methoxy-4-vinylphenol	13.702	0.42

14112	1(2H)-Naphthalenone, 3,4-dihydro-6-methoxy-	15.969	2.28
6262	Ornithine	16.264	0.33
1474	2,2'-Bipyridine	17.001	0.23
996	Phenol	17.181	0.5
222285	Erythritol	18.47	0.06
760	Glyoxylic acid	20.017	0.31
379	Octanoic acid	21.684	3.56
5364509	Methyl oleate	22.402	0.25
7057995	alpha-Chloralose	22.809	0.06
6912	Xylitol	23.306	0.19
5281	Octadecanoic acid	24.206	0.53
445638	Palmitoleic acid	24.376	0.27
261	Butanal	24.831	0.18
14408225	beta-D-Tagatopyranose	25.217	0.07
2723872	D-Fructose	25.549	0.66
18950	d-Mannose	26.045	0.67
94715	D-(+)-Glucuronic acid	26.745	7.46
445580	Docosahexaenoic acid	26.745	0.31
444899	Arachidonic acid	26.852	0.19
99459	D-(+)-Talose	27.024	8.55
6421258	Methyl arachidonate	27.032	0.26
18302	1,2,4-Butanetriol	27.241	0.98
17386	1-Pentadecanamine	27.338	0.34
5793	Glucose	27.357	2.34
985	n-Hexadecanoic acid	28.29	4.61
10349	Methylsuccinic acid	28.666	0.41
6036	d-Galactose	30.306	0.17
892	Scyllo-Inositol	30.64	0.91
5282800	10E,12Z-Octadecadienoic acid	31.447	1.72
3075922	Dimethylaminoethyl palmitate	34.461	0.94
9255	Oxazole	35.293	0.21
135191	D-Xylopyranose	35.395	0.08
101719625	alpha-D-Glucopyranoside	37.46	0.14
25310	L-Rhamnose	37.894	0.03
70095	Octadecanedioic acid	39.232	1.46
54301978	D-(+)-Turanose	39.47	0.07
11756	Ethylmalonic acid	39.831	0.16
439709	beta-D-Fructofuranose	40.728	0.04
445408	5-Methyluridine	41.378	0.28
5362793	Methyl linolelaidate	41.615	0.27
12311284	Linocinnamarin	41.762	10.74
3931	9,12-Octadecadienoic acid	42.196	1.52
5364783	Oleoyl chloride	42.287	2.42
5280450	(Z,Z)-9,12-Octadecadienoic acid	42.63	2.51
445639	(Z)-9-Octadecenoic acid	42.714	4.03
441	3-Hydroxybutyric acid	43.426	0.43
8892	Caproic acid	43.623	0.44
10314695	Rosiridin	43.633	9.55

5282714	2-Octenoic acid	43.719	0.26
5365371	13-Docosenamide	44.107	0.5
11099946	2-alpha-Mannobiose	45.697	0.08
6657	2-Aminobutanoic acid	45.803	0.65
2724705	Levoglucosan	46.111	0.53
6134	D-Lactose	46.257	0.05
10975657	Ribose	48.113	0.17
5353760	Methoprene acid	50.243	0.24
5997	Cholesterol	50.386	0.27
99474	Diosgenin	51.17	0.5
87	3-Hydroxyisobutyric acid	51.55	0.19
439507	Allose	52.663	2.66
1060	Pyruvic acid	53.211	0.06
643801	Methyl palmitoleate	53.641	0.92
743	Glutaric acid	53.757	3.42
16663321	3-Hydroxydodecanedioic acid	53.962	0.09

