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RESEARCH ARTICLE

Predicting Antioxidant, Neuroprotective, and Toxicity Activity of *Murraya koenigii* Essential Oil by in silico and ex vivo against Rotenone-induced Model

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Abstract

Objectives: To study the antioxidant capacity of Murraya koeingii essential oil (MKEO) phytoconstituents **Methods:** It was determined by using GCMS. The phytoconstituents were then exposed to molecular docking studies that targeted dopamine D2 to look for neuroprotective effects. The highest percentage content of constituents were selected as ligands and dopamine(6VMS) was retrieved from the protein data bank, the docking was performed using Schrodinger software version 2021-4. The pharmacokinetic profile associated with the selected components were predicted utilizing ADMET lab 2.0 and pkCSM which is a graph-based small molecular pk predictor along with toxicity properties of constituents. The neuroprotective activity of MKEO is also predicted by Ex vivo protocol using rotenone as an inducing agent in mice brain slices. **Findings:** The antioxidant potentials were nearer to the positive control (Ascorbic acid) which proved the antioxidant capacity of MKEO. The antioxidant enzymes of CAT and GSH were also decreased in the mice brain slices which are treated with rotenone and LPO values were decreased. The levels of dopamine, CAT, and GSH were improved in the groups supplemented with MKEO at different concentrations, along with reduced LPO levels. **Novelty:** These results are reassuring that both In silico and Ex vivo investigations had greater neuroprotective and antioxidant activities for the phytoconstituents examined from MKEO.

Keywords: Murraya koeingii; Neuroprotective; Antioxidant; Toxicity; Parkinson's Disease

1 Introduction

Age-related neurological issues, such as stroke, neuroinflammation, neurotoxicity, as well as oxidative stress, and neurodegenerative disorders, such as dementia, Parkinson's disease, and Alzheimer's disease. Among all neurodegenerative diseases, Parkinson's disease(PD) is the second most neurological disorder next to Alzheimer's. Rotenone

is a pesticide that is related to the development of Parkinson's disease symptoms by impairing dopaminergic neurons, which prevents mitochondrial electron transport chain complex I[(1)], which upregulates free radical generation and produces motor deficits[(2)]. The drugs for neurological issues exhibit a few side effects, like migraine, gastrointestinal (GI) infections, and hallucinations. Nowadays, plant-based medications are being recommended because the natural medication is safe, freely available, and affordable price.

Murraya koenigii (*M. koenigii*) (L) Spreng (Family: Rutaceae) is usually known as "curry leaves". The tropical and subtropical regions in the world have large distributions of *M. koenigii*[(3)]. Among the 14 global species belonging to the genus of Murraya, only two, *M. koenigii* and *M. paniculate*, are available in India. The traditional use of Mk essential oil(EO) and leaf powder in the seasoning of a variety of food items and ready-to foods. *M. koenigii* and its subsidiaries alleviate oxidative stress, neuroinflammation, neuronal loss, and cognitive impairments[(3)].

The current study is intended to plan and recognize the dominant activator for the dopamine receptor (protein) from the above-mentioned phytoconstituents intensifies which we call ligands in docking. The predicted analogs were oppressed for docking with the protein and the after-effects of analogs are contrasted with the best-fitted ligand to figure out the best simple which serves as a strong activator for Dopamine receptors. Concurrently the molecular properties, ADME, and toxicity profile for both ligands and analogs were examined as well.

2 Methodology

2.1 Chemicals

Curry leaf oil (Avi Naturals), glycine glycine, TBA, DTNB, DPPTH

2.2 GCMS analysis

The essential oil sample of *M. koenigii* was subjected to GCMS analysis. GCMS analysis was performed on GCMS QP2010, Shimadzu, Japan under the following conditions: injection volume 1μL with split ratio 1:50; Helium as a carrier gas with Rtx-5MS column of dimensions 30mX0.25mmX0.25μm, temperature programmed at 40 and then 280 °C with a time interval of 15mins identification was accompanied by a comparison of MS with those reported in NIST 17 libraries (Figure 1).

