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Mucuna cinerea Seeds: Levodopa Extraction Using Deep Eutectic Solvent and Its Mammalian Cell Activity

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A new class of deep eutectic solvents (DES) was used in the extraction of levodopa (*L*-dopa) present in *Mucuna cinerea*, which has been termed "green" or "designer" solvents, with notable productive and economic/environmental benefits. The seeds of *Mucuna cinerea* were prepared and submitted to extraction with heating and agitation in a solvent mixture of DES with citric acid, urea, or glycerol. The extracts were analyzed by high performance liquid chromatography (HPLC) which confirmed the efficiency of DES in extracting *L*-dopa. These extractions were carried out to determine the *L*-dopa extracts in DES with regard to the induction of cellular response activity, demonstrating the synergy between *L*-dopa and DES. The DES-glycerol extract exhibited a higher proliferative activity than commercial *L*-dopa after 24 h of treatment. The human glandular kallikrein-1 gene (hGK3) demonstrated higher expression in the treatment with the DES-urea, while the G protein-coupled receptor kinase-6 gene (GRK6) showed higher expression for all extracts compared to commercial *L*-dopa, with a higher value obtained for DES-glycerol, followed by DES-urea extract. The DES extraction method is therefore promising and unprecedented, confirming its possible use in the replacement of expensive solvents, supporting the use of ecologically viable DES in the extractions of other plant species.

Keywords: HPLC, green solvent, bioextraction, levodopa, Parkinson's disease

Introduction

Mucuna seeds biosynthesize levodopa (*L*-dopa), which is a precursor of dopamine, shown to assist in the treatment of Parkinson's disease,[1-4] a progressive neurodegenerative disease, related to brain abnormalities, whose symptoms are: tremor at rest, rigidity, gait, and change in posture. This disorder is partially defined by decreased dopamine production.[1] However, dopamine cannot be effectively used to treat the disease as it is not able to cross the blood-brain barrier.[5] Unlike dopamine, *L*-dopa can cross this barrier reaching the central nervous system, where it is converted into dopamine.[6,7]

Parkinson's disease affects 1-2% of the population over 50 years of age and is believed to be caused by environmental and genetic factors.[8-10] Catalepsy is the most common and characteristic symptom of the disease and it is a central nervous system disorder that causes muscle rigidity, immobile posture, and decreased sensitivity to pain.[10] Other symptoms of the disease are tremors at rest, gait disturbances (a debilitating factor, as it predisposes the patient to falls), postural instability, and reduction in step length.[11] The main treatment for Parkinson's disease involves the administration of synthetic *L*-dopa, which provides fast and effective control of motor system symptoms for virtually all patients.[12] When administered orally, *L*-dopa undergoes rapid metabolism by the enzyme *L*-aromatic amino acid decarboxylase (AADC) and by catechol-*O*-methyltransferase (COMT), which contributes to its crossing of the blood-brain barrier, and may cause dyskinesia (drug-induced involuntary muscle movement) when using synthetic *L*-dopa.[9]

Mucuna seeds have a high concentration, ranging from 4 to 6%, of *L*-dopa, a direct precursor of the neurotransmitter dopamine.[13] Traditional extraction methods of plant-derived *L*-dopa involve the use of conventional solvents, which for the most part are volatile, toxic, and require

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a long extraction process with an additional treatment time, resulting in the need for large volumes of solvents for successive extractions.[14] The alcoholic extract of *L*-dopa with propanol showed significant neuroprotective activity.[8,15] The acidification of the solution to pH 3 permits the extraction of *L*-dopa present in *Mucuna* seeds, with particle size close to 1 mm in approximately 8 h.[16] In all *L*-dopa quantification studies reported in the literature,[17] the extraction procedures were long, exhaustive, and performed in several steps.

A new category of biodegradable solvents, termed deep eutectic solvents (DES), was discovered in 2002 and is considered an eco-friendly alternative to conventional solvents.[18] DES are composed by an organic salt (such as choline chloride or choline acetate) and a compound capable of hydrogen interaction (such as amides, amines, alcohols, and carboxylic acids). Mixing these compounds causes a decrease in the entropy associated with phase transitions and, consequently, a significant reduction in the melting temperature of the mixture, when compared to the melting temperatures of the two separate constituents. Due to the abrupt drop in the melting temperature of the mixture, it is possible to obtain DES in a liquid state below room temperature.[19]

DES offer several important benefits, such as simple preparation, low cost, low toxicity, and high biodegradability.[18,20,21] In addition, within biological systems, they have storage and protective functions for metabolites and, in some organisms, they also exhibit a protective effect for cells in extreme conditions, such as in lack of water or low temperatures. Therefore, DES have aroused great interest in the scientific community and are promising solvents contributing to new horizons in bioextraction.

