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Fabrication and Characterization of Persea Gratissima Oil Loaded Chitosan Nanoparticles and Investigation of Its Neuroprotective Effects

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Abstract

Persea gratissima known as avocado is a valuable plant. *P. gratissima* (PgO) oil is used in traditional medicine to treat several health problems because of its numerous biological properties. *P. gratissima* is a source of phytosterols and has effects as antimicrobial, anti-inflammatory, anti-oxidant and neuroprotective activities for *in vitro* and *in vivo* models. The aim of this study is to synthesize and characterize the PgO loaded chitosan nanoparticles and investigate the neuroprotective effects *in vitro*. According to neuroprotective effects, we prepared nanocapsulation of *P. gratissima* with chitosan using by ionic gelation method. Mitochondrial activity of *P. gratissima*, chitosan nanoparticles and PgO loaded chitosan nanoparticles were investigated by XTT method on SH-SY5Y and L929 cell lines comparatively. Obtained results showed that PgO loaded chitosan nanoparticles have a proliferative effect for SH-SY5Y cell line as a neuroprotective agent and no side effect for both SH-SY5Y and L929 cell lines. In conclusion PgO loaded chitosan nanoparticles are promising for neural regeneration and candidate for further in vitro and *in vivo* evaluation as a potential neurodegenerative disease drug formula.

1. Introduction

Persea sp. are in *Lauraceae* family which are evergreen trees, distributed in tropical and subtropical regions around the world. *P. gratissima* is a valuable kind of genus, and its fruit known as avocado has numerous biological activities such as antimicrobial [1-4] and anti-oxidant properties [5, 6]. The biological activities of *P. gratissima* are due to the existence of various classes of natural products mentioned in Table 1 [7]. It has a high potential for phytochemicals such as phytosterols and their analogues [8]. *P. gratissima* and its derivatives have been used by many communities for local medicine to treat several health problems [9] and many investigations highlight the effectiveness of this fruit as an antifungal [1], antibacterial [2, 3], antiviral [4], analgesic, anti-inflammatory, anti-haemolytic, hepatoprotective [10], and especially anti-oxidant activities [11]. Anti-oxidant properties of plants are due to their high potential for phytochemicals such as phytosterols and their analogues [8]. It is known for several decades that chemicals obtained from plants, such as vegetables, herbs and fruits, are known to treat potential in several traditional local medicines [12, 13]. Another potential to treat is PgO and it is declared as a source of phytosterols (analogues of cholesterol and β-sitosterole) [14] and these compounds have been associated with different pharmacological activities such as anti-inflammatory, antioxidant, and anti-apoptotic [10, 15]. The β-sitosterole in *P. gratissima* has a special effect on neuroprotection, contributing to the treatment of neurodegenerative diseases such as Alzheimer disease. In relation to neuroprotection, it works as a

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chemoprotection agent [13, 15-17]. Also, the promise of this compound for the treatment of various diseases defines it as one of the notable components of the future, making it the major drug of the future [15].

Table 1. Bioactive compounds present in PgO [5].

Class of Natural Product	Name of the Composition
Temperature	Linoleic acid
	Oleic acid
	Palmitic acid
	Palmitoleic acid
Phytosterols	Compesterol
	β-sitosterol
	Stigmasterol
	Δ^5-avenasterol
	Δ^7-avenasterol
Tocopherols	α-tocopherol
	Y-tocopherol
	Vitamin E

While determining the effects of active substances on diseases, on the other hand, scientists has started to be applied an advantageous method for herbal pharmaceuticals called micro/nanocapsulation. This method is a process of enclosing a substance as the core material inside a membrane to protect against environmental factors. Encapsulation is a widely used technique in many fields such as textile and food industries, medical areas for cell immobilisation, cell transplantation, fermentation, drug delivery and many other fields [18]. The structure of the nanoparticles is composed of the core and the wall. The core is the active part and the covering material of capsules is also known as membrane, carrier or shell [19]. There are several synthetic and natural polymers and also organic materials used as wall in micro/nanoparticles. The selection of wall material is very important and related to the physical properties of the core material, correspondingly a hydrophilic polymer wall is necessary for a lipophilic core and an aqueous core needs an insoluble polymer wall [18]. A wide range of polymers and various polymer combinations are suitable for the encapsulation of nanostructures. Chitosan is commonly used as a wall material polymer because it does not show toxicity. Further advantages of chitosan are its biocompatibility, due to its physical and chemical properties, biodegradability, and stability in micro- and nano-sized particles, and under high-temperature conditions. For these reasons, Cht-NPs have great importance in controlled drug delivery systems [20].

