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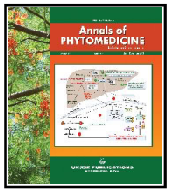

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Identifying potential phytoactives from *Glycine max* (L.) Merr. and their role in NADPH oxidase inhibition

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Abstract

The resurgence of herbal practices globally underscores a shift toward herbal medicine for the management of neurodegenerative disorder, with concerns about pharmaceutical safety driving this trend. Various synthetic drugs are available in the market aimed for the treatment of neurodegenerative disorder but these drugs come with the lot of side effects which causes the shifting of over 80% population towards the plant based medicine. This research investigates the inhibitory potential of *Glycine max* (L.) Merr. (Soybean) constituents on NADPH oxidase which plays a key role in neurodegenerative disorder using molecular docking and pharmacokinetic studies. The aim is to contribute to knowledge on the therapeutic applications of soybean compounds targeting oxidative stress. Molecular docking with AutoDock Vina/ CADD labshare and pharmacokinetic predictions using the Swiss ADME web tool were employed to assess the interactions and properties of *G. max* compounds affecting NADPH oxidase. The study reveals significant interactions between soybean constituents and NADPH oxidase, suggesting potential inhibitory effects. Pharmacokinetic analyses provide insights into the drug likeness and ability of the constituents to cross blood brain barrier. This research advances our understanding of *G. max* constituents' therapeutic potential in inhibiting NADPH oxidase, offering valuable insights for herbal medicine applications. The observed interactions highlight a promising avenue for addressing oxidative stress-related conditions, emphasizing the need for further exploration of plant-based remedies in contemporary healthcare.

1. Introduction

In the contemporary milieu, there is a global resurgence in herbal practices, signifying a robust revival as an increasing number of individuals are turning to herbal medicine for diverse ailment management (Warrier, 2021). Growing apprehensions regarding the safety of pharmaceutical drugs and surgical interventions have underscored the pivotal role of herbal medicines (Verma *et al.*, 2022). A notable paradigm in modern pharmaceuticals is the prevailing emphasis on symptom suppression, often sidelining the imperative of addressing the fundamental etiology of diseases. Importantly, the World Health Organization (WHO) reports that over 80% of the global population relies on plant-based herbal medicines for primary healthcare needs (Polak *et al.,* 2015).

Herbs and spices, renowned for their intrinsic antioxidant properties, have been integral components as natural antioxidants and food additives. Among these, soybeans emerge as a distinctive plant-based entity, harboring high-quality protein replete with essential amino acids, phospholipids, carbohydrates, isoflavones, and phytosterols, each showcasing well-documented pharmacological activities. The array of phytochemicals in *G. max* , including sitosterol, stigmasterol (Marsan *et al.,* 1998), campesterol, Δ7-avenasterol, and brassicasterol (Choong *et al.,* 1999), significantly contribute to their notable cholesterol-lowering effects (Anderson *et al.,* 1995) Moreover, G. *max* oil encompasses α-, γ-, and β-tocopherols (vitamin E), acting as potent antioxidants (Evans *et al.*, 2002).

Isoflavones (ISOs), such as genistein and daidzein (Fang *et al.,* 2002), structurally mirroring mammalian estradiol. The health benefits associated with soybean products have been extensively documented. Growing evidence suggests that *G. max* consumption may contribute to the prevention of certain cancers (Anthony *et al.,* 1996; Arjmandi *et al.,* 1998), a reduction in the risk of osteoporosis (Arjmandi *et al.,* 1998; Barnes *et al.,* 1994) and a positive impact on chronic renal disease (Fico *et al.,* 2000; Ranich *et al.,* 2001). Additionally, *G. max* products have been linked to lower plasma cholesterol levels (Franke *et al.,* 1995), antiatherosclerotic activity (Hillis and Isoi, 1965; Huff *et al.,* 1982), and a decreased risk of coronary heart disease (Lucas *et al.,* 2001). Despite these observed benefits, the precise active components responsible for these effects remain to be precisely defined. Current understanding posits that isoflavones, specifically genistein and daidzein, are believed to be key contributors to the positive outcomes associated with soy products (Markham *et al.,* 1978). However, ongoing research is necessary to further elucidate the molecular mechanisms and specific compounds responsible for these health-promoting effects. Additionally, *G. max* manifest immune system modulation, antioxidant effects, and hold promise in the realm of neurodegenerative disorders (Das *et al.*, 2021; Ma *et al.*, 2009).