Although *L*-dopa treatment reduces tremor, rigidity and other symptoms, its prolonged use contributes to the development of fluctuations in motor response and involuntary movements known as *L*-dopa-induced dyskinesia (LID).[22,23] G protein-coupled receptor kinase (GRK) genes play an important role in the signaling cascade of pathways activated by *L*-dopa, with decreased expression of the G protein-coupled receptor kinase-6 gene (GRK6), and GRK3 isoforms associated to worsening of LID. Therefore, these genes have been studied as therapeutic targets in the treatment of Parkinson's disease.[24,25] Thus, the purpose of this work was to prepare some deep eutectic solvents based on choline chloride and compare their potential for extracting *L*-dopa present in *Mucuna cinerea* seeds with traditional and non-toxic solvents in order to optimize yield and to test the cell activity, including the expression of biomarker genes associated with cell signaling pathways triggered by *L*-dopa.

Experimental

Materials and chemicals

All chemicals and reagents were analytical grade used without further purification and purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All solutions were prepared using deionized water. All glassware was soaked in 10% HNO_3 solution for at least 24 h, and then ultrasonic cleaned. After repeated moistening with deionized water, they were dried in a constant temperature drying oven. *Mucuna cinerea* seeds were purchased from BRseeds (São Paulo, Brazil). *M. cinerea* was registered in SisGen Number A3EDB3D.

DES preparation

The deep eutectic solvents were prepared in the following mixtures: choline chloride with citric acid, choline chloride with urea, and choline chloride with glycerol. Initially, DES were prepared with choline chloride, anhydrous citric acid and water, in proportions of 2:1:6.55, respectively.[26] The mass needed to prepare an aliquot of 15 g was calculated based on the molar masses of each component 7.1070 g/4.8897 g/3.0032 g. The three components were weighed into a beaker. Soon after, the beaker containing all the reagents was transferred to a stirring plate and heated in glycerin bath at 80 °C for 2 h. After this time, the heating was turned off and stirring continued until the next day, so the mixture cooled slowly and homogeneously. DES choline chloride/urea/water, and choline chloride/glycerol/water were prepared using the same conditions and proportions.[19,27] All the prepared DES were allowed to cool to room temperature and dried in a vacuum oven at 50 °C for 24 h. The solvents were stored in sealed laboratory vials and kept in a desiccator.

L-Dopa extraction and quantification

In these experiments, 1 g of *Mucuna cinerea* seeds were ground to the granulometry of 1.180 nm, and 2 mL of deionized water and 1 mL of DES were used. The DES used for extraction were DES-citric acid, DES-glycerol, and DES-urea. Extractions were performed in closed tubes at the ultrasound Elmasonic (Elma Sonic, San Jose, USA) with 100 W of power and 37 Hz of frequency, at 65 ºC, for 50 min. The total volume of solvents was 3 mL, with 2 mL of water and 1 mL of DES. The extractions were performed in triplicates.

Proceeding the extraction time, an aliquot of 20 μL of the solution was removed from each vial, and deionized

water was added, totaling 5 mL of sample to be analyzed by high-performance liquid chromatography (HPLC) to detect peak areas related to the *L*-dopa concentration in each aliquot. The samples obtained from the extractions were identify by HPLC, and the standard analytical curve of *L*-dopa was determined. A Shimadzu LC-10AD system (Shimadzu, Tokyo, Japan) consisting of detector UV SPD-10AV was used. A C18 column (25 cm × 4.6 mm, 5 μm particle size) Supelco (Sigma-Aldrich, St. Louis, Missouri, USA) was used as a stationary phase. A solution of deionized water and acetic acid (99:1) was used as the mobile phase. The wavelength used in the equipment was 284 nm, with a flow of 0.7 mL min^{-1}, pressure of 114 kgf cm^{-2} at 27 ºC. 20 μL of extract were injected. After the extraction and the analysis in HPLC, all the solutions were lyophilized and the extracts were prepared for the biological tests.