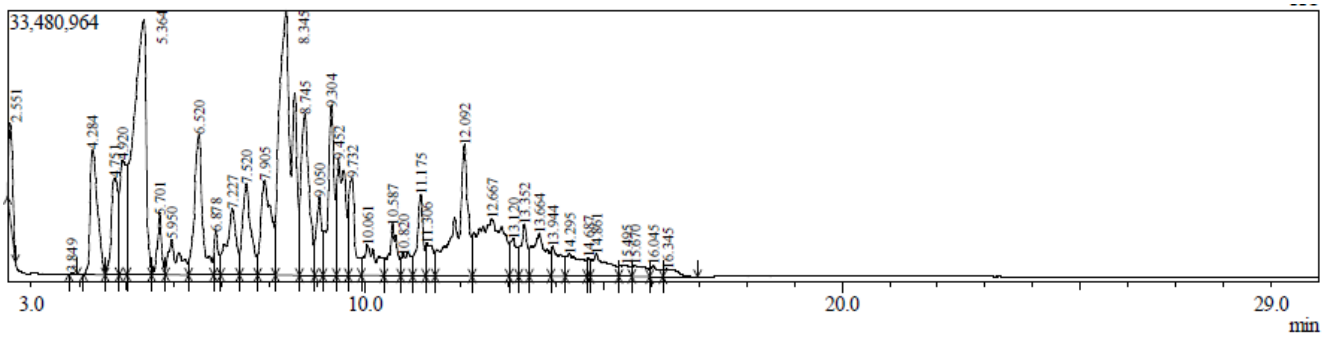

Fig 1. GCMS analysis of *M. koenigii*

2.3 DPPH activity[(4)]

2ml of oil was dissolved and was subjected to 0.1mM DPPH at various concentrations which were dissolved in methanol and kept in the dark for 30 mins. The absorbance was noted at 517nm in the UV spectrometer, against DPPH and methanol as blank. Ascorbic acid was utilized as the positive control. The amount of DPPH that can scavenge free radicals has been calculated as follows:

$$\text{DPPH radical scavenging percentage } (IC_{50}\%) = \left[\left(A_{control} - A_{sample}\right)/A_{control}\right] \times 100$$

where A sample represents the absorbance of the DPPH solution associated with the EOs sample, A control represents the absorbance of the DPPH solution without the EOs sample, as well as A sample blank represents the absorbance of the EOs sample without the DPPH solution. The IC50 values are the concentrations of the sample needed to scavenge 50% of free radicals.

2.4 In silico

2.4.1 ADME toxicity screening

The ADMET screening and drug-likeness prediction of the phytoconstituents obtained from GCMS analysis of MKEO using ADMETlab 2.0 (https://admetmesh.scbdd.com/service/evaluation/index) and pkCSM (https://biosig.lab.uq.edu.au/pkcsm/prediction) which helps to predict pharmacokinetic (A-absorption, D-distribution, M-metabolism, E- elimination) and toxicity properties were redeemed with RDkit cheminformatics tool kit (Table 1).

Table 1. Distribution of ADME predictors in pkCSM [5]

Property	Model Name	Unit	Predicted Value
Absorption	Water solubility(A1)	Numeric (log mol/L)	–
	Caco2 permeability(A2)	Numeric (log Papp in 10^{-6} cm/s)	>0.9
	Intestinal absorption (human) (A3)	Numeric (% Absorbed)	>30%
	Skin Permeability(A4)	Numeric (log Kp)	≥-2.5
	P-glycoprotein substrate(A5)	Categorical (Yes/No)	–
	P-glycoprotein I inhibitor(A6)	Categorical (Yes/No)	–
	P-glycoprotein II inhibitor(A7)	Categorical (Yes/No)	–
Distribution	VDss (human) (D1)	Numeric (log L/kg)	≥-0.15
	Fraction unbound (human) (D2)	Numeric (Fu)	–
	BBB permeability(D3)	Numeric (log BB)	≥-1
	CNS permeability(D4)	Numeric (log PS)	≥-3
Metabolism	CYP2D6 substrate(M1)	Categorical (Yes/No)	–
	CYP3A4 substrate(M2)	Categorical (Yes/No)	–
	CYP1A2 inhibitor(M3)	Categorical (Yes/No)	–
	CYP2C19 inhibitor(M4)	Categorical (Yes/No)	–
	CYP2C9 inhibitor(M5)	Categorical (Yes/No)	–
	CYP2D6 inhibitor(M6)	Categorical (Yes/No)	–
	CYP3A4 inhibitor(M7)	Categorical (Yes/No)	–
Excretion	Total Clearance(E1)	Numeric (log ml/min/kg)	Higher is better
	Renal OCT2 substrate(E2)	Categorical (Yes/No)	–
Toxicity	AMES toxicity(T1)	Categorical (Yes/No)	–
	Max. tolerated dose (human) (T2)	Numeric (log mg/kg/day)	
	hERG I inhibitor(T3)	Categorical (Yes/No)	
	hERG II inhibitor(T4)	Categorical (Yes/No)	
	Oral Rat Acute Toxicity (LD50) (T5)	Numeric (mol/kg)	
	Oral Rat Chronic Toxicity (LOAEL) (T6)	Numeric (log mg/kg_bw/day)	
	Hepatotoxicity(T7)	Categorical (Yes/No)	
	Skin Sensitisation(T8)	Categorical (Yes/No)	
	T.Pyriformis toxicity(T9)	Numeric (log ug/L)	> -0.5
	Minnow toxicity(T10)	Numeric (log mM)	< -0.3

