Multi-Knowledge Electronic Comprehensive Journal for Education and Science Publications (MECSJ)

ISSUE (33), June (2020)

ISSN: 2616-9185

www.mecsj.com

The Analysis of Polyphenol-Derived Metabolites as Anti-Amyloidogenic Agents

Alshehri, HA[1]; Hugel, HM[2]; Blanch, E[3]

[1] School of science, Al-Imam Mohammad Ibn Saud Islamic University (IMSIU), Riyadh, Saudi Arabia

[2] School of Applied Sciences and Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3001 Australia

[3] School of Applied Sciences and Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3001 Australia

Abstract

A class of molecules that treats multiple age-related diseases would have a major impact on global healthy aging and economics. Polyphenols and related natural products that are abundant in food and beverages are arguably the most promising family of preventative and protective compounds for the alleviation of multiple aging-related diseases or disabilities, including AD. Their extensive metabolism by gut microbiota has revealed that, ultimately, the polyphenol-gut microbial derived products are attributed to simple phenolic acids.

Our study investigated whether the anti-AD effects of the human dietary intake of polyphenols may confirm the efficacy of the major or highest median levels of urinary excretion of phenolic acids in inhibiting amyloid fibrils. Therefore, ThT assays were performed on a library of simple phenolic acids and related compounds to study the efficacy of their prevention of amyloid fibril generation.

Four of the analyzed polyphenolic acids, namely 3-hydroxyphenyl acetic acid, caffeic acid, 2,4-dihydroxycinnamic acid, and L-dopa, showed significant ThT fluorescence inhibition, which is an indication of significant anti-amyloidogenic activities.

The second stage, which was based on the previous ThT results, entailed the re-designing and synthesizing of structures related to the phenolic metabolites. From the ThT assay, the two synthesized compounds in this study showed a higher activity than that of EGCG. Furthermore, our ThT assay results highlighted the importance of the phenolic hydroxyl groups in the structure and also indicated the effect of position and number of the hydroxyl groups and other moieties linked to the phenyl ring in the structure.

Keywords: Alzheimer; phenolic compounds; amyloid beta; polyphenol; urine metabolites; caffeic acid; antioxidant.

تحليل المستقلبات المشتقة من البوليفينول كعوامل مضادة للأميلويدوجينك

الملخص

إن فئة المركبات التب تعالج الأمراض المتعددة الناتجة عن الشيخوخة سيكون لها تأثير كبير على الشيخوخة الصحية والاقتصاد العالميين. ويمكن المجادلة بأن البوليفينول والمنتجات الطبيعية المتوفرة في الأطعمة والمشروبات هي من أسر المركبات الواعدة بمكافحة والوقاية من الأمراض والإعاقات المرتبطة بالتقدم بالعمر، بما في ذلك AD. كشف استقلابها الواسع بواسطة الميكروبات المعوية، أن منتجات البوليفينول المشتقة بواسطة الميكروبات المعوية تنسب إلى أحماض الفينول البسيطة في نهاية المطاف.

بحثت دراستنا فيما إذا كانت التأثيرات المضادة لـ AD لتناول البشر غذائياً للبوليفينول قد تؤكد فعالية أعلى أو أكبر متوسط لمستويات الإفراز البولي لأحماض الفينول في تثبيط الألياف الأميلودية. ولذلك تم إجراء فحوصات THT على مجموعة من أحماض الفينول البسيطة والمركبات ذات الصلة لدراسة فعاليتها في منعها لتوليد الليف الأميلودي. أظهرت أربعة من أحماض البوليفينول التي تم تحليلها، وهي حمض ثلاثي هيدروكسيفينيل أسيتيك، حمض الكافيك، ٢، رباعي ديهيدروكسيسيناميك، ول-دوبا، أظهرت تثبيطاً ملحوظاً لتوهج الـ THT، الأمر الذي يؤشر على الأنشطة الهامة المضادة للأميوليد.

تضمنت المرحلة الثانية، التي استندت إلى نتائج THT السابقة، إعادة تصميم وتجميع الهياكل المتعلقة بالمستقلبات الفينولية. من خلال اختبار THT، أظهرت المركبتان المجمعتان في هذه الدراسة نشاطاً أعلى من نشاط EGCG. علاوة على ذلك، أظهرت نتائج THT لدينا أهمية مجموعات هيدروكسيل الفينول في الهيكل وأيضاً أشارت إلى أهمية موضع وعدد مجموعات الهيدروكسيل وغيرها من الجزئيات المرتبطة بحلقة الفينيل في الهيكل.