Efficiency of *L*-dopa in extract for the treatment of Parkinson's disease

Initially, a commercial *L*-dopa solution 40 μg μL^{-1} in saline phosphate buffer (PBS) pH 7.5 containing 2.5% dimethylsulfoxide (DMSO) was prepared. *L*-dopa extracts in DES-urea, DES-glycerol, and DES-citric acid were diluted in PBS to obtain 40 μg mL^{-1} of *L*-dopa and 2.5% of DMSO. For cytotoxicity testing, 3 different concentrations of a serial dilution in a factor of 5 were used, resulting in 40, 8, and 1.6 μg mL^{-1} for all experimental groups.

Study of the cytotoxicity of the tested compounds

The toxicity of the compounds and DMSO was evaluated in HEK 293 (human embryonic kidney cells) cultivated using Dulbecco's Modified Eagle Medium (DMEM), with the addition of 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% *L*-glutamine for cell maintenance, and throughout the process, the cells were incubated at 37 °C and 5% CO_2.

At a confluence between 40-70% (1×10^5 cells *per* well), the procedure of counting and observing their viability was performed by the technique of exclusion of cellular viability using Trypan blue with the aid of a Neubauer chamber.[28]

The cells (1×10^4 cells *per* well) were cultivated in each well of a 96-well plate, as described in the literature[29,30] with supplementation of 2.5% of FBS. After a period of 16 h, for the cells to adhere to the surface of the plate, the compounds were added at concentrations of 40, 8, and 1.6 μg mL^{-1}.[29,30]

The quantification of the cells that remained alive after the treatments was performed through metabolic viability analysis using the colorimetric assay of the tetrazolium salt (MTT).[31] After the treatment time, the culture medium was replaced by 10 μL of MTT solution (5.0 mg mL^{-1}) diluted in PBS.

Following 4 h of reaction, the supernatant was removed and the metabolic product of MTT was homogenized in 100 μL of dimethylsulfoxide P.A. (DMSO). The intensity of the color of formazan (a byproduct of MTT mitochondrial metabolism) was evaluated by optical density at 570 nm in an ELISA reader (SpectraMax M5, Molecular Devices, San Jose, USA).

Wells that did not receive any treatment were used as negative control while wells treated with DMSO at 2.5% were used as positive control. All concentrations were tested in quadruplicates.

Data analysis was performed with the help of the Graphpad Prism 6.0 program for Windows.[32] The data were normalized in percentage values using the mean absorbance of the negative control of their respective treatment time as a reference of 100% of proliferation. At the end of this stage, the values of the groups were submitted to the one-tailed analysis of variance (ANOVA) test followed by a *T*-test of multiple paired comparations (*L*-dopa *versus* compounds), with shielding of the Sidak test for multiple comparations.

Total RNA extraction and cDNA synthesis

The HEK 293 cells were cultured in DMEM medium supplemented with 100 international unit mL^{-1} (100 i.u. mL^{-1}) of penicillin, 100 μg mL^{-1} of streptomycin, and 10% of inactivated FBS. The cell monolayer was grown in a plastic bottle kept in a cell incubator at 37 ºC with a concentration of 5% CO_2.

When the cell monolayer reached a semi-confluence stage, the cells were mechanically disaggregated by culture medium, centrifuged for 5 min at 2000 rpm and then resuspended in 1 mL of DMEM. With the aid of a hematological counter and Neubauer chamber, 2.8×10^5 cells were sowed in each well of a 6-well cell culture plate. 24 h later, the culture medium was replaced by DMEM supplemented with 2% FBS and with additional supplementation of the compounds to be tested at a concentration of 40 mg μL^{-1}. After 12 h of treatment, the culture medium was removed and then 1 mL of TRIzol (Invitrogen, Waltham, USA) was added and the samples were incubated at room temperature for 5 min.

Subsequently, 200 μL of chloroform (Sigma, C2432, Saint Louis, USA) was added, a step from which the protocol suggested by the manufacturer was adopted. All extraction steps were performed at 4 °C and under ribonuclease-free conditions.

The quality of total ribonucleic acid (RNA) was evaluated through the integrity of ribosomal RNA bands in 2% agarose gel.[33] The purity and concentration of the samples were determined by absorbance readings at 230, 260, and 280 nm in a Spectramax M5 (Molecular Devices, San Jose, USA) apparatus.

Total RNA was added with 1 U μL^{-1} of DNAse-RNAse Free (Promega, Madison, USA) to eliminate contaminant deoxyribonucleic acid (DNA), following manufacturer's specifications. The samples were amplified by conventional polymerase chain reaction (PCR) using primers for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to confirm the absence of cellular DNA contamination.