2.5 Docking process

2.5.1 Ligand and protein preparation

The sdf files of phytoconstituents that were obtained from the GCMS analysis were downloaded from Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and the target protein Dopamine D2 was retrieved from the RCSB PDB web server (https://www.rcsb.org/) were optimized by deleting unbound water molecules which are replaced with hydrogen bonds for satisfying the valancies and side chains were stabilized by adding missed amino acids. For the intersection of phytoconstituent targets and disease, proteins were placed in open venny an online program utilized to visualize and isolate overlapping targets between drug and disease. The overall process is carried out in Schrodinger software version 2021-4 (Figure 2).

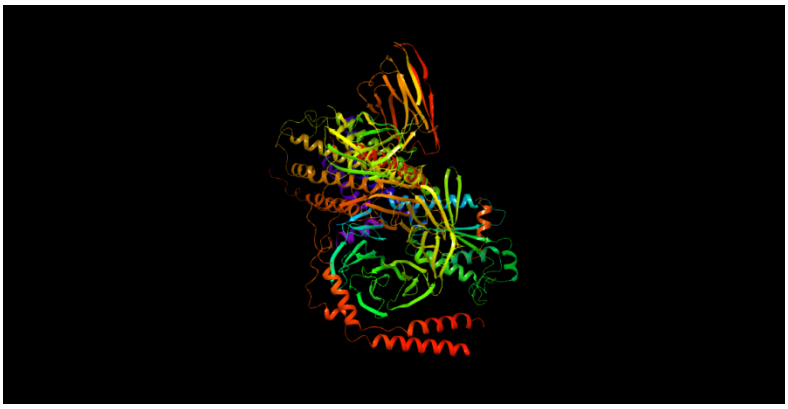

Fig 2. 3D crystalline structure of target protein Dopamine D2- PDB (6VMS)

2.6 Ex-vivo studies

2.6.1 Artificial cerebrospinal fluid (ACSF) preparation

The composition of ACSF was NaCl, KCl, $CaCl_2$, $MgSO_4$, Glucose, glycylglycine. All the salts were dissolved in distilled water and can be stored in the refrigerator. On the day of the experiment glucose and glycylglycine were mixed.

2.6.2 Collection of the brain

After immediate dissection of the brain from the anesthetized and decapitated male rat, it was rinsed with ice-cold saline and transferred to ice-cold (4°C) ACSF. After an hour, the cerebellum had been removed, and the required groups of the midbrain had been sliced[(6)].

2.6.3 Grouping and treatment

The brain was sliced to 1mm thickness. They were incubated at 37°C in ACSF at pH 7.4 and gassed with 95% O_2/CO_2 for 1hr. each slice was differentiated into groups[(7)]. Group I served as control treated with normal saline, Group II with DMSO, Group III as rotenone group 1ng/ml, standard group slice was treated with Syndopa, and another 3 groups IV, V, and VI are pre-treated with *M.koeingii* oil (10μL, 20μL, 40μL). For one hour, these groups were incubated at 37°C. Standard group and pre-treated groups were removed after 30mins to be treated with rotenone and placed in an incubator for another 30mins.

After 1hr of the incubation period, the slices were homogenized in PBS pH 7.4 as well as centrifuged at 10,000x 4°C for about ten mins. The homogenized and centrifuged content was estimated for LPO, GSH catalase, and Dopamine as per the standard procedures.

2.7 Estimation of biomarkers

2.7.1 LPO assay[(8)]

To homogenized supernatant equal volumes of 37% TBA, 15% TCA, and 0.25N HCl were added and subjected to boiling for 1hr. then it is brought to cool at room temperature. It was centrifuged at 5000 for 10mins 4°C. At 532nm, the absorbance has been measured. Results were given in terms of nanomoles per milligram of protein. Using the formula, the concentration of MDA was determined.

$$\text{Conc. of MDA } = \text{Abs}532 \times 100 \times VT / \left(1.56 \times 10^5\right) \times W\ T \times VU$$

where VT is the overall volume of the mixture (4 mL), Abs532 is the absorbance, 1.56 × 105 is the molar extinction coefficient,WT is the weight of dissected brain (1 g), and VU is an aliquot volume (1 mL).