In the literature, there are several encapsulation methods for several herbal oils (electrospraying for Peppermint oil [21], phase separation for Rosemary oil [22], emulsify-solvent diffusion for Lavender oil and spray-drying for Chia essential oil [23] are documented. Nevertheless, there is restricted knowledge about PgO encapsulation. On the other hand, there is also less study about PgO-Cht-NPs' *in vitro* investigation that comes into prominence with its antioxidant, anti-apoptotic [13], and improvement of mitochondrial function [24] properties, especially for neuroprotection.

Therefore, the aim of this study is the preparation and characterization of nanoformulations of PgO, and the investigation of neuroprotective effects of these nanoparticles.

In this study, PgO-Cht-NPs were applied and cell proliferation effects of PgO, blank Cht-NPs and PgO-Cht-NPs, were searched on SH-SY5Y neural cell line for neuroprotective observation and L929 fibroblast cell line for control, individually and comparatively, for the first time in this study.

2. Materials and Methods

2.1. Preparation of Chemicals

Chitosan (Low Molecular Weight; 50.000-190.000 kDa) and polyvinyl alcohol (PVA) (Mw = 31.000-50.000, 87-89%) were purchased from Sigma-Aldrich USA (2.5% w: v CAS# 9012-76-4). 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) was obtained from Merck (Darmstadt, Germany). All the chemicals and solvents were of analytical grade and inspired by the study of Cakir-Koc et. al (2018) [25]. *P. gratissima* oil (*Persea gratissima* - CAS# 8024-32-6, Talya herbal, Antalya, Turkey) was purchased from a pharmacy in Istanbul. Product certificate of analysis is obtained from the company. Ultrapure water from Millipore Milli-Q Gradient System was used to prepare the solutions.

2.1.1. Synthesis of Cht-NPs

Cht-NPs were prepared using a modified version of the ionic gelation method [26]. Chitosan was added to 1% (v: v) acetic acid solution and mixed on a magnetic stirrer until a homogeneous solution was obtained. The pH of the solution was adjusted to 4.6–4.8. Next, Tween 80 (CAS# 9005-65-6, Sigma-Aldrich) mixture (1:1, v: v) was combined with a solution of chitosan. In order to obtain a homogeneous solution, the mixture was stirred at room temperature for 2 h. Next, sodium tripolyphosphate (TPP, CAS# 7758-29-4, Sigma-Aldrich) was dissolved in distilled water (0.05%, w: v) and then added drop-wise.

The solution was agitated on a magnetic stirrer for 45 min, and finally, ultra-sonication was applied. The samples were filtered with a 22µm pore-sized regenerated cellulose membrane before characterization.

2.1.2. Synthesis of PgO-Cht-NPs

PgO-Cht-NPs were prepared as mentioned above. And next, PgO and Tween 80 mixture (1:1 v: v) were combined with a solution of chitosan. In order to obtain a homogeneous solution, the mixture was stirred at room temperature for 2 h. Next, TPP was dissolved in distilled water (0.05%, w: v) and then added drop-wise. The solution was agitated on a magnetic stirrer for 45 min, and finally, ultra-sonication was applied. The samples were filtered with a 22 µm pore-sized regenerated cellulose membrane before characterization each sample was filtered through a 0.2 µm regenerated cellulose membrane (Sartorius, Germany) to remove any impurities from the solutions.

2.2. Characterization of Nanoparticles

2.2.1. Dynamic Light Scattering Analysis

In order to analyze of average particle size, polydispersity index, and zeta potential of PgO-Cht-NPs, a Zeta-sizer Nano ZS (Malvern Instruments, UK) instrument equipped with a 4.0 mV He-Ne laser (633 nm) was used. Every electrophoretic light scattering measurement was performed at 25°C and each sample was prepared with phosphate-buffered saline (PBS) before filtering with 0.45 µm Polyethersulfone (PES) membrane.