As a positive control, a sample containing DNA of the same cell line was used. The purity and concentration of the samples were determined via analysis in the SpectraDrop (Molecular Devices, San Jose, USA) apparatus and the total RNA samples were stored at −80 ºC. The synthesis of the first complement deoxyribonucleic acid (cDNA) tape was performed using the ImPromII Reverse Transcriptase System (Promega, Madison, USA) kit, according to the manufacturer's recommendations, from samples containing 1 µg of total RNA.

Quantification of transcripts by PCR in real time

To evaluate the alterations in genes associated with LID, we chose the real-time PCR technique, which has the advantage of being highly sensitive, thus allowing the quantification of small changes in gene expression.[34] Real-time PCR reactions were performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Waltham, USA) device.

Based on the values of CT (cycle threshold), which is the point at which fluorescence increases appreciably above the fluorescence of noise, the relative quantification was performed using the mean of each biological replicate calculated as (mean CT/$10^{[\beta\text{-actin}]}$) + (mean CT/$10^{[GAPDH]}$), and the β-actin and GAPDH genes were evaluated for the choice of the normalizer gene (endogenous control) for quantification of the target genes. Detection was performed using the SYBR Green Master Mix (Applied Biosystems, Waltham, USA) system.

The samples were analyzed in three biological replications, quantified in independent runs, and each sample was analyzed in duplicate in each reaction plate. Initially, tests were performed to determine the optimum concentration of primers and the efficiency of the reaction.

The components for each reaction were 1 µL of cDNA, 6.0 µL of 2X SYBR Green Master Mix (Applied Biosystem, Waltham, USA), and primers at concentrations of 200, 400, 600, and 1000 nM. The efficiency test was performed in serial dilutions of 50, 25, 12.5, 6.25, 3.125, and 1.56 ng μL^{-1} of cDNA.

To calculate the slope value, the formula Efficiency PCR = $(10^{(1/\text{-slope})} - 1) \times 100$ was used. Amplification reactions were performed under the following conditions: 10 min at 95 ºC; 40 cycles of 15 s at 94 ºC and 1 min at 60 ºC. The efficiency of real-time PCR was calculated through a graph where the Ct values were arranged on the ordinate axis and the values of each dilution in the abscissa axis (Table 1).

Results and Discussion

L-Dopa quantification

The *Mucuna cinerea* seed extract samples obtained under ultrasound with a heating of 65 °C for 50 min using

Table 1. Primer oligonucleotides used in real-time PCR experiments

Gene	NCBI sequence ID	Sequence 5'-3'	Tm	Expected base pairs / bp	Reference
hGRK3	NM_005160.4	GCGATTTGCACTACCACCTT	58.83	341	35
		AGGGCTGTGACCTCTCAGAA	60.18		
GRK6	NM_001004106.3	TAGCGAACACGGTGCTACTC	59.83	101	36
		GCTGATGTGAGGGAACTGGA	59.38		
hGRK6	NM_001004105.3	CGAGAACATCGTAGCGAACA	58.11	276	35
		TCCGGGGTCACTTCATACTC	58.52		
β-Actin	NM_001614.5	TCATGAAGTGTGACGTGGACATCCGC	64.74	284	37
		TTTAGAAGCATTTGCGGTGGACGATG	65.32		
GAPDH	NM_002046.7	ATGGGGAAGGTGAAGGTCG	59.97	108	37
		GGGGTCATTGATGGCAACAATA	58.97		

GNDF and hGNDF: gene associated with neuronal growth; GRK3, hGRK3, GRK6, hGRK6: genes associated with continuous treatment with *L*-dopa and that, for this reason, become hyperactive; β-actin and GAPDH: genes expressed in a constitutive manner and that will be as endogenous control. Tm: melting temperature; GRK6: G protein-coupled receptor kinase-6 gene; NCBI: National Center for Biotechnology Information; hGRK: human glandular kallikrein-1 gene.

different solvents were analyzed by HPLC. The *L*-dopa concentration in each extraction sample was obtained from the analytical curve ($y = 2067189x - 11703$, $R^2 = 0.9971$) showing the areas related to the peak of *L*-dopa. The analytical curve was obtained with 9 concentrations of *L*-dopa standard. Subsequentially, the values of the obtained areas were replaced in the linear equation of the analytical curve to obtain the *L*-dopa concentration. The results exhibited the highest concentration of *L*-dopa in the value of 0.0579 mg mL^{-1} with DES-glycerol, 0.0514 mg mL^{-1} with DES-citric acid, and 0.0346 mg mL^{-1} with DES-urea.