2.7.2 Catalase (CAT) assay[(9)]

In simple terms, the assay combination is composed of 0.1 ml of tissue homogenate and 2.9 ml of 10 mM H2O2 with 50 mM Potassium Phosphate Buffer i.e., pH 7. The rate of decrease in the absorbance at 240 nm was recorded for 2 min. The findings were presented as CAT activity units per milligram of protein.

2.7.3 Reduced Glutathione (GSH) assay[10]

To tissue supernatant 10% TCA and DTNB were added. The absorbance was noted at 412nm. The values were expressed as nM of reduced glutathione per mg of protein:

$$\text{GSH level } = Y - 0.00314/0.0314 \times DF/$$

where Y is Abs412 of tissue homogenate, DF is dilution factor (1), BT is brain tissue homogenate (1 mL), and VU is an aliquot volume (1 mL).

2.7.4 Dopamine estimation[11]

To brain tissue 1:10 ratio of HCl and Butanol was taken and homogenized. The homogenation was centrifuged at 3000rpm for 10mins. An aqueous phase was used for the dopamine assay. The aqueous phase is prepared by mixing 2.5ml n-hexane, and 0.3ml of 0.1M HCl. To 1ml of centrifuged supernatant, 0.2ml aqueous phase is added. To 0.2ml aqueous phase mixture 0.05ml 0.4M HCl, 0.1ml sodium acetate buffer pH 6.9, 0.1ml ethanol iodine solution. In order to stop the reaction after two minutes, 0.1 ml of sodium sulphite solution and 0.1 ml of acetic acid were added. For 6 minutes, the solution was heated at 100°C. The absorbance was noted at 330-375nm in a spectrofluorimeter.

$$X_{\text{dopamine}} = \frac{\text{Sample O.D } - \text{ Blank O.D}}{\text{Standard O.D - Blank O.D}} \text{ X Conc. of standard } (500\mu\text{g/ml})$$

This revealed how much dopamine was contained in 1 ml of the sample.

2.8 Statistical analysis

All values are expressed as Mean±SEM. The analysis of all the studies were done with the help of analysis of variance (ANOVA) followed by Tukey's Multiple Comparision Test and were considered as statistically significant $p \leq 0.001$. All the data were analyzed using Graph pad Prism version 9.0.

3 Results and Discussion

The antioxidant activity as estimated in vitro by using the DPPH assay demonstrated that *M. koeingii* essential oil had strong anti-oxidant potential by scavenging free radicals (Table 2). This was indicated by reduced absorption values while increasing the dose were measured by UV spectroscopy at 517 nm. The most effective free radical scavengers were found in *M. koeingii* essential oil was 91.4% decolorization at a concentration of 1000 μg/ml whereas ascorbic acid (positive control) showed 92.5% of decolorization at 1000 μg/ml concentration. Following the trapping by the unpaired electron of DPPH, the activity of scavenging free radicals was assessed as decolorizing activity. By using the DPPH method, *M. koeingii* essential oil's capacity to scavenge free radicals showed a concentration-dependent response.

Table 2. IC50 values of MKEO at different concentrations compared with positive control

Concentration μg/ml	*M. koeingii* essential oil (%)	Ascorbic acid (%) (Positive control)
200	**24.6**	**79.1**
400	**36.03**	**90.8**
600	**54.3**	**91.25**
800	**78.5**	**91.4**
1000	**91.4**	**92.5**

37 compounds were found in the chromatogram from the GCMS analysis involving the Mk essential oil. The most prevalent compounds were Caryophyllene(13.28%) and sabinene (12.54%). (Table 3)

Table 5 shows the expected ADMET or Pharmacokinetics features for the nine chemical substances. When compared to the reference drugs Syndopa, all of these compounds had intestinal absorption values that were higher than 90% yet less than 100%. These described compounds have significant human intestinal absorption characteristics, as evidenced by the fact that their intestinal absorption values have above the threshold value of 30%. Some of the chosen ligands had BBB permeability (log BB) values that were < -1, indicating that the distribution of those substances throughout the brain is poor. Some individuals had CNS permeability (log PS) values that were greater than -2, which is regarded as penetrating the central nervous system. The described compounds' metabolic features were confirmed by the discovery that they were both CYP3A4 substrates and

Table 3. Phytoconstituents in the MKEO evaluated by GCMS analysis and Docking scores of molecular modeling

Peak	CID	% Area	R-Time	Name of the compound	Docking scores
1	5281515	13.28	8.345	Caryophyllene	-5.97
2	18818	12.54	5.364	Sabinene	-5.571
3	6450812	6.24	12.092	Gurjunene	-6.83
4	442393	5.75	12.667	Selinene	-6.22
5	10104370	5.14	8.745	Bisabolene	-5.679
6	5352485	4.48	6.520	Humelene	-5.641
7	17868	4.25	9.304	Thujene	-5.433
8	6654	3.87	7.905	α- pinene	-4.827
9	31253	3.81	9.452	Myrecene	-2.191
10	5312435	3.59	4.284	n- Heptadecanoic acid	-5.22

