



***In vitro* MAO-B Inhibitory Effects of *Citrus trifoliata* L. Fruits Extract, Self-Nano-Emulsifying Drug Delivery System and Isolated Hesperidin: Enzyme Assay and Molecular Docking Study**

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FLAVONOIDS have demonstrated neuroprotective activity. Hesperidin, the major flavonoid in the hydroalcoholic extract of *Citrus trifoliata* L. fruits was quantified quantitatively using HPLC. The calibration curve obtained by plotting different concentrations of hesperidin standard versus the area under the curve revealed that hesperidin content was up to 30 mg/g. Hesperidin was isolated and identified using ¹H and ¹³C NMR. Self-nano-emulsifying drug delivery system (SNEDDS) was prepared to improve the physical characteristics and optimize the activity of the extract. Monoamine oxidase enzyme (MAO-B) inhibitory effects of the SNEDDS, the extract, and the isolated hesperidin were evaluated. They showed significant decrease in the IC₅₀ up to 129.9008, 252.7341, and 707.7631 ng/ml, respectively, compared with selegiline, with IC₅₀ of 133.8403 ng/ml. The SNEDDS showed the highest activity, whereas the hydroalcoholic extract showed higher activity than the pure hesperidin, which could be attributed to synergistic effect of other flavonoids in extract. Hesperidin molecular docking studies were carried out. The ability of hesperidin to interact with the key amino acids in MAO-B binding site rationalizes this pronouncing activity as proven by its docking pattern and docking score compared with that of the known MOA-B inhibitor, safinamide.

Keywords: *Citrus trifoliata*, Hesperidin, Monoamine oxidase enzyme-B, Molecular docking, selegiline, Self-nano-emulsifying drug delivery system.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder associated with dopamine (DA) depletion and induction of a gradual dysfunction of the basal ganglia in the central nervous system that mainly affects the motor system[1]. Symptoms of PD include resting tremor, bradykinesia, muscle rigidity and gait impairment. Non-motor symptoms, including memory impairments, disordered sleep, and neuropsychiatric manifestations and these features are probably due to the spread of pathology beyond the basal ganglia with continuous oxidative stress[2, 3].

Flavonoids derived from natural products especially edible citrus fruits, have demonstrated

neuroprotective activity [4, 5] that may be related to their antioxidant capabilities and their capability to penetrate into the brain [6]. The bioflavonoid hesperidin is a specific flavonoid glycoside that is frequently found in *Citrus trifoliata* L. and other Citrus fruits. Several previous studies have reported the activity of hesperidin in neurodegenerative diseases such as Alzheimer's [7] and Parkinson's disease [8]. Furthermore, hesperidin has an important neuroprotective property against oxidative-induced damage[9], as well as in vitro antioxidant activity against DPPH, superoxide radical, nitric oxide radical, hydroxyl radical and hydrogen peroxide[10]. ¹H and ¹³C NMR are the most commonly used tool for identification and structure elucidation of flavonoids[11].

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Monoamine oxidases (MAOs) are an enzyme family that can oxidize several endogenous primary amines. MAOs are well-known for their role in regulating central nervous system neurotransmission. Two isoforms, MAO-A and MAO-B, have been recognized. MAO-A and MAO-B are mitochondrial-bound enzymes that are expressed throughout the brain and other tissues and catalyze the breakdown of monoamine neurotransmitters including dopamine, serotonin, and epinephrine in the CNS [12]. Monoamine Oxidase Inhibitors (MAOIs) inhibit monoamine oxidase, resulting in an increase in the concentration of monoamines in the synapse [13]. Inhibition of MAO-B, the predominant isoform in the human brain, is a therapeutic strategy for treating Parkinson's disease. The drugs Selegiline and rasagiline are selective irreversible inhibitors of MAO-B and clinically approved to relieve symptoms and postpone the need for levodopa in early PD and to be useful in the management of end-of-dose akinesia in fully developed disease [14, 15].

Development of Self-Nano-Emulsifying Drug Delivery Systems (SNEDDS) are gaining great considerations in order to stabilize herbal drugs and enhance their bio availabilities. Nano sized drug delivery systems containing herbal drugs have been considered as ideal carrier systems for the optimization of the activity of these extracts [16, 17]. SNEDDS is frequently used for the stabilization of natural products and these carrier systems may also increase the bioavailability of natural bioactive materials.