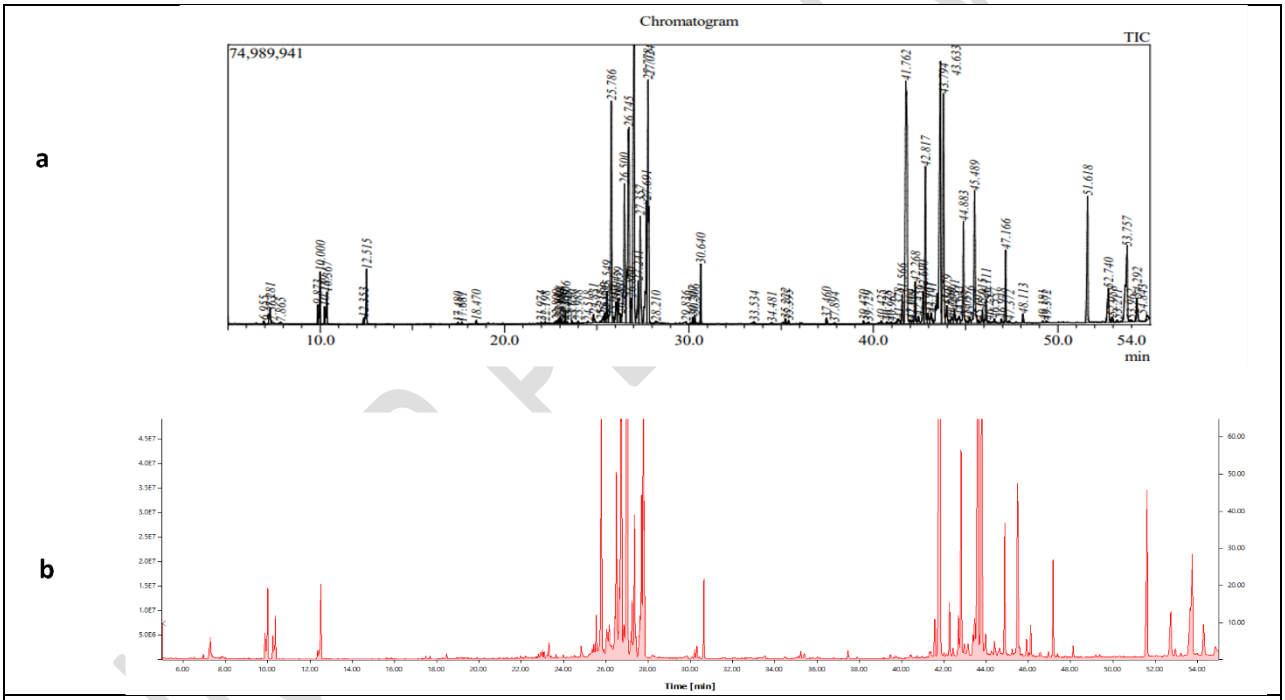


Fig. 1 Ion chromatogram profile of little millet (ATL 1) for visualization. a – represent GC-MS results, b- represent the chromatogram resulted from the openchrom tool.

3.2 *In silico* ADMET AND VIRTUAL SCREENING

Out of identified 92 bioactive phytoconstituents, 86 were screened based on the drug-likeness property. The remaining six compounds were found to violate the Lipinski-rule of five, suggesting potential challenges in their oral absorption, bioavailability, and overall development as drug candidates. Superoxide dismutase (PDB ID: 1CB4), catalase (PDB ID: 2CAG), and glutathione peroxidase (PDB ID: 2P31) were investigated using molecular docking (Fig. 2). All

the compounds from GC-MS profiles of seed extracts were docked against the superoxide dismutase, catalase, glutathione peroxidase enzymes. The top ten compounds showed the best binding affinity (Table 2, Fig. 3). Out of ten compounds, six compounds i.e Acetin, 3-Hydroxydodecanedioic acid, Hexadeconoic acid, Methyl caprylate, Octanoic acid, Methyl succinic acid were common to the three receptors. The compound Acetin showed the highest negative CDOCKER energy with glutathione peroxidase (-34.9153 kcal/mol) and superoxide dismutase (-41.0927 kcal/mol), whereas for catalase, the compound Hexadecanoic acid showed the lowest CDOCKER energy (-49.1006 kcal/mol) (Fig. 4). The two-dimensional representation was performed to clearly visualize the interactions between the proteins and the ligands (Fig. 5).

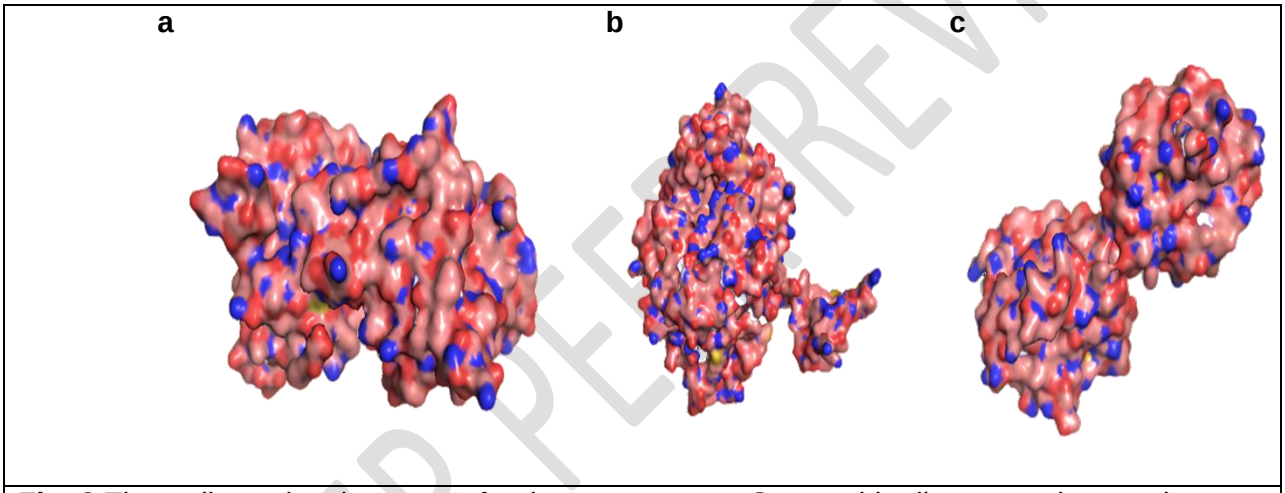


Fig. 2 Three-dimensional structure for the enzymes. a - Superoxide dismutase, b – catalase, c - glutathione peroxidase.

Table 2 Docking result of three proteins with the top 10 highest binding energy ligands. 1CB4 - Superoxide dismutase, 2CAG – Catalase, 2P31 - Glutathione peroxidase.