2.2.2. Preparation of Standard Curve of PgO

The standard curve of PgO was prepared using a UV-Vis spectrometer (Shimadzu, Japan) at 230 nm. The encapsulation and loading efficiency of PgO-Cht-NPs were determined by using the standard curve.

2.2.3. Encapsulation Efficiency and Loading Capacity of the PgO-Cht-NPs

To determine the efficiency of the encapsulation, the free PgO concentration in the supernatant taken after centrifugation was determined by using the calibration curve. After the amount of free PgO was found, the encapsulation efficiency was calculated using the following formula Eq. (1).

$$\text{Encapsulation Efficiency (\%)} = \frac{(\text{Total drug amount} - \text{Free drug amount})}{Total\ drug\ amount}\ x\ 100 \quad (1)$$

Loading capacity was calculated by following the formula Eq. (2).

$$Loading\ Capacity = \frac{\text{Encapsulated PgO}}{Total\ Cht-NPs\ weight} \quad (2)$$

2.2.4. In Vitro Release Test

1 mL of nanoparticles were placed in dialysis capsules. Once these dialysis capsules were disposed into beakers containing PBS (pH: 7.4), the beakers were placed in a 37ºC water bath under gentle agitation. A time-dependent release study was carried out at time intervals of 0; 0.5; 1; 2; 3; 6; 7; 9; 10; 24; 72; 96; 120; 144; 168; 192 and 216 h. At proper time intervals, the intake amounts of PgO-Cht-NPs were first extracted in PBS and then quantified spectrophotometric at 230 nm. These amounts of released PgO values were obtained from the calibration curve. The release of PgO was determined as per the following equation (3).

$$Release\ (\%) = \frac{Released\ PgO}{Total\ PgO} x100 \quad (3)$$

2.3. Cell Culture Experiments

2.3.1. SH-SY5Y and L929 Cell Lines' Culture

SH-SY5Y is a twice-subcloned cell line derived from the SK-N-SH neuroblastoma cell line. L929 is a mouse-derived dermal fibroblast cell line. Both of these cell lines were individually thawed and transferred into a 10% foetal bovine serum (FBS) containing DMEM/F-12 (supplemented with 0,05% penicillin-streptomycin) maintaining media. Cells were cultured in a humidified atmosphere at 37ºC and 5% CO_2.

2.3.2. Cytotoxicity Experiments

Both cell lines that are commercially available from ATTC, was used for the cytotoxicity studies. Fibroblast cultures are commonly used to understand the dermal response against new products. Cells were cultured as mentioned above. The plates were incubated at 37 °C for 3 days in a 5% CO_2 incubator until 80% confluence was attained. A trypsinization process was applied to the cells, and the detached cells were obtained by centrifugation. Then, both cell lines were seeded (10,000 cells/well) in 96-well flat-bottom microplates with 100 µL of the medium. The plates were incubated at 37 °C for 24 h, for attachment

to the good bottoms. Various concentrations (1, 5, 10, 20, 50 and 100 µg/mL) of the Cht-NPs and PgO-Cht-NPs were added, respectively, and incubated for another 24 h. The media containing chitosan or nanoparticles were then removed, and 100 µL of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, Merck, Darmstadt, Germany) solution in the fresh medium was added to the wells at 0,5 mg/mL (with 7,5 µg/mL phenazine methosulfate). The cells were incubated at 37 °C for 3 h. Afterward, the optical density was measured at 450 nm with a multi-plate reader (Thermo Labsystems Multiscan Ascent 354 Microplate Photometer). Finally, the percentage of the cell viability was calculated by the following equation.

$$Viability\ (\%) = \frac{Absorbance\ of\ experimental\ group}{Absorbance\ of\ control\ group}\ x\ 100 \qquad (4)$$

2.4. Statistical Analysis

Experiments were triplicated. GraphPad Prism 5 program was used. Homogeneity of variants was determined by ANOVA one-way. Values p<0.005 were meaningful statistically.

3. Results and Discussion

In this study, PgO-Cht-NPs were prepared, and the proliferative effects of PgO-Cht-NPs on SH-SY5Y and L929 were comparatively determined. SH-SY5Y is a neuronal cell line. L929 is a fibroblast cell line that is used to represent a comparison cell for the purpose of the study.