Molecular docking is a target structure-based molecular modeling technique that simulates the drug-target interaction [18]. Molecular docking is used to predict the binding pattern by which the

compound interacts with its biological target. It also predicts the binding affinity of this compound its target. So, molecular docking is used to explain observed compound experimental activity [19-21] or as a tool for further lead optimization [22]. In the current work, the major flavonoid of the hydro alcoholic extract of *Citrus trifoliata* L. fruit (family Rutaceae) hesperidin was quantitatively quantified by HPLC (Fig. 1) and then isolated as pure compound. Development of SNEDDS containing hydro alcoholic extract of *Citrus trifoliata* L. Fruits to improve the physical characters of the herbal extract and enhance its MAO-B inhibition activity. Molecular docking studies were performed to find out the possible binding mode of hesperidin with MAO-B, and to study its interaction with the enzyme hot spots (key amino acids) with the aim of explaining its MOA-B inhibitory activity.

Materials and Methods

Materials

Plant material

The fruits of *Citrus trifoliata* L. were collected from Horticulture Research Institute, Giza, Egypt during the fruiting stage at November 2018. Specimens were dried according to standard herbarium techniques and voucher specimens, (No.9.12.18.1) are kept in the Herbarium collection of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Methods

Preparation of extract

The whole fruits (2 kg) were Shade-dried, powdered and macerated till exhaustion in ethanol (70%). The extract (250 g) was then collected, filtered and evaporated to dryness under reduced pressure.

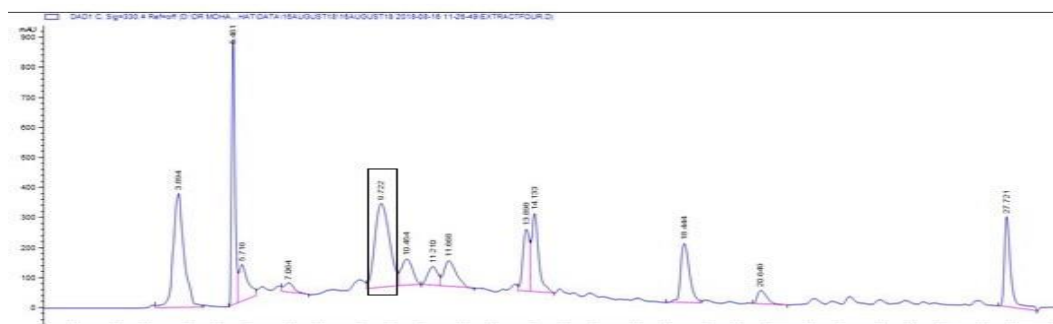


Fig. 1. HPLC Chromatogram of hydro alcoholic extract of *Citrus trifoliata* L. Fruits

HPLC assay of hesperidin

Hesperidin assay was achieved according to Menghinello and colleagues in the year 1999 with modifications on a Zobrax Eclipse plus C8 (4.6 x 250 mm, 5-Micron). Column temperature was maintained at 20°C. using a mobile phase consisting of water acidified with trifluoroacetic acid, pH 2.5 (solution A) and acetonitrile (solution B) in the following gradient at 0 min; 95% A: 5% B, 5 min; 85% A: 15% B, 10 min; 75% A: 25% B, 15 min; 65% A: 35% B, 20 min; 55% A: 45% B, 25 min; 45% A: 55% B, 30 min; 35% A: 65% B, 35 min; 75% A: 25% B, 40 min; 15% A: 85% B, 55 min; 0% A: 100% B, 60 min; 95% A: 5% B. The flow rate of the mobile phase was 1 ml/min.

Extraction and purification of hesperidin

Hesperidin was isolated according to Lahmer et al. [23] with slight modifications. 250 g dried powdered fruits were defatted using 800 mL petroleum ether. The petroleum ether extract was discarded and the powder was extracted with 800 mL methanol. After complete extraction the filtrate was acidified (pH 3-4) with 6% acetic acid, the concentrated liquid was kept in refrigerator (4-6°C) overnight. A solid crystalline substance appeared. It was filtered and the crude hesperidin was separated out as amorphous powder. The crude hesperidin was subjected to further chromatographic purification on sephadex column (1 X 23 cm).