PROTEINS	CID	NAME	CDOCKER ENERGY (-)	CDOCKER INTERACTION ENERGY (-)
2P31	**91696644**	**Acetin**	**34.9153**	**26.6745**
	16663321	3-Hydroxydodecanedioic acid	28.138	27.4269
	985	Hexadecanoic acid	26.4428	21.8462
	17386	1-Pentadecanamine	23.3986	24.198
	8091	Methyl caprylate	21.667	19.3686
	10349	Methyl succinic acid	21.3053	15.8083

	379	Octanoic acid	21.0653	17.8585
	743	Glutaric acid	20.1727	16.2499
	6262	Ornithine	18.7588	18.24
	7824	Methyl caproate	18.5965	17.0158
2CAG	**985**	**Hexadecanoic acid**	**49.1006**	**49.496**
	16663321	3-Hydroxydodecanedioic acid	46.7995	46.5005
	91696644	Acetin	45.5305	41.5605
	445638	Palmitoleic acid	35.6671	51.559
	445639	(Z)-9-Octadecenoic acid	35.4041	47.8866
	643801	Methyl palmitoleate	34.5347	48.6672
	5282800	10E,12Z-Octadecadienoic acid	33.6368	43.5033
	8091	Methyl caprylate	32.0204	31.1985
	379	Octanoic acid	30.8388	29.3319
	10349	Methyl succinic acid	29.9184	25.0309
1CB4	**91696644**	**Acetin**	**41.0927**	**39.5708**
	985	Hexadecanoic acid	39.4853	38.6622
	16663321	3-Hydroxydodecanedioic acid	38.415	43.643
	17386	1-Pentadecanamine	34.2751	34.5461
	8091	Methyl caprylate	31.1103	32.0526
	743	Glutaric acid	30.5001	29.4161
	7824	Methyl caproate	28.5516	28.0417
	379	Octanoic acid	27.9276	29.3689
	10349	Methyl succinic acid	26.2322	25.2763
	6262	Ornithine	25.3419	29.6714

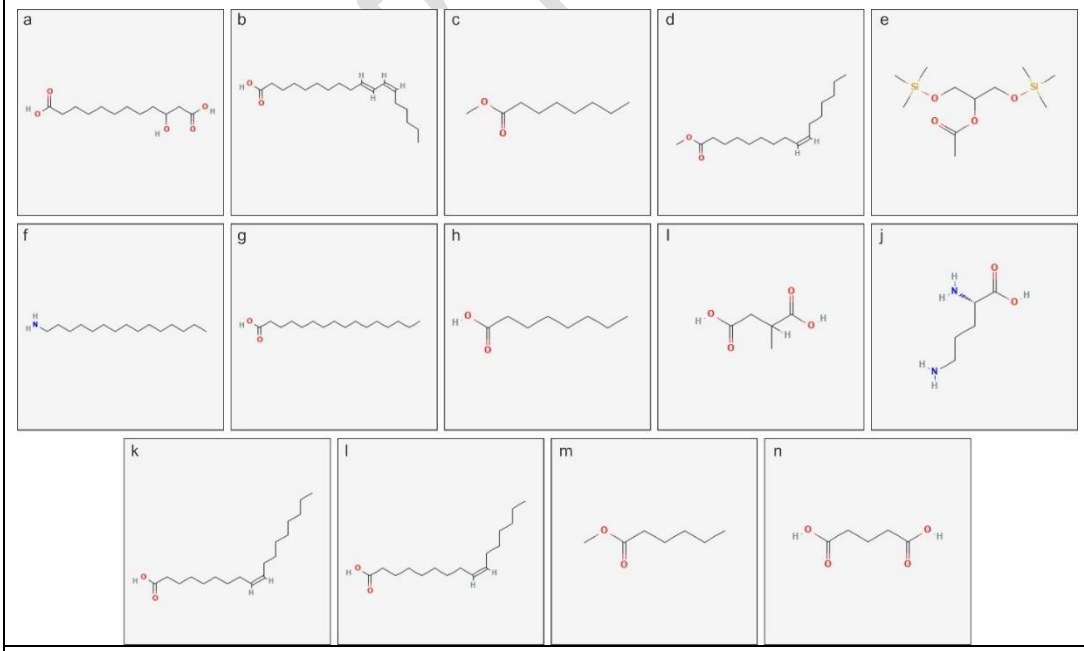


Fig. 3 The top ten compounds showed the best binding affinity with the three enzymes such as

superoxide dismutase, catalase, glutathione peroxidase. a – 3-Hydroxydodecanedioic acid, b - 10E,12Z-Octadecadienoic acid, c – Methyl caprylate, d – Methyl palmitoleate, e – Acetin, f – 1-Pentadecanamine, g – Hexadecanoic_acid, h – Octanoic acid, I – Methyl succinic acid, j – Ornithine, k – Z-9-Octadecenoic acid, l – Palmitoleic acid, m – Methyl caproate, n – Glutaric acid.