الكلمات المفتاحية: الزهايمر، مركبات فينولية، أميوليد بيتا، بوليفينول، مستقلبات البول، حمض الكافيك، مضاد الأكسدة.

ISSUE (33), June (2020)
ISSN: 2616-9185

1. Introduction

β-amyloid (Aβ) fibrils aggregates have been found in autopsied samples from the brains of patients with AD. These Aβ peptides are generated after being cleaved from amyloid precursor protein (APP) by β-secretase, which is followed by γ-secretase. Inhibition of Aβ assembly is a potential approach to treat or prevent AD (Kang, Seo & Park, 2016**)**.

AD is the most common amyloid disease, and was first described by Alois Alzheimer in 1906 (Graeber et al., 1997). This disease is characterized by the formation of amyloid plaque neurofibrillary tangles in the patients' brains. Beta-amyloid (Aβ) peptides are the major constituents of the plaques. The deposition of amyloid fibril is also associated with other diseases such as Parkinson's disease and type II diabetes (Manach, Morand, Rémésy & Jimenez, 2004). The consumption of food, which is rich in polyphenols, is an essential feature of healthy living (O'Keefe et al., 2014). However, after consumption, the polyphenolic compounds pass through the gastrointestinal tract and are absorbed into the circulatory system, becoming metabolites (Rio et al., 2013). A study by Lin et al. showed that a wide range of phenolic acids is generated after microbiotic metabolism of flavonoids (Lin, Yang, Wang & Ling, 2016). Some researchers in animal studies have reported that phenolics are generated during the metabolism of aromatic amino acids (Takahama & Murata, 2002). Furthermore, Sasot et al. (2017) and Urpi-Sarda et al. (2015) reported that there are up to 70 phenolic metabolites from wine intake that have been detected in human urine.

It has previously been shown that small quantities of polyphenols are absorbed into the bloodstream when consuming food rich in polyphenols (Gasperotti et al., 2015; Selma, Espín & Tomás-Barberán, 2009). The gut microbiota plays a major role in human health as it produces catabolites by microbial metabolism polyphenols (Gasperotti et al., 2015; Selma, Espín & Tomás-Barberán, 2009). Gasperotti et al. (2015) observed that after intravenous injection of 23 known polyphenol metabolites into a rat (see Figure 1) only 10 phenolic metabolites crossed the blood brain barrier (BBB).