Preparation of self-nanoemulsifying drug delivery system loaded with extract

One of the successful combinations between oils, surfactants and co-surfactants was selected to be formulated [24]. One of the successful combinations between oils, surfactants and co-surfactants was selected to be formulated [24]. The assay was performed in 96-well black plate. MAO-B Enzyme was reconstituted with 22 µl MAO-B Assay Buffer. MAO-B Substrate was reconstituted with 110 µl dd H₂O. Selegiline (Inhibitor Control) was Reconstituted with 250 µl dd H₂O to make a stock solution of 2 mM. Hesperidin was dissolved in DMSO and diluted with MAO-B Assay Buffer. 10 µl of hesperidin solution, working solution of Selegiline and MAO-B Assay Buffer (Enzyme Control; EC) were added into assigned wells. Final solvent concentration should not be more than 2% by volume.

magnetic stirrer (Wisd Wisestir MSH 20-D, Witeg, Germany) at 500 rpm at room temperature till complete solubilisation of the extract. After that, the mixture was treated with 100 ml distilled water (100-fold dilution) on the magnetic stirrer for 2 min. The diluted formula was visually examined for clarity against a dark background.

Self-nanoemulsifying characterization

The MDS and PDI of the formula after treatment with distilled water (100-fold) was investigated using Zetasizer Nano ZS (Ver.6.20, Malvern Instruments Ltd., Worcestershire, England) using detector at scattering angle of 90° to the incident beam at room temperature [25].

The zeta potential (ZP) of the formulation was examined after 100-fold dilution by a laser Doppler anemometer coupled with the same equipment at room temperature. A potential of ±150 mV was set. All measurements were run in duplicate.

Screening of MAO-B inhibition

Mitochondrial outer-membrane-bound MAOs produce hydrogen peroxide (H₂O₂) by using O₂ as an electron acceptor[26]. Bio Vision's Monoamine Oxidase Activity assay is a sensitive assay for detecting MAO-B isoenzyme activity in the presence of Selegiline - specific inhibitor for MAO-B[27]. The assay is based on the fluorometric detection of H₂O₂, generated during the oxidative deamination of the MAO substrate.

Screening protocol: MAO-B inhibition enzyme assay was performed using Monoamine Oxidase B (MAO-B) Inhibitor Screening Kit[28]. The assay was performed in 96-well black plate. MAO-B Enzyme was reconstituted with 22 µl MAO-B Assay Buffer. MAO-B Substrate was reconstituted with 110 µl dd H₂O. Selegiline (Inhibitor Control) was Reconstituted with 250 µl dd H₂O to make a stock solution of 2 mM. Hesperidin was dissolved in DMSO and diluted with MAO-B Assay Buffer. 10 µl of hesperidin solution, working solution of Selegiline and MAO-B Assay Buffer (Enzyme Control; EC) were added into assigned wells. Final solvent concentration should not be more than 2% by volume.

2 µl of MAO-B stock solution were added into 8 µl of MAO-B Assay Buffer to prepare MAO-B Enzyme Solution. For each well, 50 µl MAO-B Enzyme Solution were prepared as follows: MAO-B Assay Buffer (49 µl) and diluted

MAO-B Enzyme (1 μ l). After mixing 50 μ l/well were added into wells containing test inhibitors, Inhibitor Control and Enzyme Control. Incubated for 10 min. at 37°C.

For each well, 40 μ l of MAOB Substrate Solution were prepared as follows: MAO-B Assay Buffer (37 μ l), MAO-B Substrate (1 μ l), Developer (1 μ l) and OxiRed™ Probe (1 μ l). After mixing 40 μ l of the MAO-B Substrate Solution were added into each well and mixed.

Measurement: Fluorescence (Ex/Em= 535/587 nm) was kinetically measured at 37°C for 10-40 min. two points (T1 and T2) were chosen in the linear range of the plot and corresponding fluorescence values (RFU1 and RFU2) were obtained.

