Extraction, Isolation and Characterization of Novel Active Constituents from a Medicinal Plant Source

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ABSTRACT

Purpose: There are limited therapeutics for Alzheimer's disease (AD), a progressive neurodegenerative disease. We aimed to isolate and characterize novel active ingredients from a medicinal plant to identify potential therapeutics for treating AD.

Methods: The plant material was extracted using methanol followed by ethyl acetate and hexane/ACN-H_2O washes to remove salts and lipids, respectively. The ACN-H_2O fraction was subjected to flash chromatography using the C18 reversed-phase column. This yielded 13 fractions which were subjected to antioxidant assay using the stable free radical 2, 2-diphenyl-β-picrylhydrazyl (DPPH) at 0.1, 1, 5, 10, 50, and 100 µg/ml and anti-amyloid-beta (Aβ) assay at 1, 5, 10, and 25 µg/ml using the thioflavin T (ThT) based fluorescence aggregation kinetics study.

Results: The solvent systems and separation methods were optimized using several analytical methods including flash chromatography, LCMS and $^1H/^{13}C$ NMR to isolate and characterize fractions from the plant material. In the antioxidant assay, fractions F2 and F3 exhibited ~49% scavenging activity at 50 µg/ml and were not as potent as the reference agents resveratrol and trolox (90% and 96% scavenging activity). In the anti-Aβ assay, fraction F2 exhibited excellent inhibition at 10 and 25 µg/ml (90% and 95% inhibition respectively) and was much more potent compared to the reference agent resveratrol (82% and 92% inhibition at 10 and 25 µ/ml respectively).

Conclusion: Our study demonstrated that novel active constituents from a proprietary plant source, exhibit antioxidant and anti-Aβ activity. Few isolated fractions exhibited excellent antioxidant properties in the DPPH assay and also demonstrated anti-Aβ activity in the in vitro ThT assay suggesting their potential as novel therapeutics. Future studies will be carried out to complete the structural elucidation and consider potential synthetic strategies to obtained novel active ingredients for further biological evaluation.

INTRODUCTION

Natural products present in plants are significant sources of new chemical entities (NCEs) for drug discovery, as they contain compounds that are known to have therapeutic properties, such as antioxidant, anti-inflammatory, anti-viral, antimicrobial, aggregation inhibiting, to list a few.[1,2] Previous research findings suggest that NCEs from crude plant extracts exhibit promising activity in reducing neurodegeneration.[3] Specific structural classes of compounds found within natural products such as phenolic acids and flavonoids exhibit antioxidant properties and reduce Aβ neurotoxicity.[1,3] AD is a progressive neurodegenerative disease linked to the appearance of Aβ aggregation which is linked to the formation of neurotoxic species within the brain, ultimately triggering memory loss and the devaluation of cognitive skills on those affected.[4] There are multiple hypotheses regarding what exactly leads to AD, the most significantly investigated one being the Aβ aggregation hypothesis.[4] Oxidative stress is also known to promote neurodegeneration in AD.[4]

OBJECTIVES

- We aimed to **extract and purify** *Powdered Plant X* and then **isolate and characterize** the natural compounds present in the plant material
- Bioactivities of semi purified fractions derived from *Plant X* were tested using:
  (1) an antioxidant α, α-diphenyl-β-picrylhydrazyl (DPPH) assay
  (2) an amyloid-beta (aggregation kinetics) assay