In addition to the solutions of the three DES tested as solvents, extractions using ethanol, water and a water/ethanol mixture (1:1) were also tested. Three different extraction times were used: 5, 10 and 20 min. Using the ultrasound extraction method, it was observed that extraction with DES-citric acid had the highest *L*-dopa content. Water also proved to be a good extractor solvent. The DES-glycerol showed little variation in the extractions with respect to time. DES-urea proved to be the least efficient solvent in terms of extraction, but it was more selective in the analyzes carried out for the extraction of *L*-dopa. The mixture of water with ethanol was slightly more efficient than pure ethanol. After observing and analyzing all the chromatograms obtained, it was concluded that the DES are the best choices for the extraction of *L*-dopa. In addition to the DES being more selective, according to the chromatogram analyses, they also proved to be more potent solvents in the extraction of *L*-dopa than traditional solvents. Thus, the three DES were chosen as the best solvents to continue the *L*-dopa extraction studies together with the ultrasound method. These parameters were used for the next studies carried out.

Proliferation analysis in HEK-293 cells

The assays were performed with *L*-dopa extracts in DES-urea, DES-citric acid, and DES-glycerol solvents regarding the *L*-dopa pattern in HEK-293 cells. Initially, the cytotoxicity and induction of cell proliferation produced by the extracts were evaluated (Figures 1, 2 and 3).

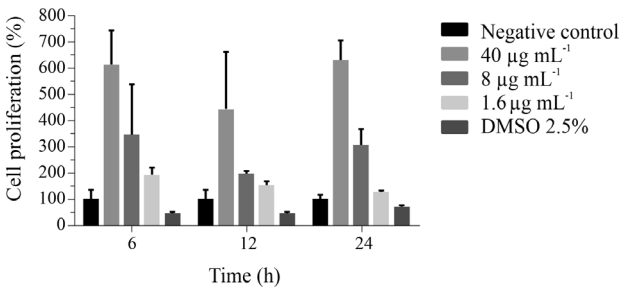

Figure 1. Proliferative activity of DES-urea at times 6, 12, and 24 h.

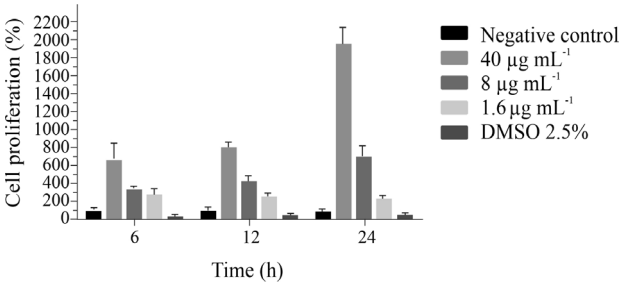

Figure 2. Proliferative activity of DES-glycerol at times 6, 12, and 24 h.

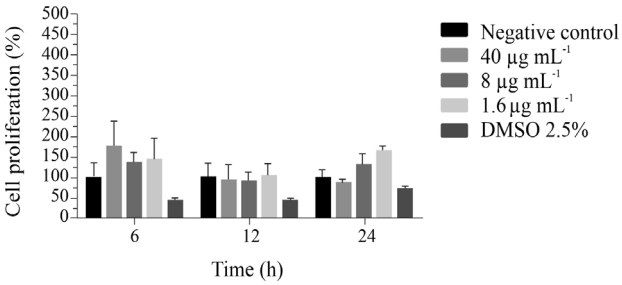

Figure 3. Proliferative activity of DES-citric acid at times 6, 12, and 24 h.

The highest concentration of DMSO used was 2.5%, which caused a reduction in cell proliferation in relation to the untreated control, with a statistically significant difference ($p < 0.05$). However, this concentration of DMSO did not interfere with the activity of the compounds, as observed in the graph expressing their activity in the presence of the highest concentration of DMSO.

The extracts produced with urea and glycerol showed higher proliferative activity than those produced with the citric acid extract. The cell proliferation induced by the extract was not dose dependent, but increased with prolongation of treatment time.

Since cell mortality was not observed, the proliferation effect at the highest concentration (40 µg mL^{-1}) was compared with the commercial *L*-dopa compound at different times (Figure 4). DES-glycerol extract showed higher activity than commercial *L*-dopa following a 24 h treatment.