Calculation: The slopes were calculated for all samples, including Enzyme Control (EC), by dividing the net Δ RFU (RFU2 -RFU1) values by the time Δ T (T2 - T1). % Relative Inhibition was calculated as follows:

$$\% \text{ Relative Inhibition} = (\text{Slope of EC} - \text{Slope of tested substance}) / \text{Slope of EC} \times 100$$

Molecular docking study

All the molecular modeling studies were carried out using Molecular Operating Environment (MOE, 10.2008) software. All minimization were performed with MOE until an RMSD gradient of 0.1 kcal·mol⁻¹Å⁻¹ with MMFF94x force field and the partial charges were automatically calculated. The X-ray crystallographic structure of the human monoamine oxidase B (MAO-B) enzyme co-crystallized with a benzyloxy substituted derivative (Safinamide) as inhibitor (IC₅₀ = 0.08 μ M) (PDB ID: 2V5Z) was downloaded from the protein data bank (<http://www.rcsb.org>) [29]. Chain B was first removed then water molecules and ligands that are not involved in binding were removed. The protein was prepared for docking study using Protonate 3D protocol in MOE with default options. The co-crystallized ligand was used to define the binding site for docking. Triangle Matcher placement method and Affinity dG scoring function were used for docking. Docking setup was first validated by redocking of the co-crystallized ligand (Safinamide) in the vicinity of the binding site of the enzyme. Redocking validation proved the suitability of the used docking protocol for the current study as indicated by the deviation between the docking pose and the co-crystallized structure binding

mode as indicated by their RMSD of 0.965 Å and with the ability of the docking protocol to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site.

Results and Discussion

HPLC assay of hesperidin

From the calibration curve (Fig. 2) obtained by plotting different concentrations of hesperidin standard versus the area under the curve, HPLC analysis (Fig. 1) revealed that hesperidin was present in considerable amount in the hydro alcoholic extract of *Citrus trifoliata* L. Fruits with concentration up to 30mg/gm. Hesperidin was isolated and identified using ¹H and ¹³C NMR

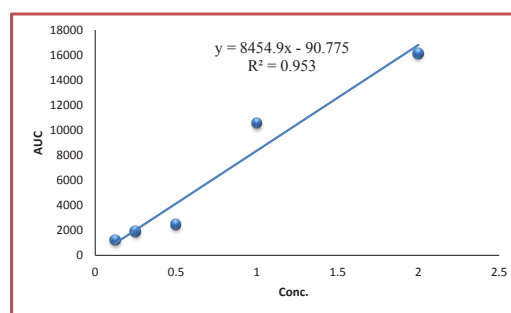


Fig. 2. Calibration curve of hesperidin standard.

Identification of hesperidin (Fig. 3)

NMR Spectral analysis were recorded at 400 MHz for ¹H and 100 MHz ¹³C by Bruker NMR spectrometer using DMSO-d₆ and chemical shifts were given on a δ (ppm) scale with tetramethylsilane as internal standard. The ¹H-NMR spectrum revealed the presence of proton signals at 12.031 (1H, s, 5-OH), 9.107 (1H, s, 3'-OH), in the aromatic region 6.959 (1H, dd, J=2 and 8, H-6'), 6.937 (1H, d, J=2, H-2'), 6.926 (1H, d, J=8, H-5') corresponding to protons B ring. Resonances at 6.1620 (1H, d, J=2, H-8), 6.148 (1H, d, J=2, H-6) are typical for the Meta coupling between H-6 and H-8 in ring A [30]. Characteristic resonances at 5.526 (1H, dd, J = 8, 4.0 Hz, H-2), 3.290 (1H, dd, J = 16, 8.0 Hz, H-3a) and 2.812 (1H, dd, J = 14, 4.0 Hz, H-3b) and at 3.83 (3H, s, 4-OCH₃) were observed. Resonances at δ 5.05 (1H, d, J=8 Hz, H-1''), 4.548 (1H, brs, H-1''') were assigned for anomeric protons and protons of rhamnosyl CH₃ at 1.105 (3H, d, J=6Hz), 2.516 (1H, brs, H-5''') while resonance at 3.6-3.2 represent signals for the rest of sugar protons [23]. The ¹³C-NMR technique revealed characteristic pattern at 197.84 (C-4), 165.6 (C-7), 163.45 (C-5), 162.9 (C-9), 148.4 (C-4'), 146.9