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This research aims to investigate the inhibitory potential of chemical constituents extracted from *G. max* (soybeans) on NADPH oxidase through an integrative approach utilizing molecular docking and pharmacokinetic studies. Molecular docking analyses were meticulously executed leveraging AutoDock Vina software, while the Swiss ADME web tool facilitated the prediction of pharmacokinetic properties of the compounds. This multifaceted investigation aims to contribute substantially to the existing body of knowledge concerning the therapeutic applications of soybean constituents targeting NADPH oxidase, thereby offering valuable insights for future drug development within the domain of herbal medicine.

2. Materials and Methods

2.1 Collection of plant material

G. max seeds were purchased from local market Prayagraj, India. The seeds were authenticated by Dr. Sunita Garg, Former Chief Scientist, Head, RHMD, CSIR- NIScPR, New Delhi, India (Authentication number- NIScPR/RHMD/Consult/2022/4071-72-73).

2.2 Extraction method

20 g of powdered soybean seeds underwent extraction with ethanol at a concentration of 96% v/v. The extraction process was conducted within a temperature range of 40-80°C, with extraction durations spanning from 30 to 150 min. The material-to-solvent ratio varied from 1:4 to 1:10 g/ml, and the extraction cycles were performed 1, 2, and 3 times. Subsequent to extraction, the solvent was meticulously evaporated from the liquid extract under vacuum conditions at 50°C, resulting in the acquisition of the dried extract (Vishnupriya and Kowsalya, 2022).

2.3 Phytochemical analysis of the extracts of *G.max* (Shareef and Bhavya, 2021; Pratap *et al*., 2021; Khandelwal, 2007)

The phytochemical analysis of *G. max* extracts involved subjecting the ethanolic extract to qualitative chemical screening to identify various classes of active chemical constituents. This screening aimed to discern the presence of specific chemical compounds within the extract, providing valuable insights into the potential bioactive components present in *G. max*.

(a) Wagner's test

In a controlled experimental setting, 4 ml of the extract underwent a reaction with 3 drops of Wagner's reagent, followed by an undisturbed incubation period lasting 5 min. The emergence of a reddish-brown precipitate during this reaction serves as an indicative marker for the presence of alkaloids within the sample.

(b) Sodium hydroxide test

For the dissolution of 0.2 g of the extract, a solution comprising cold dilute sodium hydroxide and diluted hydrogen chloride was employed. The absence of the characteristic yellow coloration during this process serves as an indicative marker for the presence of flavonoids within the sample.

(c) Copper acetate test

In a precisely controlled environment, 5 ml of the extract underwent supplementation with 12 drops of $Cu(OAc)_2$ solution, followed by a meticulous incubation period. The manifestation of a distinctive beryl green color during this process serves as a reliable indicator for the presence of terpenes within the sample.

(d) Salkowski test

In the experimental procedure, 5 ml of the extract underwent a controlled addition of 2.5 ml of $CHCl_3$ and 2.5 ml of concentrated H_2SO_4, followed by meticulous mixing. The observation of red fluorescence within the chloroform layer and greenish-yellow fluorescence within the acid layer serves as a conclusive demonstration of the presence of steroids in the sample.

(e) Foam test

In a controlled laboratory setting, 3 ml of the extract underwent the addition of 2 ml of water, followed by rapid agitation for approximately 10 min. The observation of a persistent and stable foam formation during this process serves as a reliable indicator of the presence of saponins in the sample.

(f) Ferric chloride test

Over a duration of 10 min, a blend of 0.5 ml of plant extract and 5 ml of distilled water underwent heating. Subsequently, 3 drops of a 10% ferric chloride solution were introduced to the 2 ml of the obtained filtrate. The emergence of a greenish-blue or violet coloration during this reaction serves as a conclusive indicator of the presence of phenolics within the sample.

(g) Lead acetate test

In a controlled experimental setting, 4 ml of Pb $(C_2H_3O_2)_2$ solution underwent thorough mixing with 4 ml of the plant extract. The identification of a white precipitate during this interaction serves as a reliable indicator for the presence of tannins and phenols within the sample.

(h) Borntrager's test

A 2 ml aliquot of plant extracts underwent a boiling process followed by filtration with mild sulfuric acid. The resulting filtrate was thoroughly amalgamated with chloroform and subjected to shaking. After the separation of the organic layer, ammonia was gradually introduced. The ammoniacal layer exhibited a distinct color transition from pink to red, indicating the presence of anthraquinone glycosides.

(i) Fluorescence test

A 1N solution of sodium hydroxide was combined with a 2 ml aliquot of the extract. The presence of coumarin glycosides was confirmed by the observation of a bluish-green fluorescence.

(j) Kellar Killani's test

A solution comprising glacial acetic acid, ferric chloride, and concentrated sulfuric acid was employed to dissolve a few milliliters of extracts in water. The formation of a brown ring at the aqueous-organic interface signifies the presence of cardiac glycosides.