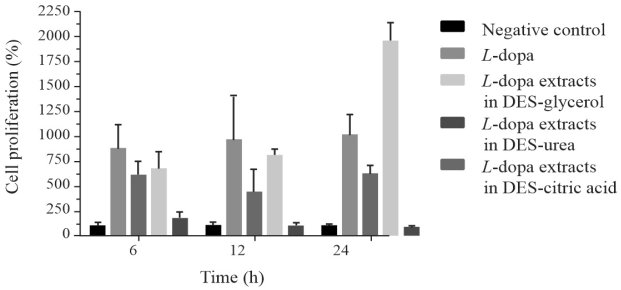

Figure 4. Activity of *L*-dopa extracts in DES at 6, 12, and 24 h.

Quantification of transcripts by PCR in real time

The results obtained from the relative expression of LID

via the HGRK3, GRK6, and HGRK6 pathway genes when cell culture was exposed to DMSO, *L*-dopa, and *L*-dopa extract in the DES at the concentration of 40 mg μL^{-1} are shown in Figure 5. Cell cultures not exposed to the compounds were used as controls. The human glandular kallikrein-1 gene (hGK3) showed higher expression in the treatment with the DES-urea, while the GRK6 gene showed high expression for all extracts compared to commercial *L*-dopa, with higher value obtained for DES-glycerol, followed by urea extract.

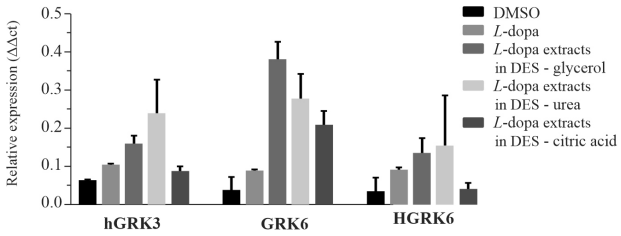

Figure 5. Relative expression of LID hGRK3, GRK6, and HGRK6 pathway genes when cell culture was exposed to DMSO, *L*-dopa, DES-glycerol, DES-urea, and DES-citric acid.

Satisfactory results of trials of this type have also been described when performed with a number of drugs such as danazol, benzoic acid, and itraconazole. The results exhibited an increase in solubility in DES from 5 to 22,000 times compared to water. In addition, the solubility test of some drugs in water, in the pure components of the extracts, and in the DES themselves in aqueous solution confirmed that the drugs were more soluble in the DES solution. These results imply that the high solubility of drugs is the result of the synergistic effect promoted by the eutectic solvent.[38]

The same synergism was observed in biochemical assays performed with *L*-dopa. The ability of DES to stabilize compositions based on RNA/DNA proteins and whole cells has been reported.[39] The same event was observed in biochemical assays performed with *L*-dopa extracts in DES solution. Thus, this study is a promising step for the biomolecular and pharmaceutical sector, because it contributes to new routes to synthesize medicines and can improve the bioavailability of drugs in biological fluids. The solubility and stability of other important drugs in DES have also been reported.[39,40]

According to the studies conducted, *Mucuna* extracts in alcohol medium did not exhibit any detectable levels of alkaloids harmful to human health. The authors state the safety of consumption of *Mucuna* extracts in the long term, and that Parkinson's disease symptoms are reduced, in addition to the decrease in side effects when compared with formulations containing the *L*-dopa pattern in its synthetic form.[13]

The present study also affirms the safety of *L*-dopa extracts for human cells, and in the extracts containing DES, the activity of *L*-dopa was significantly potentiated.

Conclusions

The results of the present study show that the obtention of *L*-dopa from *Mucuna cinerea* seeds using DES-citric acid, DES-urea, and DES-glycerol (considered green alternative solvents for extraction) present an easy method at a low cost with enhanced extraction of *L*-dopa. All samples were analyzed by HPLC and corroborated the efficiency of DES. Regarding biological activity, the DES-glycerol extract exhibited a higher proliferative activity than commercial *L*-dopa after 24 h of treatment. The hGK3 gene demonstrated higher expression in the treatment with the DES-urea, while the GRK6 gene showed higher expression for all extracts compared to commercial *L*-dopa, and a higher value was obtained for DES-glycerol, followed by urea extract. The DES extraction method is therefore promising and unprecedented, confirming its possible use in the replacement of expensive and polluting solvents, supporting the use of ecologically viable DES in the extractions of other plant species.

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Author Contributions

Antonio J. Demuner was responsible for resources, supervision, writing review and project administration; Daiane E. Blank, Bruna T. Silva and Matheus G. Campos for investigation and methodology and visualization; Tiago A. O. Mendes for the assay of Parkinson disease, writing review.