(C-3'), 131.4 (C-1'), 118.3 (C-6'), 114.6 (C-2'), 112.5 (C-5'), 103.8 (C-10), anomeric carbons 99.9 (C-1'') and 101 (C-1'''), 96.78 (C-6), 96.58 (C-8), 78.98 (C-2), 76.7 (C-5''), 75.9 (C-3''), 73.4 (C-4'''), 72.5 (C-2''), 71.1 (C-4''), 70.7 (C-3'''), 68.8 (C-2'''), 68.5 (C-5'''), 66.5 (C-6''), 56.81 (4-OCH₃), 42.5 (C-3), 18.13 (C6''') [30].

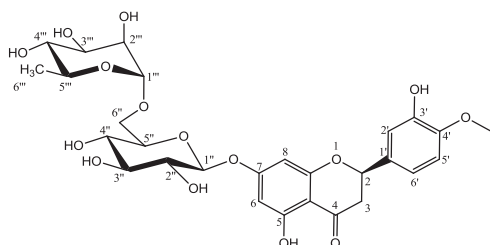


Fig. 3. Hesperidin structure.

Self-nanoemulsifying characterization

There was a good association between the globule size and the clarity of the formula. A clear nanoemulsion with small droplet size and narrow size distribution was obtained. The prepared formula recorded Mean droplet size (MDS) and zeta potential (ZP) about 18.9 ± 0.99 nm and -7.71 ± 0.14 mV, respectively (Fig. 4, 5), where the size below 100 nm indicated nanoemulsion formation after dilution. The creation of a better close-packed film of surfactants at oil/water interface leads to stabilization of the oil droplets and hence lowering of the droplet size [31].

The PDI of the formula was 0.438 ± 0.05 reflecting the nanoemulsion homogeneity. It was reported that the poly dispersity index (PDI) values below 0.5 referring to homogenous distribution with the formula [32].

MAO-B enzyme assay

The SNEDDS, hydro alcoholic extract and

isolated hesperidin showed significant decrease in the IC₅₀ up to 129.9008 ng/ml, 252.7341 ng/ml and 707.7631 ng/ml respectively compared to selegiline with IC₅₀ of 133.8403 ng/ml (Fig. 6, 7 and 8). Statistical analysis was carried out by one-way ANOVA showed that all IC₅₀ values were significantly different at $p < 0.05$.

Molecular docking study

Molecular docking studies were performed (Fig. 9, 10 and 11) to find out the possible binding mode of hesperidin with MAO-B. The X-ray crystallographic structure of the human monoamine oxidase B (MAO-B) enzyme co-crystallized with a benzyloxy substituted derivative (Safinamide) as inhibitor (IC₅₀ = 0.08 μ M) (PDB ID: 2V5Z) was used in this study. Docking setup was first validated by re-docking of the co-crystallized ligand (Safinamide) in the vicinity of the binding site of the enzyme. Redocking validation step showed the suitability of the used docking protocol for the planned docking study. The docking protocol could reproduce the co-crystallized ligand pose as indicated by the low RMSD of 0.965 Å between the docked pose and the co-crystallized ligand (energy score (S) = -8.34 kcal/mol) and by its ability to reproduce all the key interactions accomplished by the co-crystallized ligand with the key amino acids in the binding site. These interactions take place through H-bonding with the side chain amide group of Gln206 and through water molecule mediated H-bonding with Tyr60, Gln65 and Gly205. Moreover, through hydrophobic interaction with hydrophobic side chains of Pro104, Trp119, Leu164 and Ile316 (Fig. 9, 10 and 11). The validated docking protocol was then used in predicting the ligand-enzyme interactions at the binding site for the compound of interest.