Table 4. ADMET/Pharmacokinetics properties

Peak	Name of the compound	A1	A2	A3	A4	A5	A6	A7	D1	D2	D3	D4
1	Caryophyllene	-5.555	1.423	94.845	-1.58	No	No	No	0.652	0.263	0.733	-2.172
2	Sabinene	-4.629	1.404	95.356	-1.342	No	No	No	0.566	0.295	0.836	-1.463
3	Gurjunene	-5.72	1.409	96.881	-1.784	No	No	No	0.77	0.166	0.819	-1.819
4	Selinene	-6.439	1.429	95.574	-1.702	No	No	No	0.639	0.089	0.816	-1.461
5	Bisabolene	-6.057	1.419	95.232	-1.27	No	No	No	0.634	0.231	0.788	-2.131
6	Humelene	-3.741	1.511	93.439	-2.16	Yes	No	No	0.33	0.415	0.522	-1.876
7	Thujene	-4.294	1.386	95.256	-1.371	No	No	No	0.575	0.356	0.81	-1.793
8	α- pinene	-3.733	1.38	96.041	-1.827	No	No	No	0.667	0.425	0.791	-2.201
9	Myrecene	-4.497	1.4	94.696	-1.043	No	No	No	0.363	0.39	0.781	-1.902
10	Syndopa(r)	-2.89	-0.289	47.741	-2.735	Yes	No	No	-0.105	0.604	-0.843	-2.032

(r) is a reference drug that is the gold standard for the treatment of Parkinson's disease; A1 = Water solubility, A2 = Caco2 permeability, A3 = Intestinal absorption (human), A4 = Skin Permeability, A5 = P-glycoprotein substrate, A6 = P-glycoprotein I inhibitor, A7 = P-glycoprotein II inhibitor, D1 = VDss (human), D2 = Fraction unbound (human), D3 = BBB permeability, D4 = CNS permeability

Table 5. ADMET or Pharmacokinetics features

Peak No	Name of the compound	M1,M4, M5, M6, M7	M2	M3	E1	E2	T1	T2	T3,T4	T5	T6	T7	T8	T9	T10
1	Caryophyllene	No	No	No	1.088	No	No	0.351	No	1.617	1.416	No	Yes	1.401	0.504
2	Sabinene	No	No	No	0.071	No	No	0.369	No	1.549	2.309	No	No	0.788	0.726
3	Gurjunene	No	Yes	No	0.926	No	No	-0.001	No	1.538	1.405	No	No	1.425	0.225
4	Selinene	No	Yes	Yes	1.174	No	Yes	-0.03	No	1.581	1.511	No	Yes	1.736	-0.078
5	Bisabolene	No	No	No	1.458	No	No	0.418	No	1.642	1.347	No	Yes	1.943	-0.065
6	Humelene	No	No	No	1.389	No	No	0.622	No	1.825	1.168	No	Yes	1.235	1.195
7	Thujene	No	No	No	0.077	No	No	0.353	No	1.589	2.243	No	No	0.597	0.995
8	α- pinene	**No**	**No**	**No**	**0.043**	**No**	**No**	**0.48**	**No**	**1.77**	**2.262**	**No**	**No**	**0.45**	**1.159**
9	Myrecene	**No**	**No**	**No**	**0.438**	**No**	**No**	**0.617**	**No**	**1.643**	**2.406**	**No**	**No**	**0.894**	**0.736**
10	Syndopa(r)	**No**	**No**	**No**	**0.43**	**No**	**No**	**0.922**	**No**	**2.234**	**2.699**	**Yes**	**No**	**0.281**	**3.143**

M1 = CYP2D6 substrate, M2 = CYP3A4 substrate, M3 = CYP1A2 inhibitor, M4 = CYP2C19 inhibitor, M5 = CYP2C9 inhibitor, M6 = CYP2D6 inhibitor, M7= CYP3A4 inhibitor, E1 = Total Clearance, E2 = Renal OCT2 substrate, T1 = AMES toxicity,T2 = Max. tolerated dose (human), T3 = hERG I inhibitor, T4 = hERG II inhibitor, T5 = Oral Rat Acute Toxicity (LD50), T6 = Oral Rat Chronic Toxicity (LOAEL), T7 = Hepatotoxicity, T8 = Skin Sensitisation, T9 = T.Pyriformis toxicity, T10 = Minnow toxicity.

inhibitors, particularly caryophyllene, which had the highest binding affinity. They were also within the permissible limitations in terms of total clearance value. All of the reported substances were discovered to be non-toxic. The compounds are reported to have good permeability, low toxicity level, as well as high absorption value to the cell membrane in relation to the projected characteristics. It was expected that all of the described drugs would have favorable pharmacokinetic and toxicological profiles.