References

1. Margolesky, J.; Shpiner, D. S.; Moore, H.; Singer, C.; Jagid, J.; Luca, C. C.; *Parkinsonism Relat. Disord.* **2020**, *77*, 26. [Crossref]
2. Saranya, G.; Jiby, M. V.; Jayakumar, K. S.; Pillai, P.; Jayabaskaran, C.; *Phytochemistry* **2020**, *178*, 112467. [Crossref]

3. Tandon, B.; Anand, U.; Alex, B. K.; Kaur, P.; Nandy, S.; Shekhawat, M. S.; Sanyal, R.; Pandey, D. K.; Koshy, E. P.; Dey, A.; *Ind. Crops Prod*. **2021**, *169*, 113626. [Crossref]
4. Mali, P.; Kamble, P.; Aware, C.; Suryawanshi, S.; Jadhav, J.; *J. Appl. Res. Med. Aromat. Plants* **2023**, *34*, 100451. [Crossref]
5. Mischley, L. K.; Farahnik, J.; Mantay, L.; Punzi, J.; Szampruch, K.; Ferguson, T.; Fox, D. J.; *Nutrients* **2023**, *15*, 802. [Crossref]
6. Rahman, M. M.; Wang, X.; Islam, M. R.; Akash, S.; Supti, F. A.; Mitu, M. I.; Harun-Or-Rashid, M.; Aktar, M. N.; Khatun, K. M. S.; Jahan, F. I.; Singla, R. K.; Shen, B.; Rauf, A.; Sharma, R.; *Front. Pharmacol*. **2022**, *13*, 976385. [Crossref]
7. Rakesh, B.; Praveen, N.; *J. Appl. Biol. Biotechnol.* **2022**, *1*, 125. [Crossref]
8. Johnson, S. L.; Park, H. Y.; Silva, N. A.; Vattem, D. A.; Ma, H.; Seeram, N. P.; *Nutrients* **2018**, *10*, 1139. [Crossref]
9. Parrales-Macias, V.; Harfouche, A.; Ferrié, L.; Haik, S.; Michel, P. P.; Raisman-Vozari, R.; Figadere, B.; Bizat, N.; Maciuk, A.; *ACS Chem. Neurosci.* **2022**, *13*, 3303. [Crossref]
10. Sardjono, R. E.; Khoerunnisa, F.; Musthopa, I.; Khairunisa, D.; Suganda, P. A.; Rachmawati, R.; *J. Phys.: Conf. Ser.* **2018**, *299*, 012080. [Crossref]
11. Pelicioni, P. H. S.; Brodie, M. A.; Latt, M. D.; Menant, J. C.; Menz, H. B.; Fung, V. S. C.; Lord, S. R.; *Exp. Gerontol*. **2018**, *111*, 78. [Crossref]
12. Martins, H. F.; Pinto, D. P.; Nascimento, V. A.; Marques, M. A. S.; Amedoeira, F. C.; *Quim. Nova* **2013**, *36*, 171. [Crossref]
13. Cassani, E.; Cilia, R.; Laguna, J.; Barichella, M.; Contin, M.; Cereda, E.; Isaias, I. U.; Sparvoli, F.; Akpalu, A.; Budu, K. O.; Scarpa, M. T.; Pezzoli, G.; *J. Neurol. Sci.* **2016**, *365*, 175. [Crossref]
14. Halim, R.; Danquah, M. K.; Webley, P. A.; *Biotechnol. Adv.* **2012**, *30*, 709. [Crossref]
15. Misra, L.; Wagner, H.; *Indian J. Biochem. Biophys*. **2007**, *44*, 56. [Link] accessed in June 2023
16. Huisden, C. M.; Szabo, N. J.; Arriola, K. G.; Adesogan, A.; *Trop. Subtrop. Agroecosystems* **2019**, *22*, 45.
17. Mohapatra, S.; Ganguly, P.; Singh, R.; Katiyar, C. K.; *J. AOAC Int.* **2020**, *103*, 22. [Crossref]
18. Abbott, A. P.; Capper, G.; Davies, D. L.; Rasheed, R. K.; Tambyrajah, V.; *Chem. Comm*. **2003**, *20*, 70. [Crossref]
19. Smith, E. L.; Abbott, A. P.; Ryder, K. S.; *Chem. Rev*. **2014**, *114*, 11060. [Crossref]