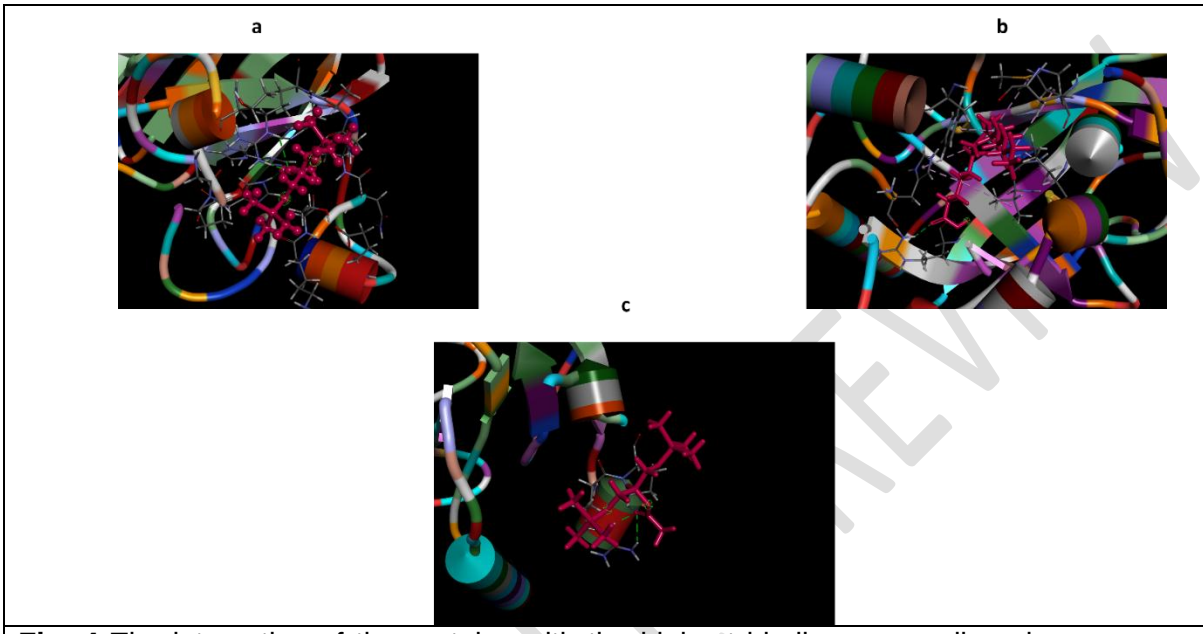


Fig. 4 The interaction of the proteins with the highest binding energy ligands. a – superoxide dismutase with Acetin ligand, b – catalase with the Hexadecanoic acid ligand, c – glutathione peroxidase with the Acetin ligand.

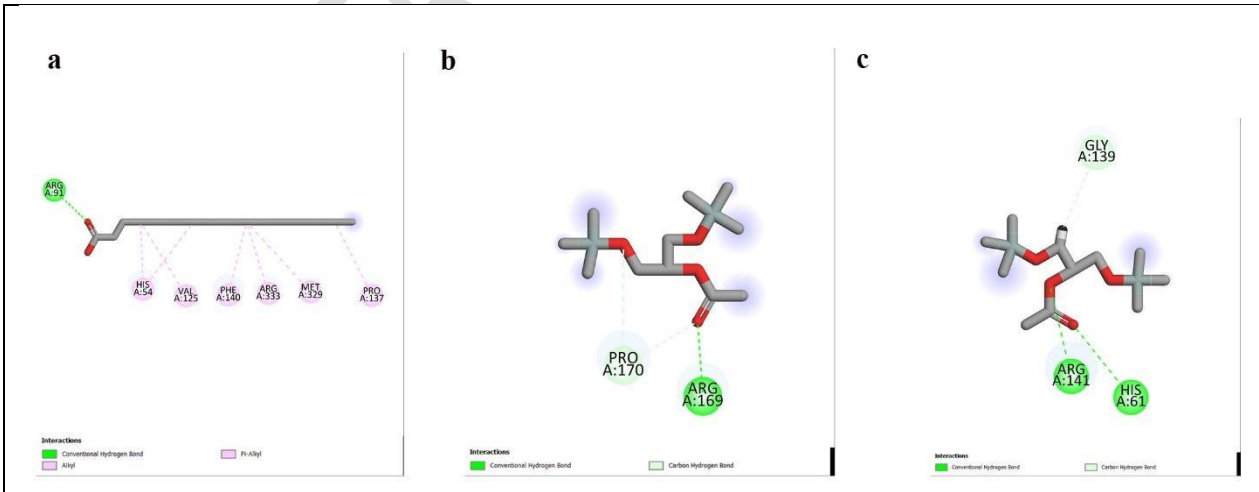


Fig. 5 The two-dimensional diagram for the interaction of proteins with the highest binding energy ligands. a – catalase with the Hexadecanoic acid, b – glutathione peroxidase with the Acetin, c – superoxide dismutase with Acetin.

3.3 BIO-MOLECULAR NETWORK

The results obtained from GC-MS analysis and the virtual screening was further carried out for construction of bio-molecular network. For this purpose, the GC-MS resulted phytochemicals were used along with the three receptors (Fig. 6a). Out of ten highest binding energy compounds, four ligands namely (Z)-9-octadecenoic acid, methyl palmitoleate, palmitoleic acid, 10E,12Z-octadecadienoic acid show the best binding affinity with the catalase protein alone. But for superoxide dismutase and glutathione peroxidase, the ligands with the best binding affinities were glutaric acid, 1-pentadecanamine, methyl caproate, ornithine found to be the common occurrence, whereas the compounds methyl succinic acid, acetin, methyl caprylate, octanoic acid, 3-hydroxydodecanedioic acid, hexadecenoic acid were shown the best binding energy with all the three proteins (Fig. 6b).