METHODOLOGY

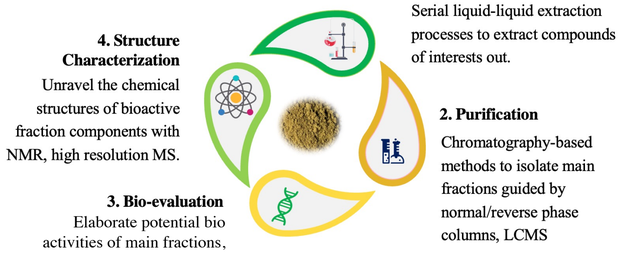

Figure 1. *Overview of Methodology*

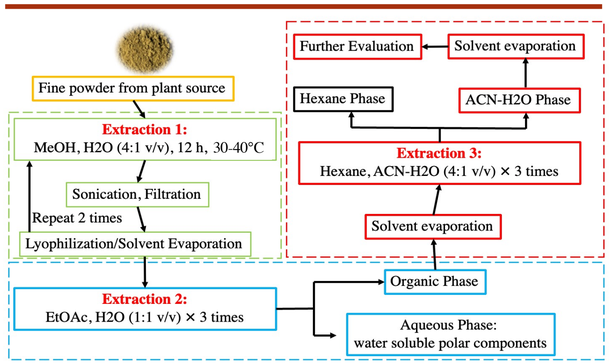

Figure 2. *Plant Extraction Procedure*

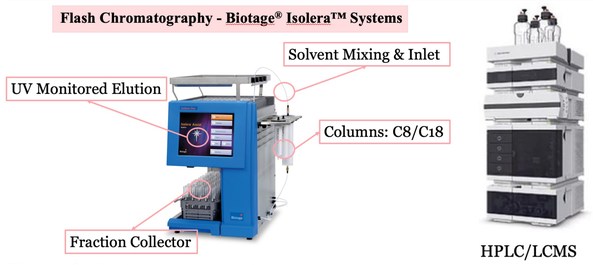

Figure 3. *Purification Machinery*

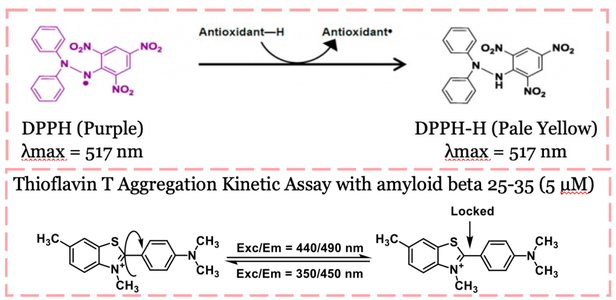

Figure 4. *Bio evaluation: DPPH Assay and Aggregation Kinetics Assay*

RESULTS

(1) DPPH Antioxidant Assay:

Table 1. *Antioxidant activity of combined fractions at six different concentrations (n = 3 based on three independent experiments)*

Fraction	% DPPH Scavenging at:					
	0.1 µg/ml	1 µg/ml	5 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml
1	1.44 ± 5%	5.93 ± 4%	8.20 ± 6%	8.68 ± 6%	27.93 ± 7%	51.80 ± 2%
2	1.43 ± 4%	5.96 ± 3%	7.35 ± 5%	19.61 ± 11%	49.44 ± 11%	89.40 ± 3%
3	1.82 ± 4%	5.13 ± 3%	7.00 ± 2%	14.23 ± 2%	48.86 ± 4%	90.73 ± 5%
4	0.76 ± 4%	1.98 ± 3%	2.78 ± 3%	8.41 ± 7%	33.82 ± 4%	46.29 ± 12%
5	13.80 ± 1%	14.76 ± 1%	15.81 ± 1%	16.99 ± 1%	28.55 ± 3%	42.49 ± 2%
6	0.94 ± 3%	1.56 ± 3%	2.34 ± 2%	6.12 ± 3%	19.14 ± 3%	32.98 ± 3%
7	5.02 ± 1%	8.43 ± 6%	8.53 ± 4%	17.32 ± 7%	42.07 ± 3%	62.37 ± 4%
8	Not Active	1.05 ± 3%	2.56 ± 3%	8.65 ± 6%	29.54 ± 8%	40.20 ± 6%

Figure 5: DPPH Antioxidant Activity at 50 µg/ml and 100 µg/ml

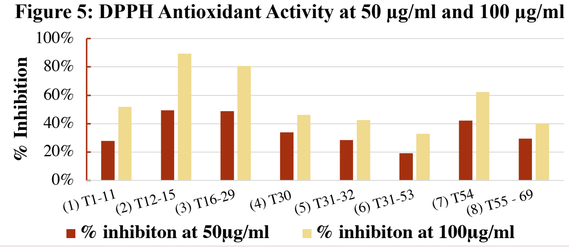

Figure 5. *The Antioxidant activity of all combined fractions (1-8) and controls (Resveratrol. Phenylalanine, Trolox) at the highest two concentrations: 50µg/ml and 100µg/ml. Data averaged from three trials (n = 3).*