For the chemical steps of the study, an ionic gelation technique was used to prepare the nanoparticles. Briefly, this method is based on an ionic gelation interaction, formed by the repulsive force between positively-charged chitosan and negatively-charged TPP. This procedure was undertaken to synthesize empty Cht-NPs and PgO-Cht-NPs. These two types of nanoparticles were examined and compared for their physicochemical properties, as well as their biological properties in cell culture.

3.1. Nanoparticle Characterization Results

3.1.1. Dynamic Light Scattering Analysis

The average size of the blank and PgO-Cht-NPs was determined using the DLS technique, with triplicate measurements (Table 2). Blank Cht-NPs has a size of 51,63 nm, a zeta potential of 10,9 nmV, and a PDI of 0,156, whereas PgO-Cht-NPs are 53,22 nm, with a zeta potential of 8,88 mV and a PDI of 0,211. This means that nanoparticles have a similar size and their diameters are formally called nanoparticles (Data not shown).

Zeta potentials of the nanoparticles are between +30 nm and -30 nm. Zeta potentials that are higher than +30 mV and lower than -30 mV mean instability for the chemical characteristics of the nanoparticles. As a result, this means that we obtained chemically stable nanoparticles.

Table 2. Zeta-average diameter results of nanoparticles.

Nanoparticles	Size (nm)	PDI	Zeta potential (mV)
Cht-NPs	51,63	0,156	10,9
PgO-Cht-NPs	53,22	0,211	8,88

3.1.2. Determination of Encapsulation Efficiency, Loading Efficiency and Release Profile

The standard curve obtained using various concentrations of PgO (Figure 1) was used to determine the encapsulation efficiency, loading capacity and release profile of the PgO-Cht-NPs. Equation 1 was used for encapsulation efficiency and it was found that encapsulation efficiency was 86%. When the literature is examined, it is seen that these rates have lower percentages ((36.2% and 17.2%, respectively) than the results we obtained [27, 28].

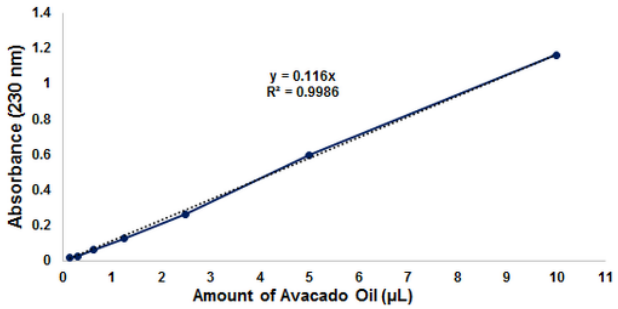

Figure 1. Calibration curve of PgO.

This ratio of encapsulation efficiency (86%) shows that encapsulation is successful. Equation 2 was used to calculate the loading efficiency of PgO-Cht-NPs. The data show that 0,625 µl of PgO is present in each 1 mg nanoparticle. It means the loading capacity was 62.5%, whereas in other studies these rates were much lower (19.2% and 3.38%, respectively) [27, 28]. When we look at the release profile, it is seen that PgO is released in a controlled and slow way from Cht-NPs. The *in vitro* release profile of PgO-Cht-NPs is given in Figure 2. *In*

vitro release profile determination was performed using PBS (pH: 7,4) solution. The first ten hours' releasing value was determined as 3,16 µl, and this rate was determined as 9,925 µl after 216 hours.

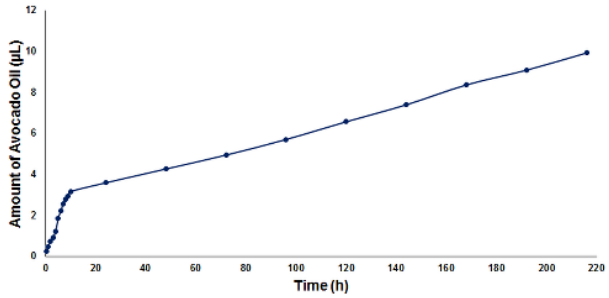

Figure 2. In vitro release profile of PgO-Cht-NPs.