ISSUE (33), June (2020)
ISSN: 2616-9185

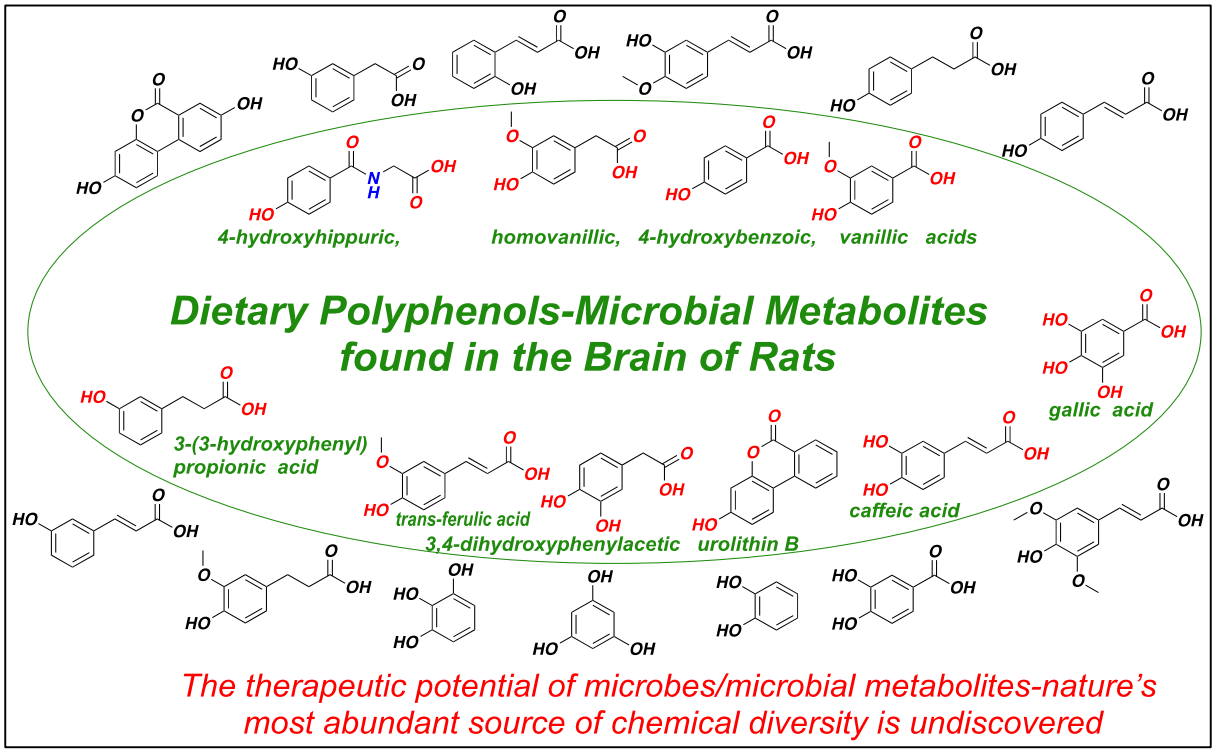


Figure 1: *Polyphenolic metabolites that cross BBB*

The Urine Metabolome Database (UMDB: http://www.urinemetabolome.ca/) contains a complete list of all the possible metabolites that have been detected in human urine. 10 phenolic compounds have been chosen in this study to measure their ability to inhibit fibrils by using the ThT assay. This study focused on testing the ability of the chosen compounds to inhibit or reduce the aggregation of HEWL amyloid and to prepare related compounds based on these chemical structures in order to increase the inhibition of amyloid fibrils.

In addition, a new study has been done in this study to draw a picture of how combining two amyloid fibril inhibitors affects the ability of the inhibitor against amyloid fibril. The ThT assay results of this combination did not provide a complete picture to understand that effect. Thus, more studies and biological tests are required to understand the effects of combining inhibitors.

ISSUE (33), June (2020)
ISSN: 2616-9185

2. Aim and objectives

In this study, a group of phenolic metabolite compounds found in human urine were screened for activity against beta amyloid formation. Furthermore, in order to explore the importance of the hydroxyl groups, their position and number in the polyphenolic structures, various phenolic compounds were synthesized and similarly investigated to study their ability to reduce or inhibit the fibril accumulation. This is the first ThT-biophysical evaluation of the consequential health status of the urinary polyphenols, most highly correlated with food intake/consumption that may reflect and mirror the relationship between polyphenol consumption and their prophylactic effect on chronic diseases, including AD. In addition, combining inhibitors was applied, in this study, between caffeic acid and natural flavonoids, and their ability to inhibit fibril aggregation was tested by using ThT assay to study the effect of combining inhibitors against fibril formation.

ISSUE (33), June (2020)
ISSN: 2616-9185

3. Material and Methods

3.1 Amyloid aggregation of lysozyme HEWL

The ability of the phenolic compounds to inhibit the aggregation of HEWL fibrils was assessed by incubating compounds with HEWL at 70 °C and pH 2.0, and measuring the fluorescence intensity changes with ThT. In order to adjust the baselines and eliminate the effect of the synthesized compounds on the ThT assay, the intrinsic fluorescence of each the compounds tested was also measured in the absence of protein (Hudson, Ecroyd, Kee & Carver, 2009).

3.2 ThT fluorescence assay

Changes in the ThT fluorescence intensity in the presence or absence of amyloid fibrils was used to examine the degree of fibril formation HEWL. Incubation of HEWL under the conditions of low pH and elevated temperature resulted in the formation of amyloid fibrils. In this study, the growth of amyloid fibrils was monitored and characterized using ThT fluorescence and the increase in ThT fluorescence emission was observed after incubating the control sample for three days.

Before incubation, at 0 h, the measured fluorescent intensity for all the test inhibitor compounds was similar to the hen egg protein in buffer solution, which means all the measured compounds did not have any fluorescence and interact with the ThT dye.

11 commercially available phenol compounds were tested for their activity against the HEWL fibril formation. Then, based on the previous results, a library of phenolic compounds was prepared and ThT assay studies were used in order to examine the inhibitory activity of these compounds. In addition, caffeic acid was mixed as equimolar mixtures with two types of flavonoids, namely hesperetin and naringenin to understand the effect of combining inhibitors and if this combination decreases or increases the ability of the tested compounds against amyloid fibrils. Moreover, ThT assays were also used in order to evaluate the ability of the flavonoids as well as the mixture of the caffeic acid and flavonoids to inhibit amyloid fibrils (see figures 2-4).