The docking results which were presented in Table 1 have shown that Gurjunene with high docking affinity valued (-6.83) ligands along with the dopamine receptor in comparison with Syndopa (-3.69) next to it selienene (-6.22) and caryophyllene (-5.97) are shown better affinity values than other ligands/phytoconstituents (Figures 3 and 4).

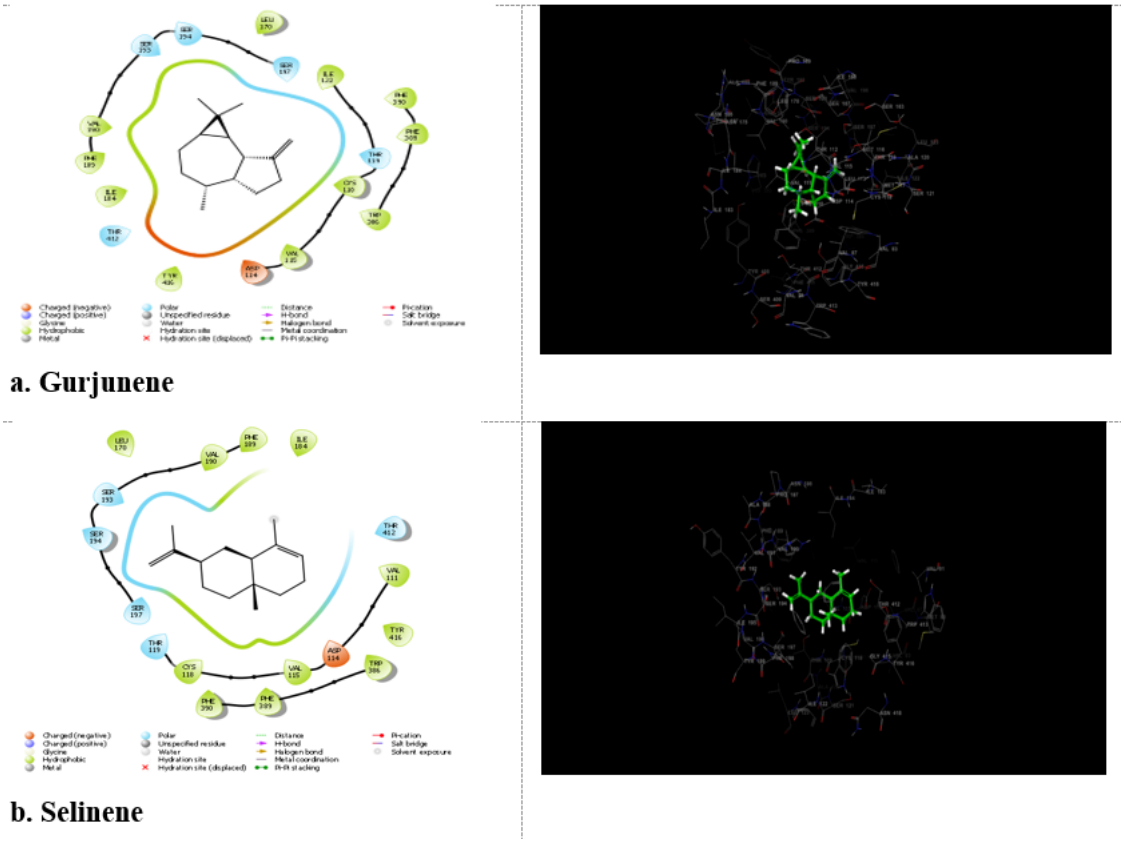

Fig 3. Ligands having the highest binding energy of 2D and 3D structures a. Gurjunene, b. Selinene (reference drug) with protein dopamine D2(PDB ID: 6VMS)

When compared to mice not receiving ROT treatment, it was discovered that the amount of lipid peroxidation within the brain slices was considerably higher in the ROT-treated group ($P < 0.001$) whereas mice treated with MKEO treatment at the dose of at different doses (10, 20 and 40 μg/ml) significantly ($P < 0.001$) attenuated the increase in MDA as compared to the ROT-treated group's concentration (Figure 5)

The level of antioxidant enzymes including CAT and GSH were evaluated for their free radicals scavenging activity and are shown in Figure 5. ROT causes the generation of free radicals and which results in a significant decrease of CAT ($P < 0.001$) activities and levels of GSH ($P < 0.001$) in ROT treated group of mice as compared to control group mice. Mkeo treatment at doses of 10, 20, and 40 mg/kg significantly attenuated the ROT-induced depletion of CAT ($P<0.001$) and GSH ($P<0.001$) as compared to ROT treated group of mice brain slices.