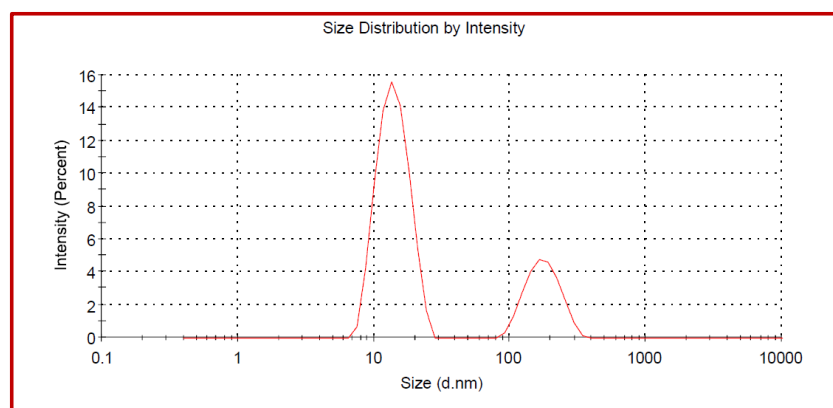


Fig. 4. Size distribution by intensity.

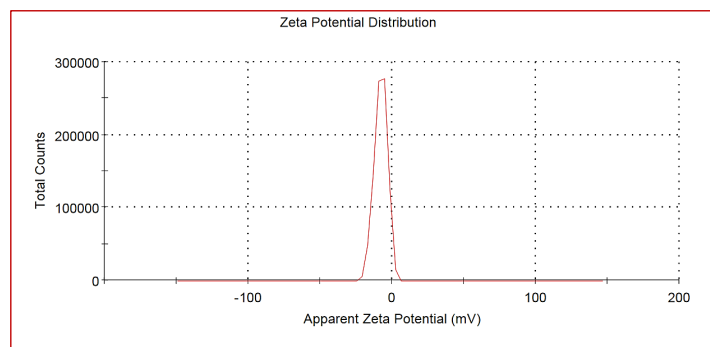


Fig. 5. Apparent zeta potential (mV).

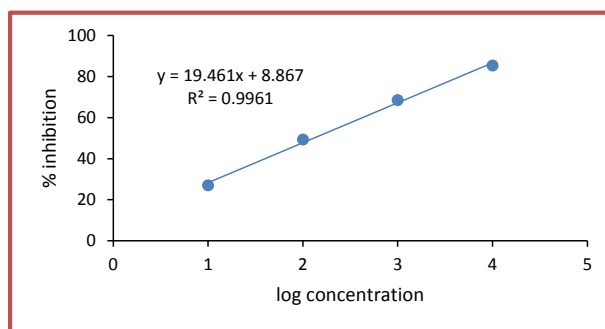


Fig. 6. Relative inhibition of the Nano Formula.

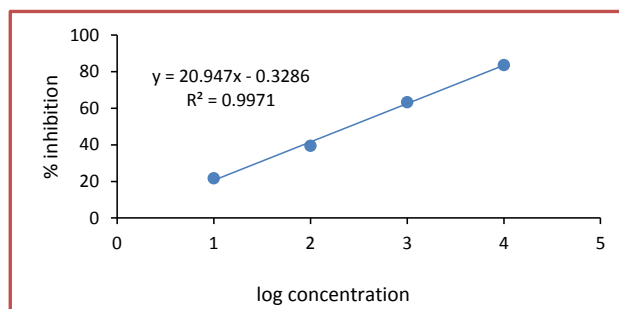


Fig.7. Relative inhibition of the extract.

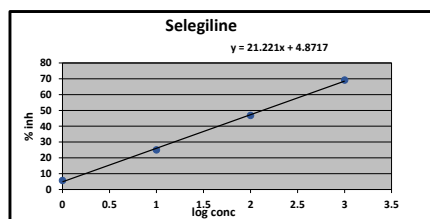
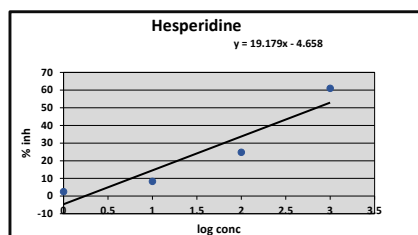


Fig. 8. MAO-B enzyme assay of hesperidin compared to selegiline.