4. Results and Discussion

A family of 11 polyphenolic metabolites in human urine was screened for anti-amyloidogenic activity against amyloid fibril. In this study, we used HEWL as it can be converted to typical amyloid fibrils under the conditions used. Although there was a significant reduction in the fluorescence intensities associated with all the compounds after 144 h of incubation, four phenolic compounds, namely caffeic acid, 2,4-dihydroxycinnamic acid, L-dopa and 3-hydroxyphenyl acetic acid, were the most effective in inhibiting HEWL fibril formation. These results suggest that these compounds are efficient amyloid inhibitors. Furthermore, our results support the outcome of the study conducted by Porzoor et al. in 2015, which stated that the main factor for anti-amyloid-aggregation properties is not the number of the phenolic hydroxyl groups but the position of them on the chemical structure (Porzoor et al., 2015).

ThT assays monitor the formation and growth of amyloid fibrils. The fluorescence intensity of ThT significantly increased when the test compound did not bind or inhibit the highly ordered β-sheet structure of amyloid fibrils. In order to increase the rate of fibril formation of HEWL, we used heat and acidic conditions (Chaari, Chevillot-Biraud & Rholam, 2015). The increase in the ThT fluorescence emission was observed after incubating the control sample for three days. The results pertaining to ThT assay are shown in Figure 3.3. In the presence of vanillin, homovanillic acid, ferulic acid, 3,4-dihydroxybenzoic acid, vanillic acid, cinnamaldehyde , and 4-hydroxyphenyl acetic acid, the ThT profiles showed that these compounds had a slight effect on lysozyme fibrillation. In contrast, the caffeic acid, 2,4-dihydroxycinnamic acid, L-dopa, and 3-hydroxyphenyl acetic acid presented significant inhibitory effects on the amyloid formation of lysozyme, with the greatest inhibition of fibrils being for caffeic acid (Figure 2).

In general, all the tested compounds in this study differed in their ability to inhibit the fibril formation. Therefore, they can be presented in the following order:

Caffeic acid > 2,4-dihydroxycinnamic acid > L-dopa > 3-hydroxyphenyl acetic acid > vanillin > homovanilic acid > ferulic acid >3,4-dihydroxybenzoic acid > vanillic acid > cinnamaldehyde > 4-hydroxyphenyl acetic acid

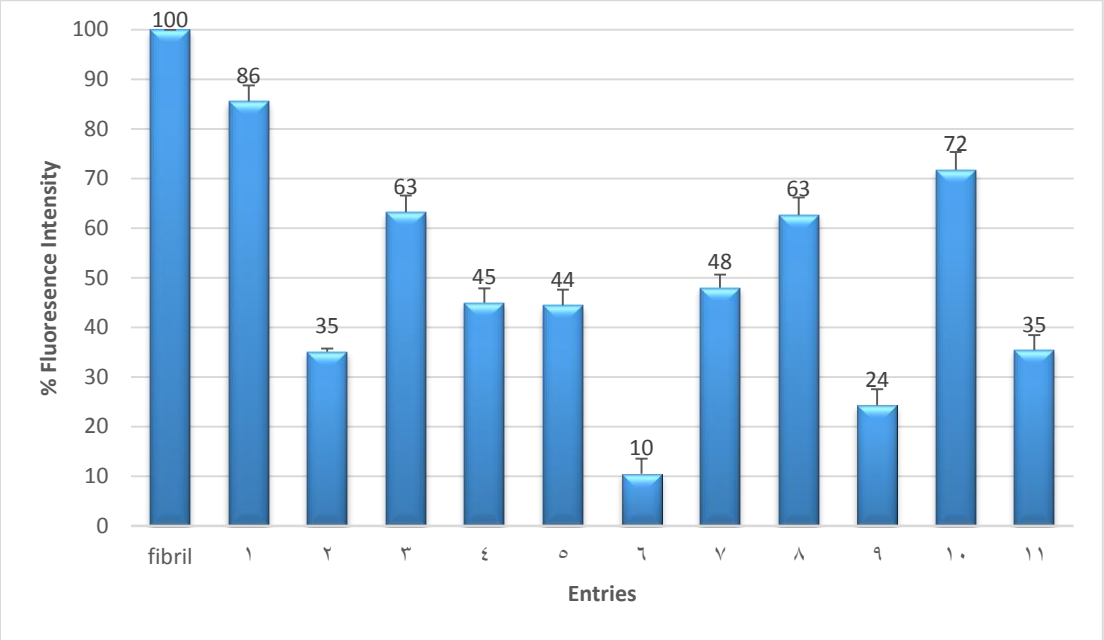


***Figure 2**: The ThT Fluorescence intensities of urinary metabolites.Error bars are the standared deviation of the fluorescence measurement based on triplicate samples.*

In the second set of ThT assays, a library of compounds has been synthesized based on the previous results. Caffeic acid in the first assay shows the best ability to inhibit fibrils and that directed us to synthesize compounds based on the chemical structure of caffeic acid. Only very pure compounds have been used in the second ThT assay, and EGCG was tested with our compounds as a reference, to make a comparison between the synthesized compound and EGCG.