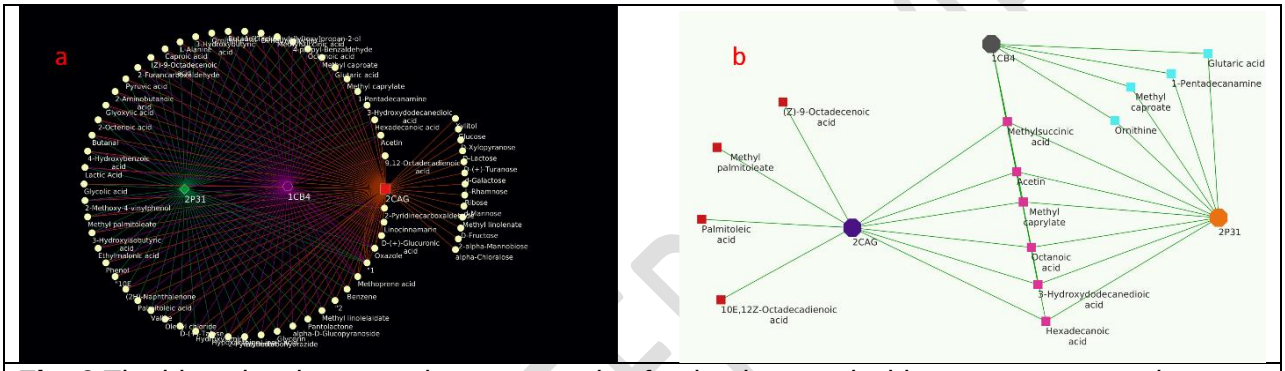


Fig. 6 The biomolecular network representation for the three antioxidant receptors. **a** – three antioxidant receptors with the complete identified phytochemicals of little millet, **b** – three antioxidant receptors with the highest binding energy resulted phytochemicals.

3.4 SIMULATION

The docked complexes were taken for the molecular dynamic simulation studies. Throughout the simulation, the NPT ensemble maintained constant particle, pressure, and temperature levels, while GPU acceleration enhanced computation efficiency. The dynamics of the system were recorded while maintaining the goal temperature of 300K using a pair list distance of 14 to allow for nonbonded interactions and a precise 2 frames per seconds time step. A molecular dynamic simulation study was performed on the most effectively docked superoxide dismutase, catalase, glutathione peroxidase complexes to examine its structural stability over a predetermined time frame. The CHARMm forcefield analysis successfully characterized the stable molecule, supported by the Momany-Rone parameter. The system comprises 5042, 4020, 15143 water molecules for glutathione peroxidase, superoxide dismutase, catalase respectively. The Molecule-CHARMm force field effectively guided the molecule through various stages of the dynamic cascade, achieving successful system conformation optimization, as evident from extensive potential energy decreases. The superoxide dismutase and glutathione peroxidase with acetin and catalase with hexadecanoic acid complex was subjected to a thorough trajectory analysis to investigate its dynamic behaviour, stability, interactions, and conformational changes. To evaluate structural changes and atom fluctuations, important parameters including Root Mean Square Deviation (RMSD)

and Root Mean Square Fluctuation (RMSF) and Radius of gyration (Rg) were used. The RMSD analysis revealed that the SOD complex exhibited significant deviations ranging from 0.1 to 0.35 nm over the initial 15 ns, followed by stability until 20 ns, while the GPx complex displayed considerable variations from 0.1 to 0.25 nm up to 16 ns, maintaining stability thereafter, and finally, the CAT complex exhibited substantial and persistent deviations in RMSD throughout the entire 20 ns simulation (Fig. 7). For the SOD complex, the RMSF analysis revealed maximum, minimum, and average RMSF values of 29 nm, 13 nm, and 27 nm, respectively, while the GPx complex exhibited corresponding values of 30 nm, 13 nm, and 29 nm; additionally, the CAT complex displayed values of 50 nm, 8 nm, and 43 nm for its maximum, minimum, and average RMSF, respectively (Fig. 8). Comparatively, while the radius of gyration remained stable for the GPx and CAT complexes until 17 ns and throughout the 20 ns simulation, respectively, the SOD complex demonstrated instability and increased fluctuations, becoming less stable after 11 ns (Fig. 9).

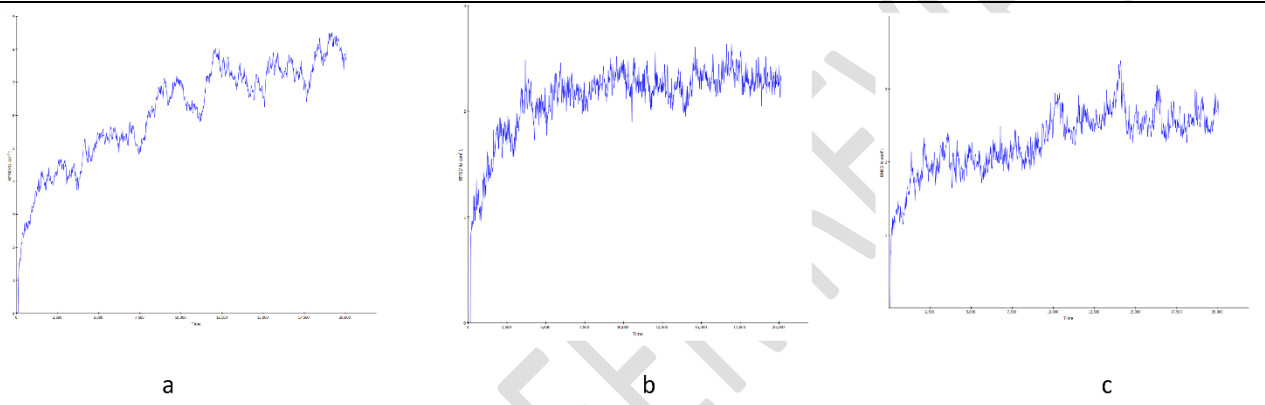


Fig. 7 RMSD. **a** – CAT complex, b – GPx complex, c – SOD complex.