(k) Spot test

A 2 ml aliquot of the extract was enclosed between layers of Whatman paper and subjected to compression for approximately 2-3 min. The residual oil observed on the paper serves as an indication of the presence of fixed oil..

2.4 Identification of phytoactives using LC-MS/MS

To identify the potential constituents of the *G. max* extract LC-MS/MS analysis has been performed at SAIF CDRI, Lucknow. The selected extracts were dissolved in methanol at a concentration of 1 mg/ml and were filtered using a 0.22 µm syringe filter to remove any suspended particulates. Subsequently, the samples (5 µl each) were carefully injected into the column for further analysis. SDBS application was used for identification of mass fragmentation through spectrum database.

2.5 Performing molecular docking

Docking studies were conducted utilizing the AutoDock Vina software, a widely employed molecular docking program. The crystal structure of NADPH oxidase (PDB ID: 2CDU, Figure I) was obtained from the Protein Data Bank and prepared for docking procedures using AutoDock Tools. Compounds derived from *G.max* were formatted in 3D and subjected to docking within the active site of NADPH oxidase under default parameters.

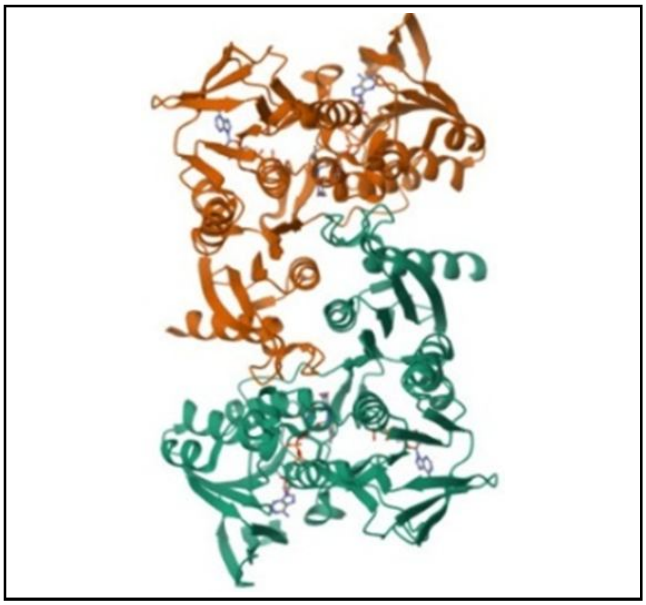


Figure 1: The crystal structure of NADPH oxidase.

Compounds exhibiting high binding affinity and favorable interactions with NADPH oxidase were subjected to further assessment of their pharmacokinetic properties using Swiss ADME. In summary, this study integrated *in silico* molecular docking and pharmacokinetic analyses to assess the potential of *G. max* chemical constituents in modulating NADPH oxidase activity, with potential implications for the treatment of neurodegenerative disorders.

3. Results

3.1 Qualitative screening of phytochemicals

The qualitative screening of ethanolic extract (ee) of *G. max* were determined. The results revealed that alkaloids, flavonoids, terpenoids, amino acids, cardiac glycosides, phenolics, saponins and tannins are present. Whereas, both, carbohydrates, fats and oils and steroids were absent as shown in the Table 1.

Table 1: Qualitative screening of phytochemical of *G.max*

S.No.	Chemical test	Test name	Result
1	Alkaloids	Wagner's test	++
2	Flavonoids	Sodium hydroxide test	+
3	Terpenoids	Copper acetate test	+
4	Carbohydrates	Molisch's test	–
5	Proteins	Millon's test	+
6	Amino acids	Ninhydrin test	+
7	Fats and oils (Fixed)	Soponification	–
8	Steroids	Salkowski tests	–
9	Cardiac glycosides	KellarKillani's test	+
10	Phenolics	Ferric chloride test	+
11	Saponins	Foam test	+
12	Tannins	Ferric chloride test	+

3.2 LC-MS/MS analysis

The LC-MS/MS analysis of the ethanolic extract of *G.max* seeds reported nearly 13 compounds based on their different retention time (rt) . The first hit was identified at retention time (rt) of 7.314 min was inositol, whereas cholecalciferol was the last hit with 900 min RT as given in the Table 2. Daidzin was identified at 245.4 RT in ethanolic extract of *G.max* seeds. The phytoconstituents predicted from the LC-MS/MS analysis of the ethanolic extract of *G.max* were shown in Table 2.