20. Radosevic, K.; Bubalo, M. C.; Srcek, V. G.; Grgas, D.; Dragicevic, T. L.; Redovnikovic, I. R.; *Ecotoxicol. Environ. Saf.* **2014**, *112*, 46. [Crossref]
21. Zhang, Q.; Vigier, K. O.; Royer, S.; Jerome, F.; *Chem. Soc. Rev*. **2012**, *41*, 7108. [Crossref]
22. Guigoni, C.; Aubert, I.; Li, Q.; Gurevich, V.; Benovic, J.; Ferry, S.; Mach, U.; Stark, H.; Leriche, L.; Håkansson, K.; Bioulac, B. H.; Gross, C.E.; Sokoloff, P.; Fisone, G.; Gurevich, E.V.; Bloch, B.; Bezard, E.; *Parkinsonism Relat. Disord*. **2005**, *11*, S25. [Crossref]
23. Zahra, W.; Birla, H.; Singh, S. S.; Rathore, A. S.; Dilnashin, H.; Singh, R.; Keshri, P. K.; Singh, S.; Singh, S. P.; *Phytomedicine Plus* **2022**, *2*, 100343. [Crossref]
24. Ahmed, M. R.; Bychkov, E.; Li, L.; Gurevich, V. V.; Gurevich, E. V.; *Sci. Rep*. **2015**, *5*, 10920. [Crossref]
25. Ahmed, M. R.; Bychkov, E.; Gurevich, V. V.; Benovic, J. L.; Gurevich, E. V.; *J. Neurochem*. **2008**, *104*, 1622. [Crossref]
26. Zhao, B. Y.; Xu, P.; Yang, F. X.; Wu, H.; Zong, M. H.; Lou, W. Y.; *ACS Sustainable Chem. Eng*. **2015**, *3*, 2746. [Crossref]
27. Vieira, V.; Prieto, M. A.; Barros, L.; Coutinho, J. A. P.; Ferreira, I. C. F. R.; Ferreira, O.; *Ind. Crops Prod*. **2017**, *107*, 341. [Crossref]
28. Strober, W.; *Curr. Protoc. Immunol.* **2015**, *111*, A3.B.1. [Crossref]
29. Hansen, J.; Bross, P. A.; *Methods Mol. Biol.* **2010**, *648*, 303. [Crossref]
30. Al-Sheddi, E. S.; Farshori, N. N.; Al-Oqail, M. M.; Musarrat, J.; Al-Khedhairy, A. A.; Siddiqui, M. A.; *Pharm. Biol*. **2016**, *54*, 314. [Crossref]
31. Meerloo, J. V.; Kaspers, G. J.; Cloos, J.; *Methods Mol. Biol*. **2011**, *731*, 237. [Crossref]
32. *GraphPad Prism*, version 6.0; GraphPad Software, San Diego, California, USA, 2016.
33. Sambrook, J.; Russell, D. W.; *Molecular Cloning a Laboratory Manual 2*, 3rd ed.; Cold Spring Harbor Laboratory Press: New York, 2001.
34. Pfaffl, M. W.; *Nucleic Acids Res*. **2001**, *29*, e45. [Crossref]
35. Teli, T.; Markovic, D.; Levine, M. A.; Hillhouse, E. W.; Grammatopoulos, D. K.; *Molec. Endocrinol.* **2005**, *19*, 474. [Crossref]
36. Balabanian, K.; Levoye, A.; Klemm, L.; Lagane, B.; Hermine, O.; Harriague, J.; Baleux, F.; Arenzana-Seisdedos, F.; Bachelerie, F.; *J. Clinical Investigation* **2008**, *118*, 1074. [Crossref]
37. Woerner, B. M.; Luo, J.; Brown, K. R.; Jackson, E.; Dahiya, S. M.; Mischel, P.; Benovic, J. L.; Piwnica-Worms, D.; Rubin, J. B.; *Molec. Cancer Res.* **2012**, *10*, 156. [Crossref]
38. Mbous, Y. P.; Hayyan, M.; Hayyan, A.; Wong, W. F.; Hashim, M. A.; Looi, C. Y.; *Biotechnol. Adv*. **2017**, *35*, 105. [Crossref]
39. Goldsborough, A. S.; Bates, M. R.; *US Pat 20140295404*, **2014**.
40. Mokhtarpour, M.; Shekaari, H.; Moattar, M. T. Z.; Golcoun, S.; *J. Mol. Liq*. **2020**, *297*, 111799. [Crossref]

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