MKEO is medicinally used to treat osteoporosis, chemotherapy treatments of cancers, and wound healing along with antimicrobial, antioxidant, antioxidant, antifungal, and antibacterial activity[12,13]. Monoterpenes and sesquiterpenes were identified in GCMS analysis of MKEO among which ß- Caryophyllene(13.28%), Sabinene(12.54%), Gurjunene(6.24%) were shown in Table 3 . In recent studies ß- Caryophyllene improved dopaminergic neuronal loss due to oxidative stress and MPTP-induced murine model[14]. Sabinene is a monoterpene compound of many herbs and plants, with many biological activities like anti-inflammatory, antifungal, and antioxidant[15]. Against a rotenone-induced model for PD, nerolidol, a sesquiterpene

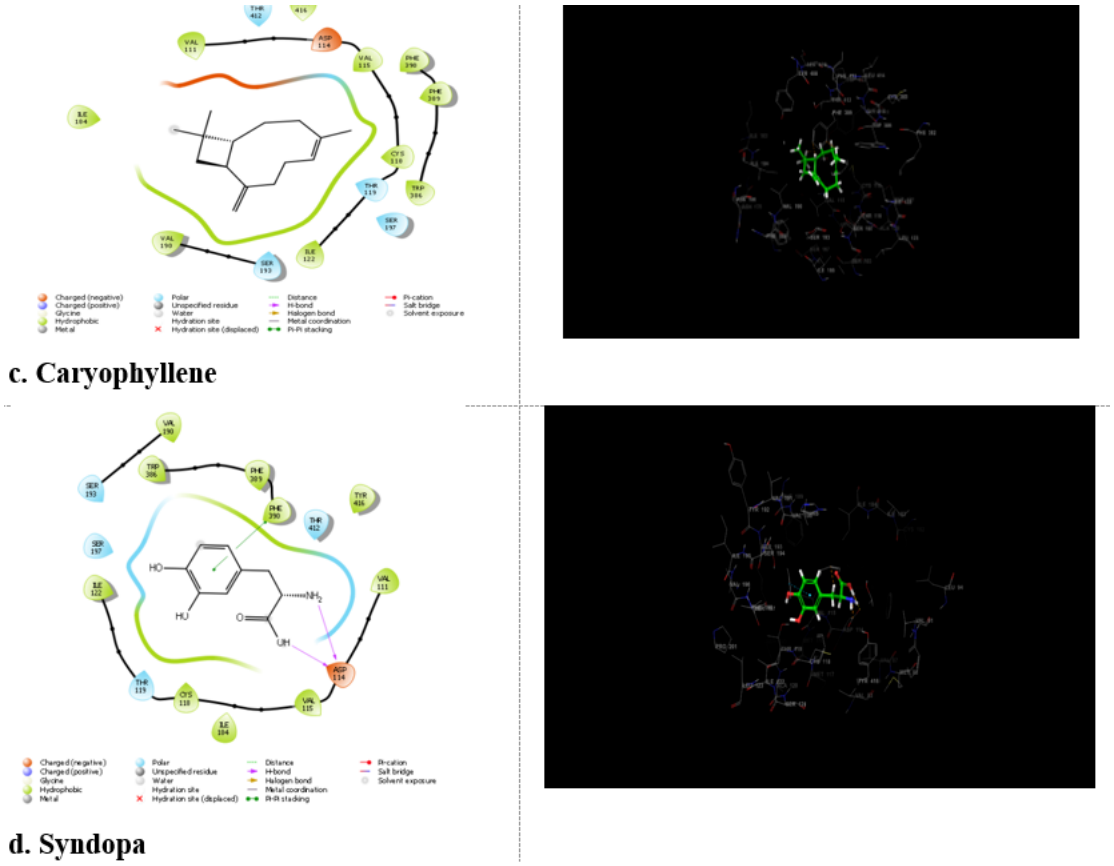

Fig 4. Ligands having the highest binding energy of 2D and 3D structures c. Caryophyllene and d. Syndopa (reference drug) with protein dopamine D2(PDB ID: 6VMS)

alcohol, exhibits neuroprotective activities it also inhibits oxidative stress and AChE activity[16].