Table 2: LC-MS/MS data interpretation

S.No.	Compound	Molecular weight	Retention time	M/Z
1	Daidzin	254.23	245.4	253.23
2	Kaempferol	286.23	239.52	285.23
3	Daidzein	254.23	12.82	253.23
4	Formononetin	268.26	267.1	267.26
5	Chlorogenic acid	354.31	60.84	353.31
6	Coumestrol	268.21	580.2	267.21
7	Cholecalciferol	384.64	900	383.64
8	Inositol	180.15	7.53	179.15
9	Palmitic acid	256.43	21.24	255.43
10	Glycitin	446.4	212.4	445.04
11	Lysophosphatidylcholine	299.26	150	298.26
12	Oleic acid	282.46	8.76	281.46
13	Linolenic acid	278.43	399.48	277.43

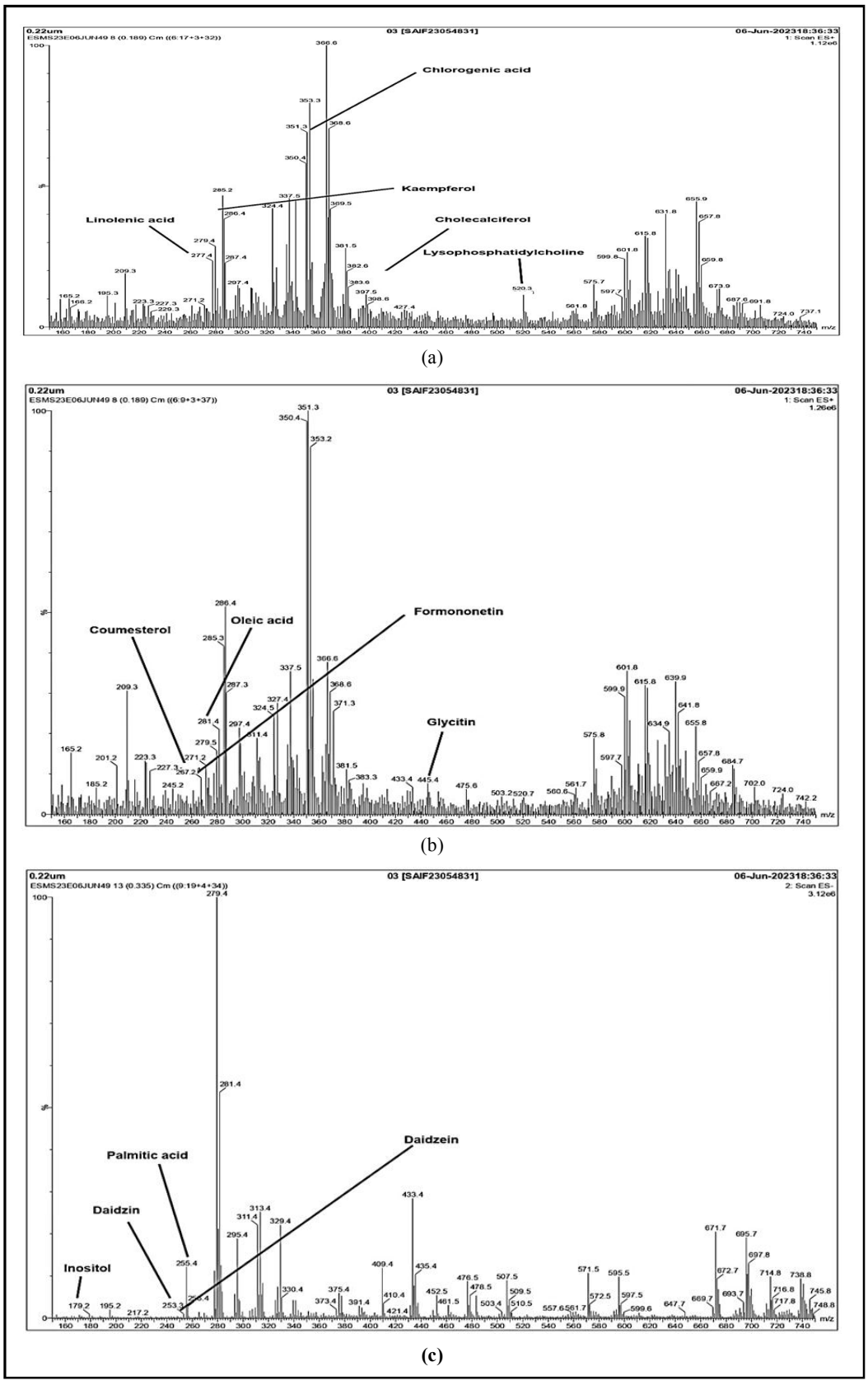


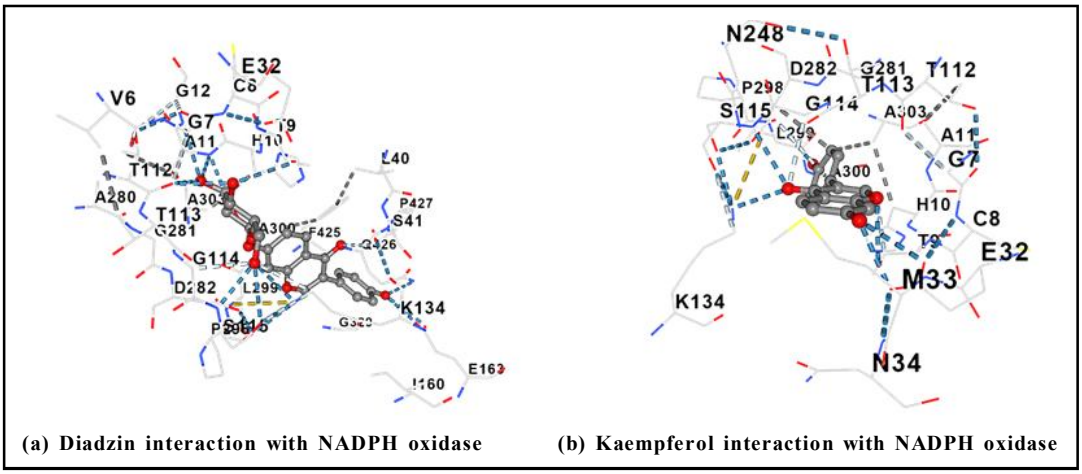
Figure 2: LC-MS/MS chromatogram of *G. max* ethanolic extract.