3.1.3. Cell Culture Results

After the characterization experiments of PgO, PgO-Cht-NPs, and CNs, we applied six different concentrations (1, 5, 10, 20, 50, and 100 µg/mL) in cell culture experiments. For this purpose, thawed cells were adapted into the cell culture conditions. Both cells lines were grown in 37 ºC and 5% humidified incubator for 4-7 days (Figure 3). After they reached 80% confluence cells were used for the toxicity experiments.

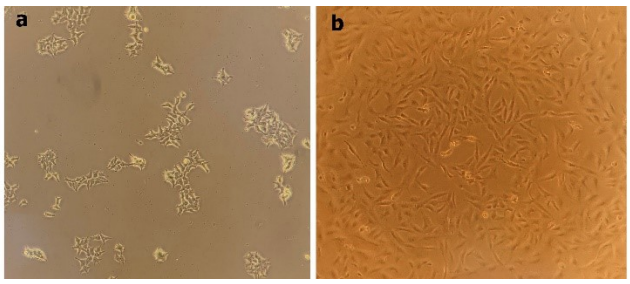

Figure 3. Observation of SH-SY5Y (a) and L929 (b) cell lines culture at inverted microscope (20X).

According to the results non-capsulated PgO concentrations, showed a significant toxic effect on the SH-S5Y5 cell lines. Although cell viability started to decrease from the lowest concentrations to higher for SH-S5Y5, while L929 cell line survived at a stable level. The blank CNs presented no significant decrease in cell viability for both SH-S5Y5 and L929 cell lines at the investigated concentrations. Whatever, it is known that chitosan is a biocompatible polymer and does not provoke a toxic effect on cells [37]. And our cell culture results also support this literature data. Finally, PgO-Cht-NPs showed a significant induction for SH-S5Y5 cell line approximately 77% (min 54%, max 90%) while its lower concentrations (1 and 5 µg/mL) induced L929 cell line proliferation up to 27%, and higher concentrations (10, 20, and 50 µg/mL) do not affect but 100 µg/mL showed a decrease in cell proliferation (24%). PgO is used in many different cosmetical products such as well skin and hair care products. Vitamins A, D, E, lecithin, Omega 9 fatty and phytosterols acids promote healing, skin regeneration, collagen augmentation and protection from the aging effects of UV light and pollution [29]. In addition, cytotoxicity [10, 30], nitric oxide and superoxide generation inhibition [10, 31], anti-cardiovascular disease [32], acetyl-CoA carboxylase inhibition [33], skin lysyl oxidase inhibition [34], and liver injury suppressing [35] effects have also been demonstrated, and suggest *P. gratissima* fruits are a healthy natural product to consume [6].

3.1.4. Cytotoxicity Results

Effects of different concentrations of PgO, blank Cht-NPs and PgO-Cht-NPs were performed using with XTT assay. Initial results showed that PgO has an inhibitory effect on SH-SY5Y for all concentrations. On the other hand, PgO did not decrease the cell proliferation rate for L929 (Figure 4).

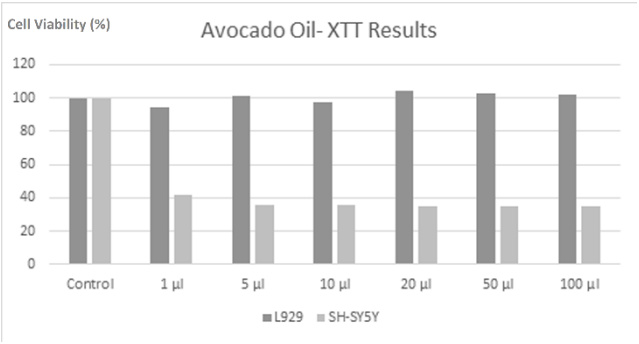

Figure 4. XTT results for PgO on L929 and SH-SY5Y cell lines.

Cht-NPs effect on both cell lines was measured. As known in literature [20], Cht-NPs showed no inhibitory or activator effects on cell lines (Figure 5).