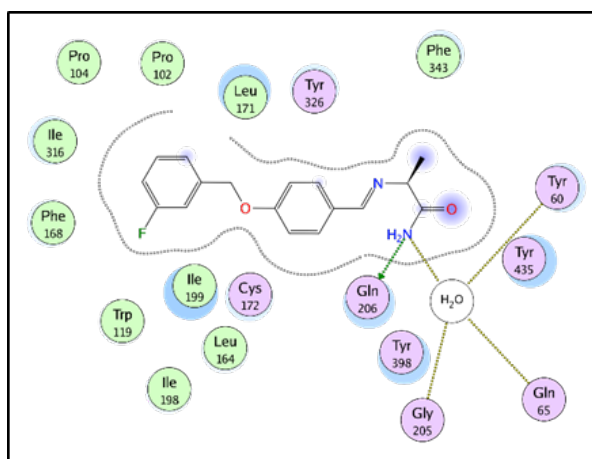
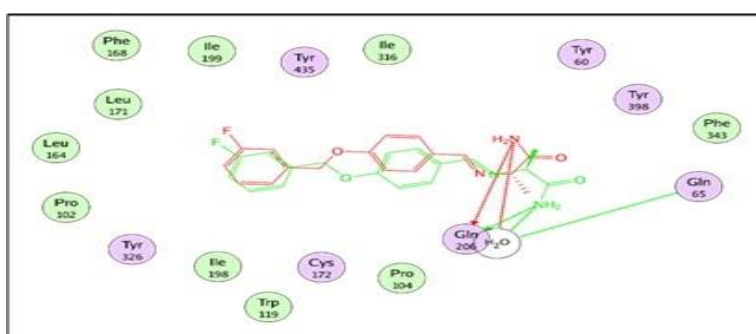
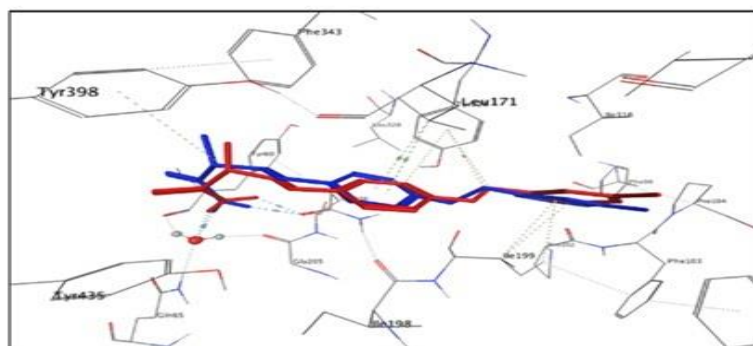


Fig. 9. 2D interaction diagram showing safinamide docking pose interactions with the key amino acids in the MAO-B binding site.



A



B

Fig. 10. A) 2D representation and B) 3D representation of the superimposition of the co-crystallized (red) and the docking pose (blue) of safinamide in the MAO B binding site with RMSD of 0.965Å. (Ligand non-polar Hydrogen atoms were removed for clarity).

The ability of hesperidin to interact with the key amino acids in the binding site rationalizes its good activity as indicated by its docking pattern and docking score compared to that of safinamide. (Fig.11 and Table 1)

Hesperidin interacts by its sugar part through hydrogen bonding with the key amino acid Ser59, Tyr60, Cys172, Tyr188 and Met436. In addition, by its aglycon part through hydrophobic interaction with hydrophobic side chains Pro104, Trp119, Leu164 and Ile316. (Fig. 11).