3.3 Molecular docking

Docking studies were performed to evaluate the binding affinity of chemical constituents of *G.max* against the NADPH oxidase enzyme. The docking scores for the selected compounds from LC-MS/MS are presented in Table 3. The binding affinities ranged from -5.5 to -9.8 kcal/mol, indicating good interactions between the ligands and the active site of NADPH oxidase. Among the top-scoring compounds, daidzin showed docking score of -9.8, coumestrol showed -9.1 and glycitin showed -9.8 docking score the interaction for each constituent is indicated in Figure 3.

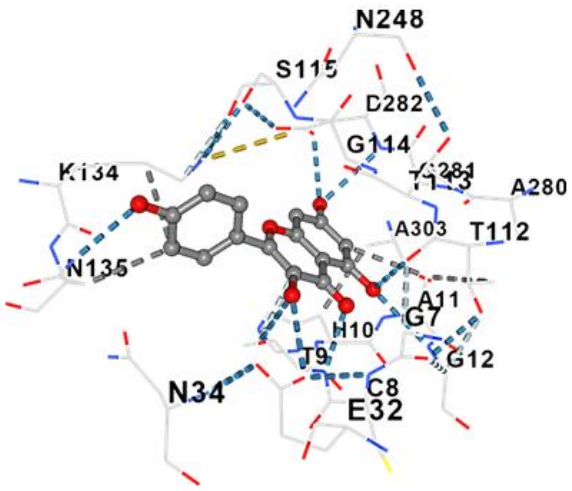
Table 3: Docking score with interactions of the selected constituents of *G. max*