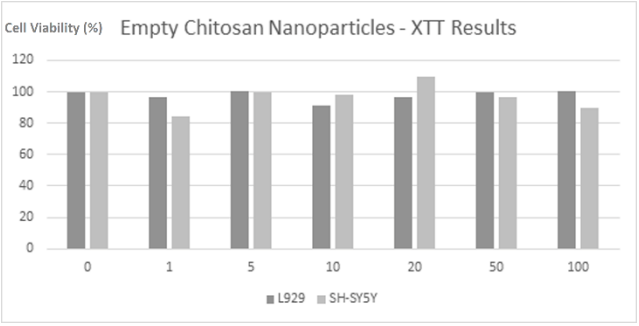

Figure 5. XTT results for Cht-NPs on L929 and SH-SY5Y cell lines.

While PgO decreased cell proliferation and blank Cht-NPs did not affect cell proliferation of SH-SY5Y, PgO-Cht-NPs showed a higher inductive effect (appr. 90%). Compared to L929, as seen in Figure 6, all concentrations of PgO-Cht-NPs were seen to have an increased cell viability. Although there is a decrease in cell viability at increasing concentrations (1, 5, 10, 20, 50, and 100%), it has been observed that the resistance of SH-SY5Y cells to this is significantly high between 54 – 90%.

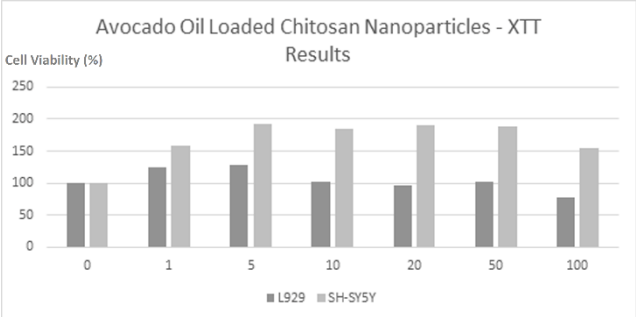

Figure 6. XTT results for PgO-Cht-NPs on L929 and SH-SY5Y cell lines.

When we search PgO's efficiency on various kinds of cell lines related to diseases, the neurodegenerative effect of β- sitosterol came across as an alternative model for neuronal diseases probably with its rich amount of phytosterols and its analogs especially β-sitosterol. As a hypothesis mitochondrial dysfunction has been declared to be a key factor in the progression of hyperglycaemia-mediated neuronal damage [24] and a study reported that β-SITO is a tubulin-binding unit and its interaction with tubulin involves several interesting features [13]. Additionally, the maintenance of oxidative phosphorylation capacity is extremely important in the central nerve system (CNS) since about 90% of the energy required for the healthy function of neurons is provided by mitochondria. Thus, mitochondrial dysfunction may cause loss of neuronal metabolic control and, consequently, neurodegeneration. This approach is supported by data demonstrating mitochondrial function decline with aging and in age-related diseases [24]. Thus, we cultured SH-SY5Y cell line and investigated the mitochondrial function degree (related to cell proliferation rate) by XTT method for PgO, CNs, and PgO-Cht-NPs compared to the L929 cell line.

As we mentioned above, this study is an initial investigation to observe the neuroprotective effects of PgO-Cht-NPs. Our results showed similar results for Cht-NPs on cell proliferation declared in literature [20, 36, 37] and a new perspective (approach, modality) was offered to use as a protective formula for neurodegenerative diseases.

4. Conclusions

A major challenge associated with several diseases such as cancerogenic and neurodegenerative diseases is the side effects or insufficient drug efficacy [38]. Despite the many benefits of essential herbal oils, they cause some biochemical and physicochemical negations such as high volatility and uncontrollable dosage. Profusion is also a disadvantage that is thought to be easily solved through the encapsulation method. Regarding the advantages of encapsulation techniques, it is expected that the practice of micro/nanoparticles containing essential herbal oils will widely increase in the next years [18]. Our results showed that PgO-Cht-NPs have a proliferative effect for cell proliferation for SH-SY5Y cell line as a neuroprotective agent and no side effect for the fibroblast cell line. In this study, a new perspective was offered to use as a protective formula for neurodegenerative diseases. Given the health benefits of PgO that has important phytosterols such as β-sitosterol, including neuroprotection [17], are proposed as a suitable candidate for further *in vitro* and *in vivo* evaluation as a potential neurodegenerative disease drug formula.

Declaration of Ethical Standards

The authors of this article declare that the materials and methods used in this study do not require ethical committee permission and/or legal-special permission.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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