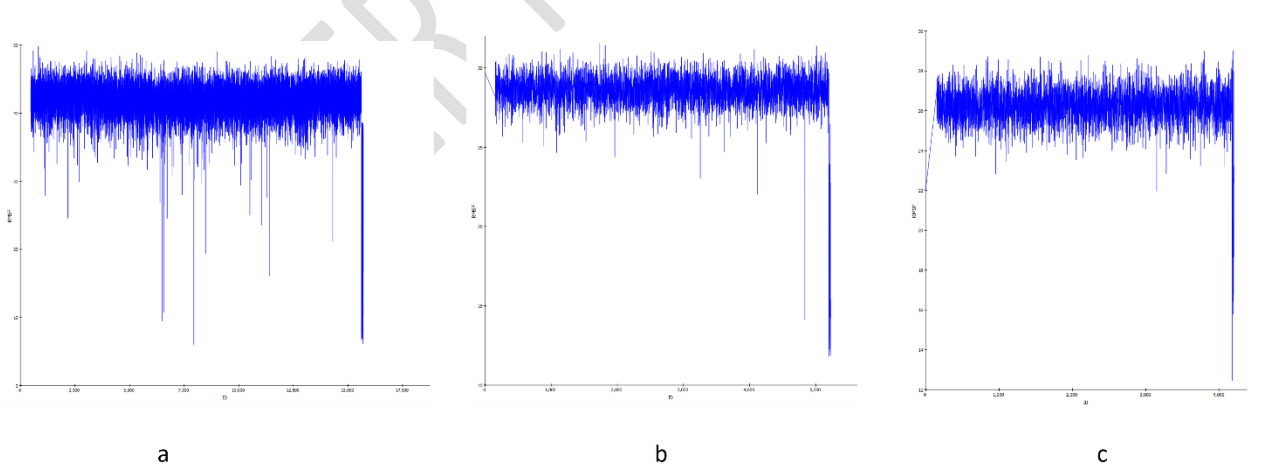
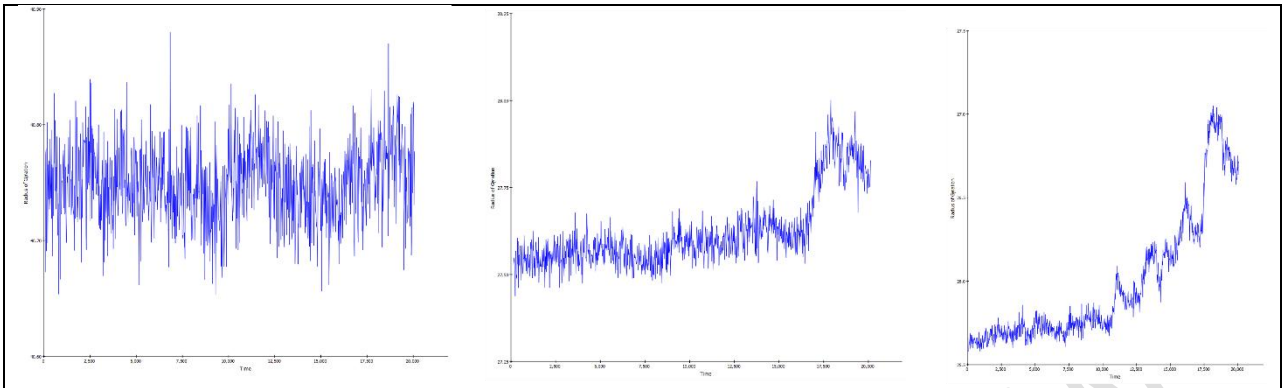


Fig. 8 RMSF. **a** – CAT complex, b – GPx complex, c – SOD complex.



a b c

Fig. 9 Radius of gyration. **a** – CAT complex, b – GPx complex, c – SOD complex.

4. DISCUSSION

Despite the existence of various phytochemicals such as saponins, tannins, terpenoids, and alkaloids being reported in studies, the exact characterization of these compounds has yet to be examined and as a result, the phytochemicals present in minor millets, particularly barnyard millet, little millet, remain undiscovered, but the data provided by [2] highlighting the overall minor millet phytochemicals that contribute to promoting health advantages. [4] identified the list of Phenols, flavonoids in little millet (CO-Samai-4) variety. Due to lack of research interest and unexplored about phytochemicals in little millet, we have performed the GCMS analysis for the little millet variety (ATL1) and found some of the compounds which includes phenols, flavonoids, and so on (Table 1). As a result, the compounds vanillic acid, kaempferol, hydroxybenzoic acid were found to be common between the two varieties. The prominent nutraceuticals which include resistant starch, phytates, phenolics, sterols, lignans, gamma-aminobutyric acid are present in little millet[24]. [25]reported that significant retention of phytates has a positive effect on health due to their anti-diabetic and anti-cancer effects, as well as their antioxidant qualities. Epidemiological data show that a high intake of bioactive compounds and antioxidants such as vitamins, phytochemicals, and predominantly phenolic compounds such as flavonoids and carotenoids have a positive impact on health and may help reduce the risk of various ailments such as cancer, Alzheimer's, cataracts, heart disease, stroke, diabetes, and age-related functional degeneracy[26],[27]. The antioxidant activity was attributed primarily to phenolic molecules containing methoxy substituents[28]. Methoxyphenols and 1-hydroxy-4-unsubstituted benzenoids were the two phenolic compounds reported for our sample. [29]identified that (E)-4-(3-(3,5-dimethoxyphenyl) allyl)-2-methoxyphenol having the potential of inhibiting the growth of colon tumours in mice, so methoxyphenols can have the inhibitory activity against cancer. Little millets are high in bioactive substances such as gallic acid, vanillic acid, p-hydroxybenzoic acid, sinapic acid, chlorogenic acid, caffeic acid, ferulic acid, and p-coumaric acid [7] and according to our results, vanillic acid was reported.