S.No.	Compound	Docking score	Interaction
1	Daidzin	-9.8	**Chain A:** GLY7 CYS8 THR9 HIS10 ALA11 GLY12 THR13 GLU32 LEU40 SER41 GLY43 THR112 THR113 GLY114 SER115 LYS134 GLU163 ALA280 ASP282 PRO298 LEU299 ALA300 ALA303 GLY329**Chain B:** PHE425 GLN426 PRO427
2	Kaempferol	-8.4	GLY7 CYS8 THR9 HIS10 ALA11 GLY12 GLU32 ASN34 THR112 THR113 GLY114 SER115 LYS134 ASN135 ASN248 ALA280 GLY281 ASP282 ALA303
3	Daidzein	-8.0	GLY7 CYS8 THR9 HIS10 ALA11 GLU32 MET33 ASN34 THR112 THR113 GLY114 SER115 LYS134 ASN248 GLY281 ASP282 PRO298 LEU299 ALA300 ALA303
4	Formononetin	-7.9	**Chain A:** THR9 HIS10 ALA11 PHE39 LEU40 SER41 GLY43 GLU163 GLY281 ASP282 LEU299 ALA300 ALA303**Chain B:** PHE425 GLN426 PRO427
5	Chlorogenic acid	-8.6	**Chain A:** VAL6 GLY7 CYS8 THR9 HIS10 ALA11 GLY12 GLU32 SER41 THR112 THR113 GLY114 SER115 LYS116 LYS134 ASN135 ASN248 ASP282 PRO298 LEU299 ALA300 ALA303
6	Coumestrol	-9.1	**Chain A:** GLY7 CYS8 THR9 HIS10 ALA11 GLY12 GLU32 MET33 THR112 THR113 GLY114 SER115 LYS134 ASP282 PRO298 LEU299 ALA300 ALA303
7	Cholecalciferol	-8.8	**Chain A:** SER41 GLY43 ILE44 GLY158 TYR159 ILE160 GLY161 GLU163 TYR188 CYS242 ILE243 GLY244 PHE245 PRO298 LEU299 ALA300**Chain B:** PHE425 GLN426 PRO427
8	Inositol	-6.0	**Chain A:** GLY7 CYS8 THR9 HIS10 TYR31 GLU32 MET33 ASN34 THR112 THR113 GLY114 SER115 LYS116 LYS134 ASN248
9	Palmitic acid	-5.5	**Chain A:** TYR159 ILE160 LYS187 TYR188 PHE245 TYR288 TYR296 ILE297 PRO298 LEU299 GLN325 SER326 SER328 SER339 THR340 GLY341 ILE342 ASN343 LEU346 GLY390
10	Glycitin	-9.8	**Chain A:** GLY7 CYS8 THR9 HIS10 ALA11 GLY12 GLU32 MET33 ASN34 LEU40 SER41 GLY43 THR112 THR113 GLY114 SER115 LYS116 LYS134 ASN248 ALA280 GLY281 ASP282 SER283 LEU299 ALA300 ALA303**Chain B:** PHE425 PRO427
11	Lysophosphatidylcholine	-6.6	**Chain A:** GLY7 CYS8 THR9 HIS10 ALA11 GLY12 THR13 GLU32 ASN34 PHE39 SER41 THR112 THR113 GLY114 LYS134 ASN135 ALA280 GLY281 ASP282 ALA303
12	Oleic acid	-5.7	**Chain A:** TYR159 LYS187 TYR188 TYR296 ILE297 PRO298 LEU299 GLN325 SER326 SER327 SER328 SER339 THR340 GLY341 ASN343 LEU346
13	Linolenic acid	-6.2	**Chain A:** TYR159 LYS187 TYR188 PHE189 TYR296 ILE297 PRO298 LEU299 SER326 SER328 SER339 GLY341 ILE342 ASN343 LEU346

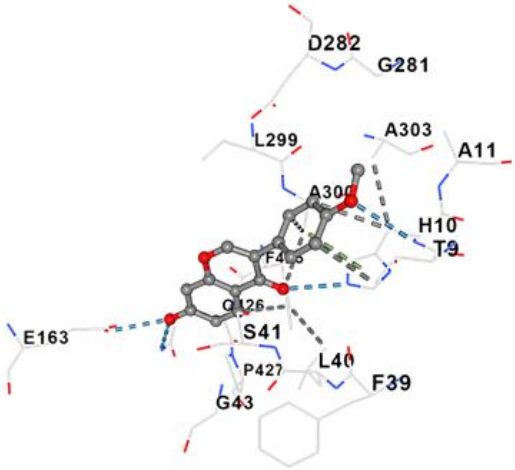


(a) Diadzin interaction with NADPH oxidase

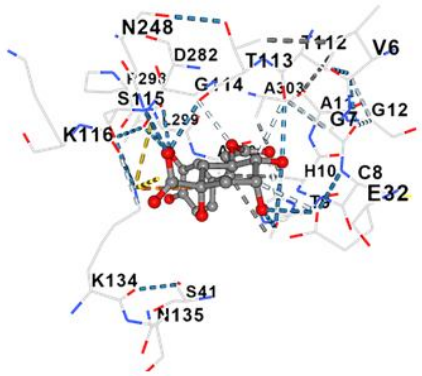
(b) Kaempferol interaction with NADPH oxidase



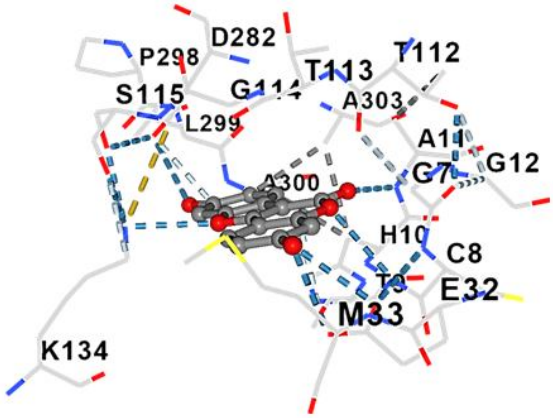
(c) Diadzein interaction with NADPH oxidase