The MKEO has excellent antioxidant activity, according to the DPPH (antioxidant) assay, which shows significant free radical scavenging activity with IC50 values that are comparable to those of ascorbic acid (Table 2). The current research indicated that Gurjunen, Selinen, Caryophyllene, as well as Sabinene, have greater binding affinity when compared with the reference drug Syndopa (Table 3Figures 3 and 4). All the selected phytoconstituents followed the Lipinski rule of 5 in ADMET lab 2.0 and the pharmacokinetic profile along with toxicity in pkCSM showed better potential constituents for neuroprotection.

In Ex vivo studies LPO was increased in rotenone treated group and decreased in MKEO treated group compared to Syndopa. The oxidative enzymes CAT, GSH, and neurotransmitter Dopamine were diminished in rotenone treated group compared to the control group. Significant improvements in CAT, GSH, and dopamine levels were observed in MKEO-treated groups compared to Syndopa. The findings of the present study showed that MKEO had greater neuroprotective activities based on Insilico as well as Ex vivo values.

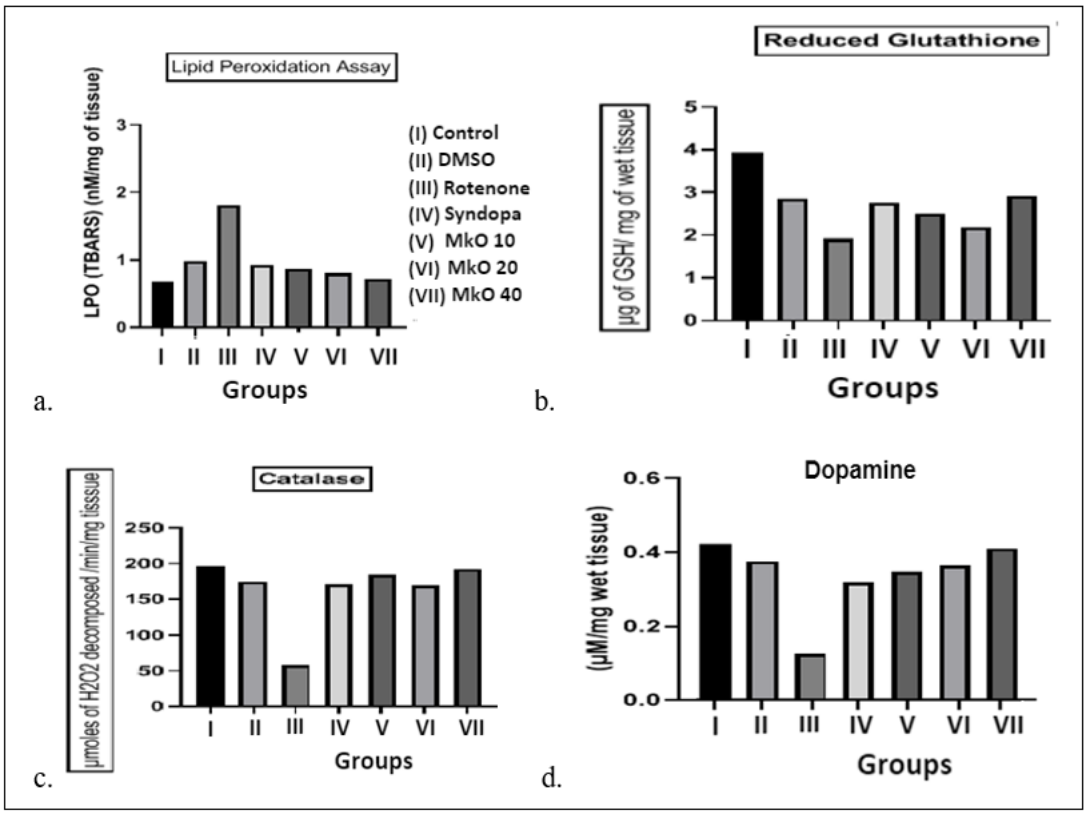

Fig 5. Statistical analysis of decreased MDA levels (a), increased GSH levels(b), CAT levels(c), and Dopamine levels(d) compared to rotenone with MKEO and Syndopa

4 Conclusion

The present study investigated, in-silico and ex-vivo neuroprotective activity of MKEO. Flexible docking found that gurjunene has high docking affinity valued ligands along with the dopamine receptor in comparison with reference drug Syndopa. The antioxidant activity of MKEO was comparable with the reference standard ascorbic acid. When compared to reference drug syndopa, MKEO showed absorption values higher than 90%. Exvivo studies observed significant improvement in CAT, GSH and dopamine levels in MKEO treated groups compared to Syndopa. We conclude that, the essential oil of MKEO were found to possess appreciable neuroprotective activity. Further clinical studies in future can be carried out to establish its safety and efficacy in humans through clinical trials.

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