When compared to the native sample, the radical scavenging activity of processed millet extracts increased, and roasted millet extract has the best radical scavenging activity (95.5%) and improved nutraceuticals properties in contrast with the germinated (91.7%) and steamed

(93.4%)[4]. It is due to the existence of highest total phenolic content in the roasted millet, where %DPPH inhibition is strongly connected to TPC[30]. [31]have reported that roasting of coffee brews has high antioxidant property and these findings were similar to the roasted little millet. Rutin has linked to the treatment of haemorrhoids, varicosis, microangiopathy and also acts as an antioxidant; it was found to be the strongest when compared to quercetin, acacetin, morin, hispidulin, hesperidin, and naringin, however, in other experiments, the effects of rutin were lower or non-existent when compared to quercetin[32],[33].

Triticum aestivum, *Carica papaya*, and *Citrus limon* phytochemicals may be promising superoxide dismutase (SOD) agonists and in particular, the docking score reveals that gliadin in *Triticum aestivum* has high binding affinity and demonstrates antioxidant activity, whereas tea extract from *Punica granatum* had the second highest binding score against SOD[13]. As for our results, the superoxide dismutase shows the best binding affinity such as -41.0927, -39.4853, -38.415 with the phytochemicals acetin, hexadecanoic acid, 3-hydroxydodecanedioic acid respectively from little millet (ATL1). Catalase activity would be an important characteristic for synthetic ROS scavenging chemicals, some efforts have been directed towards the synthesis of catalysts with dual SOD/CAT activity for potential therapeutic applications[34]. In terms of catalase protein, Terrestribisamide from *Triticum aestivum* has shown the highest binding affinity, whereas Aurochrome (*Carica papaya)*, Vitamin P (*Carica papaya*), (1E,6E)-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl) hepta-1,6-diene-3,5-dione (*Curcuma Longa*) and Demethoxycurcumin (*Curcuma Longa*) have shown equivalent binding scores[13]. But for our results, hexadecanoic acid shows the promising highest binding affinity with the catalase protein. Generally, hexadecanoic acid and octadecadienoic acid are fatty acids. Hexadecanoic acid maintain a biological activity such as antioxidants, hypocholesterolemic, nematicide, and pesticide[35]. Hexadecanoic acid or Palmitic acid shows highest percentage in the seed extract (48.84%) of *Sterculia quadrifida* was similar to the palmitic acid found in *Sterculia foetida* seed (52%)[36]. *Punica granatum* tea extract has once again demonstrated promising binding scores with glutathione peroxidase (GPx), whereas the tea extract and superoxide dismutase have identical highest binding scores and this observation supports *Punica granatum* (Tea extract) can be responsible for its antioxidant actions against superoxide dismutase and glutathione peroxidase[13]. Our findings have proven that the phytochemical acetin shows the promising binding affinity scores with both glutathione peroxidase and superoxide dismutase enzymes.

5. CONCLUSION

The current study aimed at inspecting the antioxidant enhancers of little millet's phytochemicals against superoxide dismutase, catalase, glutathione peroxidase proteins. Compounds like acetin and hexadecanoic acid had the highest binding affinity for the antioxidant proteins. Dynamics investigations were used to validate the complexes structural stability and found glutathione peroxidase exhibited to be a stable complex. These findings will serve as a starting point for future pharmacological treatments and helps to enhance the plant defence mechanisms by withstanding environmental stresses.

References:

1. Saleh, A.S., et al., *Millet grains: nutritional quality, processing, and potential health benefits.* Comprehensive reviews in food science and food safety, 2013. **12**(3): p. 281-295.
2. Pujari, N. and J.H. Hoskeri, *Minor millet phytochemicals and their pharmacological potentials.* Pharmacognosy Reviews, 2022. **16**(32): p. 101.
3. Soutade, V. and P. Raundal, *Response of Little Millet Varieties to Different Levels of Fertilizers Under Rainfed Condition.* Journal of Agriculture Research and Technology, 2022. **47**(2): p. 131.
4. Pradeep, S. and M. Guha, *Effect of processing methods on the nutraceutical and antioxidant properties of little millet (Panicum sumatrense) extracts.* Food chemistry, 2011. **126**(4): p. 1643-1647.
5. Chandrasekara, A. and F. Shahidi, *Bioactivities and antiradical properties of millet grains and hulls.* Journal of Agricultural and Food Chemistry, 2011. **59**(17): p. 9563-9571.
6. Brzozowska, I., et al., *Healing of chronic gastric ulcers in diabetic rats.* Journal of physiology and pharmacology, 2004. **55**(4): p. 773-790.
7. Kaur, P., et al., *Millets: A cereal grain with potent antioxidants and health benefits.* Journal of Food Measurement and Characterization, 2019. **13**: p. 793-806.
8. Vertuani, S., A. Angusti, and S. Manfredini, *The antioxidants and pro-antioxidants network: an overview.* Current pharmaceutical design, 2004. **10**(14): p. 1677-1694.
9. Kumar, A., et al., *Nutritional significance and antioxidant-mediated antiaging effects of finger millet: molecular insights and prospects.* Frontiers in Sustainable Food Systems, 2021. **5**: p. 684318.
10. Urquiaga, I. and F. Leighton, *Plant polyphenol antioxidants and oxidative stress.* Biological research, 2000. **33**(2): p. 55-64.
11. Leopoldini, M., N. Russo, and M. Toscano, *The molecular basis of working mechanism of natural polyphenolic antioxidants.* Food chemistry, 2011. **125**(2): p. 288-306.
12. Di Meo, F., et al., *Free radical scavenging by natural polyphenols: Atom versus electron transfer.* The Journal of Physical Chemistry A, 2013. **117**(10): p. 2082-2092.
13. Rana, S., S. Dixit, and A. Mittal, *In silico target identification and validation for antioxidant and anti-inflammatory activity of selective phytochemicals.* Brazilian Archives of Biology and Technology, 2019. **62**.
14. Yu, Q. and Z. Rengel, *Micronutrient deficiency influences plant growth and activities of superoxide dismutases in narrow-leafed lupins.* Annals of Botany, 1999. **83**(2): p. 175-182.
15. Beauchamp, C. and I. Fridovich, *Superoxide dismutase: improved assays and an assay applicable to acrylamide gels.* Analytical biochemistry, 1971. **44**(1): p. 276-287.
16. Aebi, H., *[13] Catalase in vitro*, in *Methods in enzymology*. 1984, Elsevier. p. 121-126.
17. Flohé, L. and W.A. Günzler, *[12] Assays of glutathione peroxidase*, in *Methods in enzymology*. 1984, Elsevier. p. 114-120.
18. Nakano, Y. and K. Asada, *Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts.* Plant and cell physiology, 1981. **22**(5): p. 867-880.
19. Lisec, J., et al., *Gas chromatography mass spectrometry–based metabolite profiling in plants.* Nature protocols, 2006. **1**(1): p. 387-396.
20. Sussman, J.L., et al., *Protein Data Bank (PDB): database of three-dimensional structural information of biological macromolecules.* Acta Crystallographica Section D: Biological Crystallography, 1998. **54**(6): p. 1078-1084.
21. Studio, D., *Discovery studio.* Accelrys [2.1], 2008.

22. Daina, A., O. Michielin, and V. Zoete, *SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules.* Scientific reports, 2017. **7**(1): p. 42717.
23. Shannon, P., et al., *Cytoscape: a software environment for integrated models of biomolecular interaction networks.* Genome research, 2003. **13**(11): p. 2498-2504.
24. Guha, M., Y.N. Sreerama, and N. Malleshi, *Influence of processing on nutraceuticals of little millet (Panicum sumatrense)*, in *Processing and impact on active components in food*. 2015, Elsevier. p. 353-360.
25. Kushwaha, A., et al., *Effect of hydrothermal treatment and milling parameters on milling and nutritional qualities of finger millet (eleusine coracana).* Journal of Agricultural Engineering, 2019. **55**(4): p. 34-46.
26. Dey, S., et al., *Understanding the antinutritional factors and bioactive compounds of kodo millet (Paspalum scrobiculatum) and little millet (Panicum sumatrense).* Journal of Food Quality, 2022. **2022**: p. 1-19.
27. Carnauba, R.A., et al., *Assessment of dietary intake of bioactive food compounds according to income level in the Brazilian population.* British Journal of Nutrition, 2022. **127**(8): p. 1232-1239.
28. Matos, M., et al., *Acetone: Water fractionation of pyrolytic lignin improves its antioxidant and antibacterial activity.* Journal of Analytical and Applied Pyrolysis, 2021. **156**: p. 105175.
29. Zheng, J., et al., *(E)-4-(3-(3, 5-dimethoxyphenyl) allyl)-2-methoxyphenol inhibits growth of colon tumors in mice.* Oncotarget, 2015. **6**(39): p. 41929.
30. Alothman, M., R. Bhat, and A. Karim, *Antioxidant capacity and phenolic content of selected tropical fruits from Malaysia, extracted with different solvents.* Food chemistry, 2009. **115**(3): p. 785-788.
31. Nicoli, M., et al., *Antioxidant properties of coffee brews in relation to the roasting degree.* LWT-Food Science and Technology, 1997. **30**(3): p. 292-297.
32. Chow, J.-M., et al., *Quercetin, but not rutin and quercitrin, prevention of H2O2-induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages.* Biochemical pharmacology, 2005. **69**(12): p. 1839-1851.
33. Bais, S., et al., *Evaluation of effects of rutin on oxidative stress in diabetic rat.* International Journal of Pharmacy and Pharmaceutical Sciences, 2012. **4**(Suppl 5): p. 140-145.
34. Signorella, S., C. Palopoli, and G. Ledesma, *Rationally designed mimics of antioxidant manganoenzymes: Role of structural features in the quest for catalysts with catalase and superoxide dismutase activity.* Coordination Chemistry Reviews, 2018. **365**: p. 75-102.
35. Sheela, D. and F. Uthayakumari, *GC-MS analysis of bioactive constituents from coastal sand dune taxon-Sesuvium portulacastrum (L.).* Bioscience discovery, 2013. **4**(1): p. 47-53.
36. Siswadi, S. and G.S. Saragih. *Phytochemical analysis of bioactive compounds in ethanolic extract of Sterculia quadrifida R. Br*. in *AIP Conference Proceedings*. 2021. AIP Publishing.