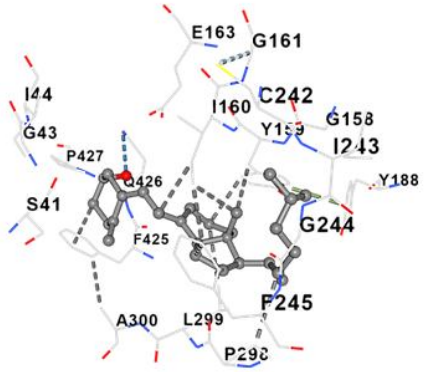
(d) Formononetin interaction with NADPH oxidase



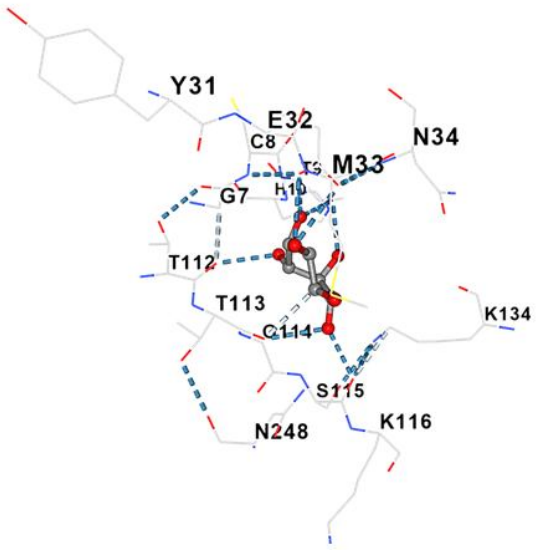
(e) Chlorogenic acid interaction with NADPH oxidase



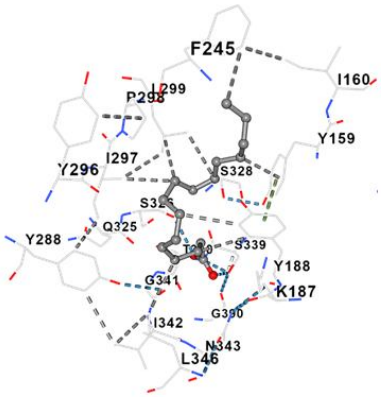
(f) Coumesterol interaction with NADPH oxidase



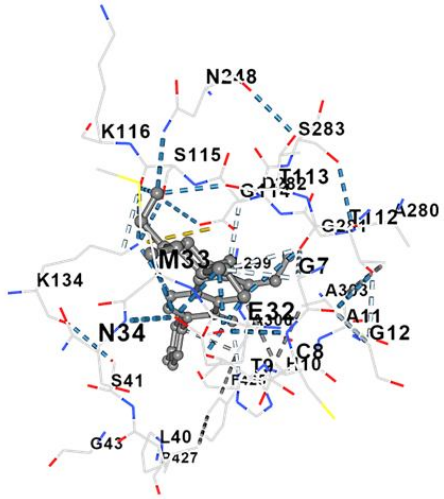
(g) Cholecalciferol interaction with NADPH oxidase



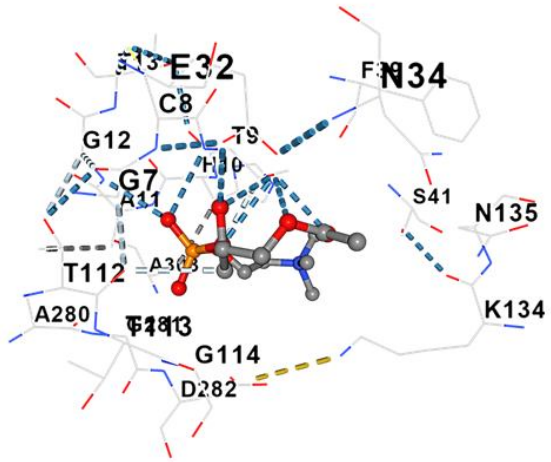
(h) Inositol interaction with NADPH oxidase



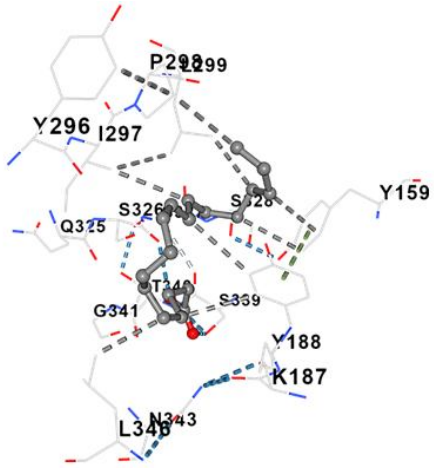
(i) Palmitic acid interaction with NADPH oxidase