The majority of the synthesized compounds exhibited high levels of activity against fibril formation (see Figure 3). Entry **16**, Henry product which has two hydroxyl groups in the structure linked to the benzene rings exhibited the greatest fibril inhibition, better than EGCG. This was followed by compound entry **12**, and compared to caffeic acid, there is no significant difference between caffeic acid and dihydrocaffeic acid. However, compound entry **13**, presented a high level of inhibition compared to the parent compound, ferulic acid. In entry **15**, the Henry product which has a chloro substituent in the *para* position of the benzene ring, also showed good activity in reducing fibril accumulation.

The Henry compound (entry **14**) that has a methoxy group in the *para* position of the benzene ring does not display that effect in the ThT assay.

From these results, small synthesized compounds in this study presented a good ability to reduce fibril formation. This study presented the importance of the hydroxyl groups in the structure in reducing the accumulation of amyloid beta oligomers.

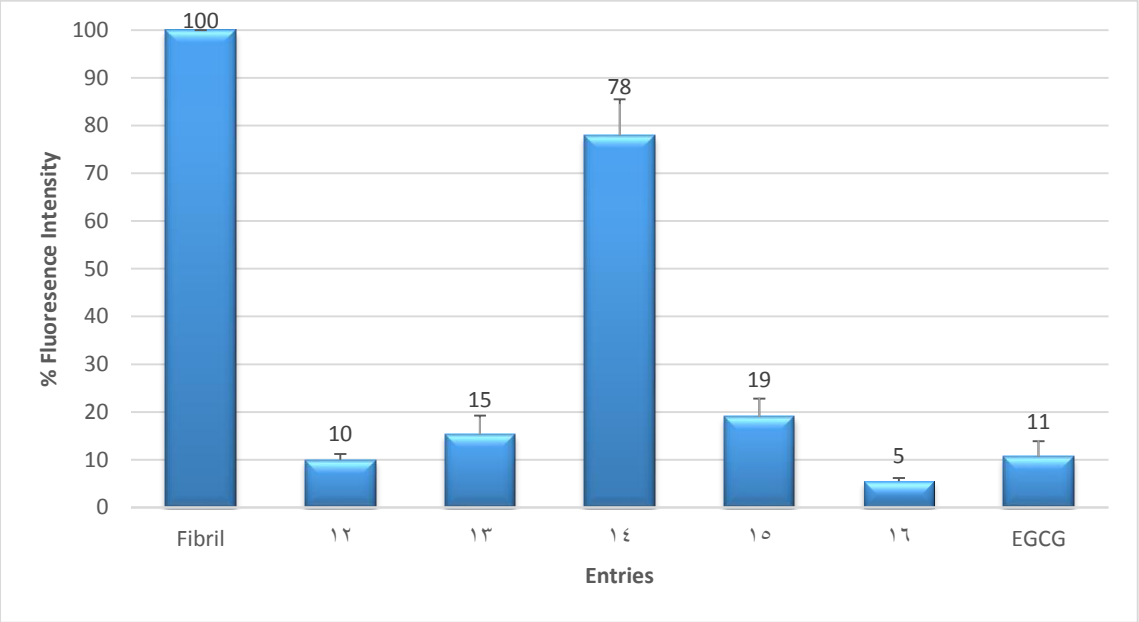


***Figure 3**: The fluorescence intensities of best polyphenols showing anti-amyloidogenic activitie. Error bars are the standared deviation of the fluorescence measurement based on triplicate samples.*

In the third set of ThT assays (Figure 4), we studied the effect of mixing caffeic acid with two types of flavonoids. Thus, equimolar mixtures of caffeic acid and hesperetin or naringenin have been tested by the ThT assay to examine the ability of the mixture of the caffeic acid and flavonoids to inhibit the amyloid fibrils. The fluorescence intensity of the flavonoids before mixing with caffeic acid showed low activity. However, after mixing, the mixture of naringenin or hesperetin and caffeic acid demonstrated better activity in reducing the fluorescent intensity. The mixture that contains naringenin and caffeic acid exhibited better activity compared to the mixture of hesperetin and caffeic acid and that may be related to the chemical structure of both flavonoids.