(2) ThT Aggregation Kinetics Assay with Aβ(25-35):

Table 2. *Aβ(25–35) aggregation inhibition activity of Fractions: 2, 3, 7, 8 and Resveratrol (n = 3 based on three independent experiments)*

[ ]	Fraction				Control
ug/ml	F2	F3	F7	F8	Resveratrol
1	55.79 ± 16%	41.29 ± 21%	20.13 ± 6%	15.04 ± 8%	64.72 ± 10%
5	72.70 ±16%	57.68 ± 7%	64.84 ± 10%	29.90 ± 9%	85.39 ± 9%
10	90.16 ± 4%	71.19 ± 15%	81.20 ± 8%	57.65 ± 19%	82.52 ± 8%
25	94.66 ± 1%	87.00 ± 10%	89.99 ± 4%	83.97 ± 1%	91.58 ± 9%

Figure 6: Aggregation kinetics profile of Aβ(25–35) of Control

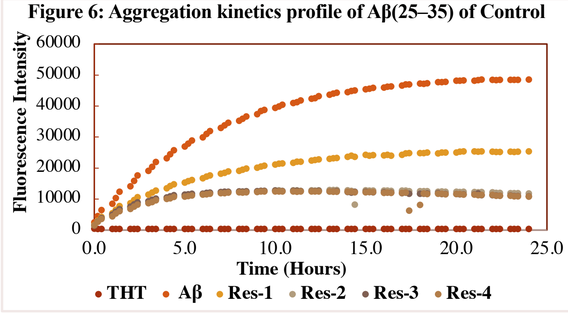

Figure 6. *Aggregation kinetics profile of Aβ(25–35) of Resveratrol (Positive Control) at 1, 5, 10, 25 µg/ml in the presence of Aβ(25–35) at 5 µg/ml over a period of 24 hours in phosphate buffer (pH 7.4) at 37°C. Data averaged from three trials (n =3).*

Figure 7: Aggregation kinetics profile of Aβ(25–35) of Fraction 2

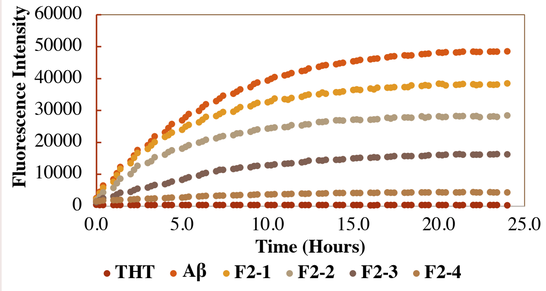

Figure 7. *Aggregation kinetics profile of Aβ(25–35) with Fraction 2 at 1, 5, 10, 25 µg/ml in the presence of Aβ(25–35) at 5 µg/ml over a period of 24 hours in phosphate buffer (pH 7.4) at 37°C. Data averaged from three trials (n =3).*

CONCLUSION

- The amyloid cascade is known to promote oxidative stress in Alzheimer's disease
- This research project was able to develop and optimize the extraction, purification and analytic methodology to separate active ingredients from the proprietary *Plant X*
- We were able to demonstrate that the active constituents present in the proprietary *Plant X* exhibit antioxidant and anti-Aβ activity with **Fraction 2 identified as a promising lead (antioxidant activity = 89% @ 100 ug/ml; anti-Aβ activity = 95% inhibition @25 ug/ml)**
- The results suggest that *Plant X* active ingredients possess biological activities that could be useful to develop novel molecules to treat Alzheimer's disease

FUTURE DIRECTIONS

- The Fraction 2 will be evaluated using full-length amyloid peptides (Aβ40/42) to study their anti-aggregation properties
- Cell culture studies will be carried out in mouse hippocampal neuronal cells to evaluate the ability of Fraction 2 to reduce or prevent amyloid-induced cytotoxicity
- Additional analytical studies will be carried out to determine the structures of active constituents present in Fraction 2

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