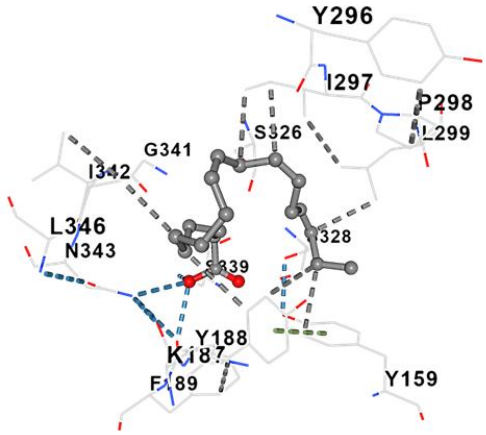
(j) Glycitin interaction with NADPH oxidase



(k) Lysophosphatidylcholine interaction with NADPH oxidase



(l) Oleic acid interaction with NADPH oxidase



(m) Linolenic acid interaction with NADPH oxidase

Figure 3: Interaction of phytoconstituents of *G.max* with respective binding sites of NADPH oxidase.

In addition, the physicochemical and ADME properties of the selected compounds were evaluated using the Swiss ADME online tool. The predicted ADME properties of the compounds are summarized in Table 4. The results indicate that the amongst the selected compounds daidzein, formononrtin, palmitic acid and linolenic acid have good drug-like properties and blood-brain barrier permeability, which shows that these may be responsible for the neuroprotective effect of *G. max* paving the way for further investigation.

Table 4: ADME properties of selected phytoconstituents of *G.max*

S.No.	Phytochemical	Mol. wt.	Qplog po/w (–2.0 – 6.5)	Qplog S (–6.5 – 0.5)	BB permiability	Qplog kp (–8.0 - –1.0)
1	Daidzin	416.38	0.50	-3.31	No	-8.36
2	Kaempferol	286.24	1.58	-3.31	No	-6.70
3	Daidzein	254.24	2.24	-3.53	Yes	-6.10
4	Formononetin	268.26	2.66	-9.73	Yes	-5.95
5	Chlorogenic acid	354.31	-0.38	-1.62	No	-8.76
6	Coumestrol	268.22	2.46	-3.87	No	-5.98
7	Cholecalciferol	384.64	6.85	-6.84	No	-3.0
8	Inositol	180.16	-2.67	1.38	No	-10.03
9	Palmitic acid	246.42	5.20	-5.02	Yes	-2.77
10	Glycitin	446.40	0.50	-3.05	No	-8.57
11	Lysophosphatidylcholine	299.26	-2.27	0.03	No	-9.33
12	Oleic acid	282.46	5.71	-5.41	No	-2.60
13	Linolenic acid	278.43	5.09	-4.78	Yes	-3.41

4. Discussion

The findings of this study offer promising insights into the potential therapeutic effects of *G. max* extract against neurodegenerative disorders. Our *in silico* analysis identified several constituents within the extract exhibiting strong binding affinities towards the NADPH oxidase enzyme, a key contributor to neurodegeneration. Furthermore, the presence of these compounds in *G. max* extract was confirmed through LC-MS/MS analysis, bolstering the credibility of our computational results. Notably, daidzein, formononrtin, palmitic acid, and linolenic acid emerged as potential lead compounds, demonstrating favorable pharmacokinetic profiles and high binding affinities towards NADPH oxidase. These findings align with previous studies highlighting the neuroprotective properties of *G. max* extract and its constituents. For instance, daidzein has been shown to exert anti-inflammatory and antioxidant effects, both of which are crucial in mitigating neurodegenerative progression. Similarly, formononrtin possesses antioxidant and antiapoptotic properties, potentially safeguarding neurons from oxidative stress and cell death (Ma and Wang, 2022). Palmitic acid and linolenic acid, while exhibiting contrasting functionalities, both contribute to maintaining neuronal cell membrane integrity and function, thereby offering potential neuroprotective benefits (Moon *et al.,* 2014; Tofighi *et al.,* 2021). Despite the promising nature of our findings, it is crucial to acknowledge limitations inherent in computational studies. *In silico* analyses, while valuable tools for initial screening and identification of potential therapeutic candidates, require corroboration through rigorous experimental verification. Therefore, further investigations involving animal models and subsequent clinical trials are essential to validate the efficacy and safety of *G. max* extract and its identified constituents in managing neurodegenerative disorders.

5. Conclusion

This study employed computational tools to explore the binding interactions between *G. max* extract constituents and NADPH oxidase, a key enzyme implicated in neurodegenerative diseases. Our analysis identified several promising compounds, particularly daidzein, formononrtin, palmitic acid, and linolenic acid, as potential candidates for further exploration. While our findings provide valuable preliminary insights, further experimental validation through animal models and clinical trials is crucial to translate these *in silico* results into tangible therapeutic applications for neurodegenerative disorders.

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Conflict of interest

The authors declare no conflict of interest relavant to this article.

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