In other words, naringenin and caffeic acid may interact better with the amyloid beta structure and that may be because of the number and position of the hydroxyl groups in the structures. The study of the effects of combining inhibitors is a new subject and we are not aware of any prior research having been done on this combination.

The results in this chapter indicate a potential new approach to inhibit fibril formation through cooperativity between two inhibitors, though more research is required to understand that effect. Thus, more studies and biological tests are required to understand the effects of combining inhibitors.

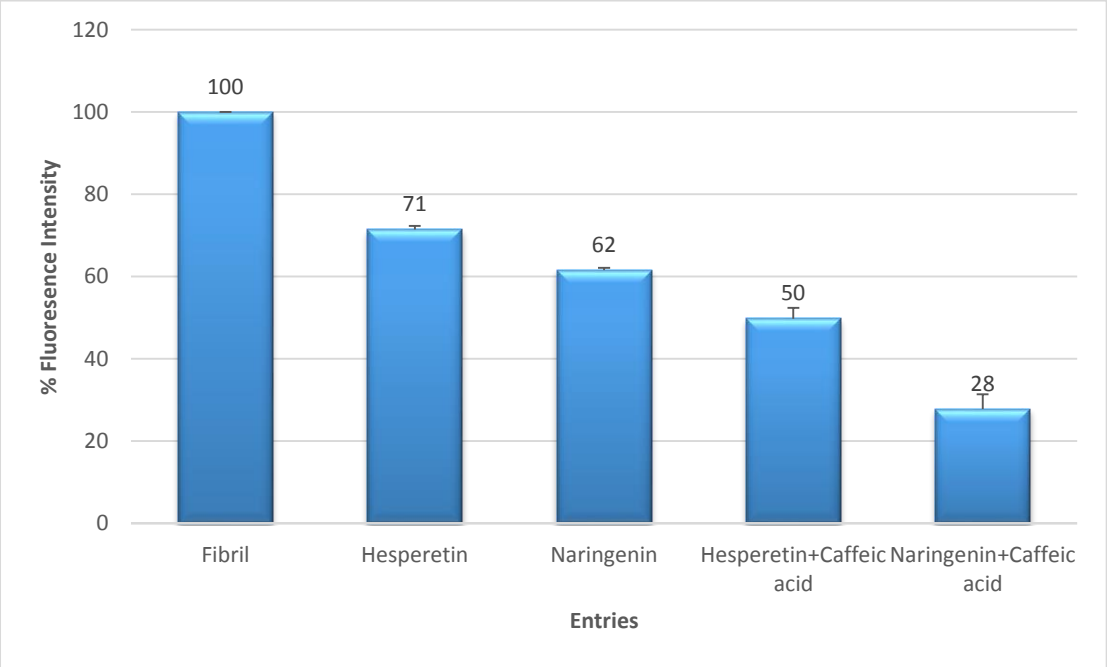
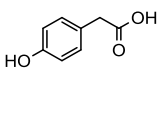
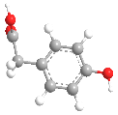
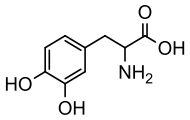
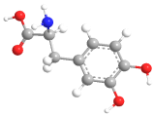
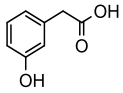
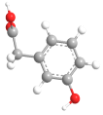
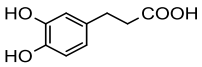
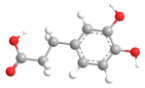
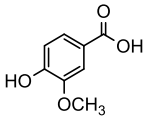
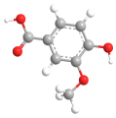
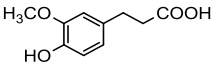
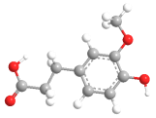
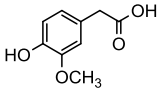
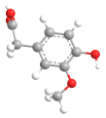
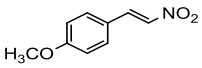
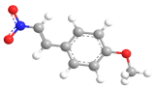
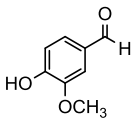
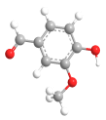
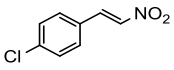
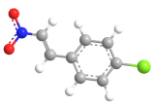