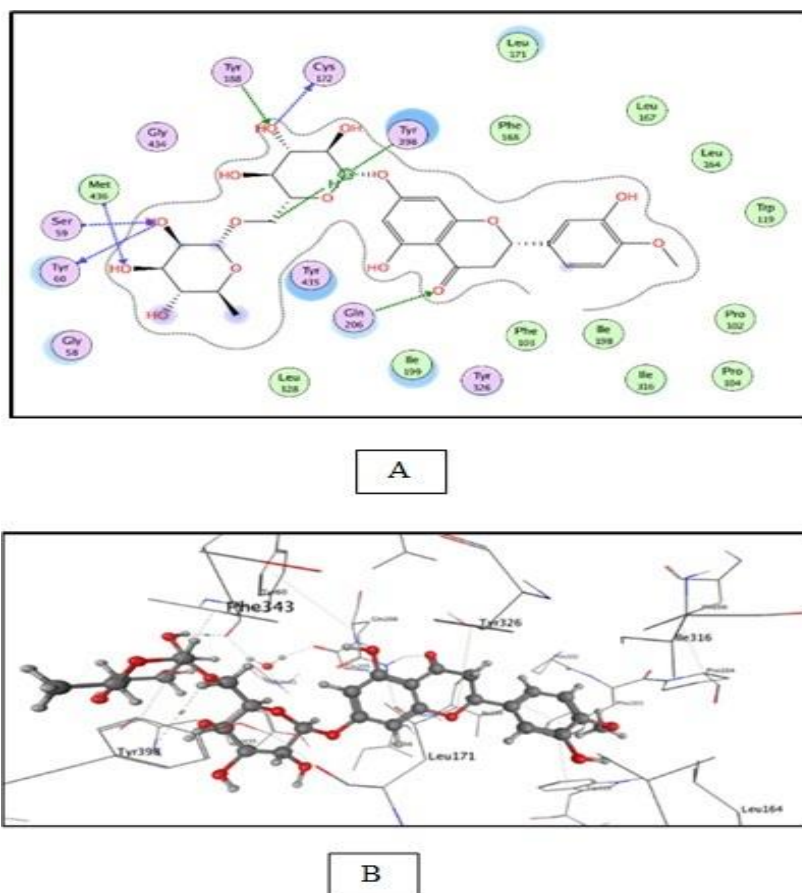


Fig. 11. 2D diagram (A) and 3D representation (B) of Hesperidin in the MAO B binding site.

TABLE 1. Docking energy scores (S) in kcal/mol and IC_{50} for the reference and tested compounds.

Number	Compound	Docking Score (Kcal/mol)	IC_{50} μ M
1	Safinamide	-8.34	0.08 (Bindaet al., 2007)
2	Hesperidin	-11.27	0.00116

Conclusion

HPLC assay showed that the fruits of *Citrus trifoliata* L. are rich in hesperidin that can protect neurons against various types of insults associated with many neurodegenerative diseases. The hydro alcoholic extract of the fruits can be used as it is or in nano form to inhibit MAO-B enzyme activity. The SNEDDS improved the MAO-B inhibitory effect with significant decrease in IC_{50} and can be used in prevention of neurodegenerative diseases in humans such as Parkinson disease. While the SNEDDS showed the highest MAO-B inhibitory activity, the hydro alcoholic extract showed higher activity than pure hesperidin which could be attributed to the synergism of other citrus flavonoids existing in the hydro alcoholic extract. The ability of hesperidin to interact with the key

amino acids in (MAO-B) enzyme binding site rationalizes the pronouncing activity to inhibit human monoamine oxidase B (MAO-B) enzyme as proven by its docking pattern and docking score compared to that of the known MOA-B inhibitor, safinamide which explains hesperidin's anti-Parkinson's activity. Finally, we concluded that *Citrus trifoliata* L. fruits can be eaten as neuroprotective and anti-Parkinson's food.

Conflicts of Interest

There are no conflicts of interest.

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دراسة التأثير المثبط لانزيم الماو ب لكل من ثمار السترس ترايفولياتا ونظام النانو لتوصيل الدواء والهسبيريدين المفصول عن طريق فحص التأثير على الانزيم ودراسة الالتحام الجزيئي معه

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تعد الفلافونويدات من المواد التي تؤثر وافي للجهاز العصبي. تعيين كمية للهسبيريدين والذي يعتبر الفلافونويد الاكبر كمية في هذه الثمار بواسطة كروماتوجرافيا السائل. تم عمل منحنى قياسي للهسبيريدين واتضح ان كمية الهسبيريدين في الثمار تصل الى ٣٠ مجم/جم. تم فصل مادة الهسبيريدين والتعرف عليها بواسطة طيف الرنين المغناطيسي للبروتون والكربون. تم عمل نظام نانو لتوصيل مستخلص الثمار لزيادة التأثير البيولوجي للمستخلص. التأثير المثبط لانزيم الماو ب جميعهم شديد الفعالية بالمقارنة بالسيلجلين ووجد ان نظام النانو اعلاهم ثم يليه المستخلص ثم الهسبيريدين. تم دراسة الالتحام الجزيئي بين الهسبيريدين وانزيم الماو ب ووجد ان الهسبيريدين يتفاعل معه بقوة مما يؤكد تأثيره المثبط على الانزيم.