***Figure 4**: ThT fluorescence assays for the flavonoids and the mixtures of caffeic acid and flavonoids. Error bars are the standared deviation of the fluorescence measurement based on triplicate samples.*

Entry	Chemical Name	Chemical Structure	3D structure	Entry	Chemical Name	Chemical Structure	3D structure
1	4-Hydroxyphenyl acetic acid			11	L-Dopa		
2	3-Hydroxyphenyl acetic acid			12	3-(3,4-Dihydroxyphenyl) propanoic acid		
3	Vanillic acid			13	(E)-2-Methoxy-4-(2-nitrovinyl) phenol		
4	Homovanillic acid			14	(E)-1-Methoxy-4-(2-nitrovinyl) benzene		
5	Vanillin			15	(E)-1-Chloro-4(2-nitrovinyl) benzene		

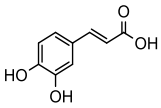
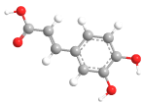
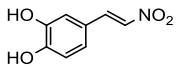
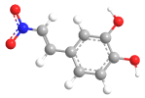
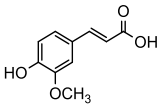
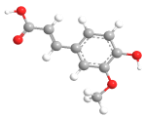
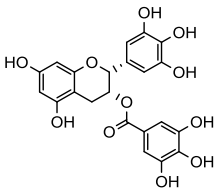
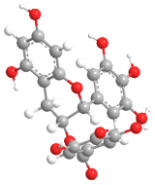
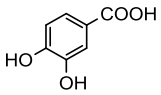
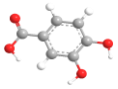
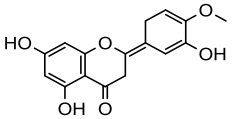
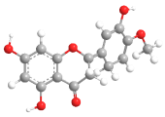
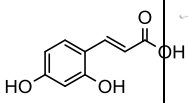
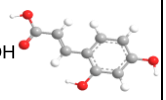
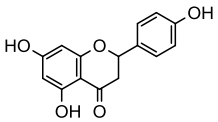
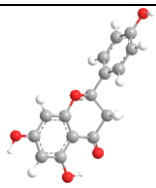
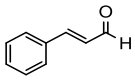
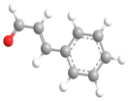
6	Caffeic acid			**16**	(E)-4-(2-nitrovinyl) benzene-1,2-diol		
7	Ferulic acid			**17**	EGCG		
8	3,4-Dihydroxybenzoic acid			**18**	Hesperetin		
9	2,4-Dihydroxycinnamic acid			**19**	Naringenin		
10	Cinnamaldehyde						

Table 1: *Chemical names and structures of ThT-assayed compounds shown in Figures 2, 3, 4*

5. Experimental

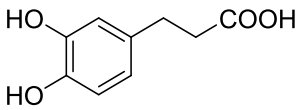
Starting materials and reagents were obtained from Sigma Aldrich and AK Scientific and were used without further purification. In some experiments, the solvents were dried by using standard methods. Thin layer chromatography (TLC) on Merck 60 F240 pre-coated silica gel polyester plates was used to follow the progress of the reaction, and products were visualized with 254 nm ultraviolet irradiation. Flash column chromatography was performed on Merck silica gel 60, 40-63 µm (mesh).

Nuclear magnetic resonance spectra were recorded on a Brucker-DPX, for proton (1H) NMR spectra at 300MHz and for carbon (13C) NMR spectra at 75. Mass spectral data was collected on a Varian Saturn 2200 analytical mass spectrometer. All compounds are named in accordance with ChemBioDrow Ultra.

5.1 Synthesis of compounds (12 & 13):

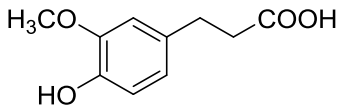
Compounds **(12 & 13)** were prepared according to the procedure described by Gaire, *et al*. [15]. Palladium on charcoal (10 mol%) was added to a solution of α, β-unsaturated acids (1.0 mmol) in methanol (1.0 mmol). The reaction mixture was stirred under H_2 gas (balloon) atmosphere for about 2 h at room temperature. The progress of the reaction was monitored by TLC and once the reaction was complete, the mixture was filtered and the filtrate was then dried under vacuo. The product was purified by column chromatography to give a pure desired product with yield 60 & 50 % respectively.

3-(3,4-dihydroxyphenyl) propanoic acid (12)



Molecular formula: $C_9H_{10}O_4$; MM: 182.06 g/mol; Yield: 60%, white crystals. ^{1}H NMR (300 MHz, d_6-D_2O): δ 6.75 (1H, d, *J*=9 Hz, ArH), δ 6.73 (1H, s, ArH), δ 6.60 (1H, d, *J*=9 Hz, ArH), δ 2.69 (2H, t, -CH_2), δ 2.52 (2H, t, -CH_2). ^{13}C NMR (150 MHz, d_6-D_2O) 178.07, 143.82, 142.13, 133.50, 120.43, 116.22, 116.04, 35.58, 29.52. GC-MS:m/z (%) 182 M+.

3-(4-hydroxy-3-methoxyphenyl) propanoic acid (13)



Molecular formula: $C_{10}H_{12}O_4$; MM: 196.07 g/mol; Yield: 55%, white crystals. ^{1}H NMR (300 MHz, d_6-D_2O): δ 6.79 (1H, s, ArH), δ 6.74 (1H, d, *J*=9 Hz, ArH), δ 6.63 (1H, d, *J*=9 Hz, ArH), δ 3.73 (3H, s, -OCH_3), δ 2.72 (2H, t, -CH_2), δ 2.53 (2H, t, -CH_2). ^{13}C NMR (150 MHz, d_6-D_2O): 178.04, 147.22, 143.03, 133.33, 120.84, 115.41, 112.62, 55.82, 35.62, 29.83. GC-MS:m/z (%) 196 M+.

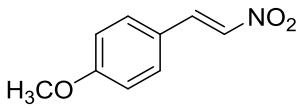
5.2 General procedure for the synthesis of Henry products (14–16, 20):

Henry products were synthesized according to the procedure used in Luzzio (2001). A round bottom flask was charged with Aldehyde (1 equiv), nitroethane (1.5 equiv) and ammonium acetate (1 equiv) and acetic acid (10 mL). The reaction mixture refluxed for 24h and the resulting mixture was extracted with ethyl acetate concentrated in vacuo and recrystallised from ethanol.

Or:

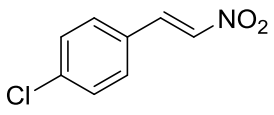
A 10 mL microwave reaction vessel was charged with aldehyde (1 equiv), nitroethane (1.5 equiv) and ammonium acetate (1 equiv). The reaction mixture refluxed for 24h and the resulting mixture was extracted with ethyl acetate concentrated under vacuo and recrystallised from ethanol.

(E)-1-methoxy-4-(2-nitrovinyl) benzene (14)



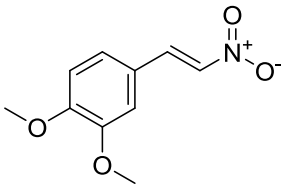
Molecular formula: $C_9H_9NO_3$; MM: 179.06 g/mol; Yield: 87%, yellow crystals. ^{1}H NMR (300 MHz, d_6-$CDCl_3$): δ 7.99 (1H, d, *J* = 12 Hz, -CH), δ 7.55 (2H, d, *J*=9 Hz, ArH), δ 7.51 (1H, s, -CH), δ 6.68 (2H, d, *J*=9 Hz, ArH), δ 3.89 (3H, s, -OCH_3). ^{13}C NMR (150 MHz, d_6-$CDCl_3$): 162.97, 139.04, 135.06, 131.18, 122.57, 114.95, 55.55. GC-MS:m/z (%) 179 M+, 180 M+1.

(E)-1-chloro-4-(2-nitrovinyl) benzene (15)



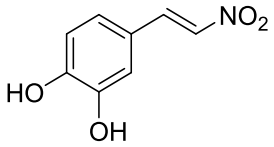
Molecular formula: $C_8H_6ClNO_2$; MM: 183.01 g/mol; Yield: 80%, yellow crystals. ^{1}H NMR (300 MHz, d_6-$CDCl_3$): δ 7.99 (1H, d, *J*=12 Hz, -CH), δ 7.55 (2H, d, *J*=9 Hz, ArH), δ 7.51 (1H, s, -CH), δ 6.68 (2H, d, *J*=9 Hz, ArH), δ 3.89 (3H, s, -OCH_3). ^{13}C NMR (150 MHz, d_6-$CDCl_3$): 162.97, 139.04, 135.06, 131.18, 122.57, 114.95, 55.55. GC-MS:m/z (%) 183 M+, 184 M+1.

(E)-1,2-dimethoxy-4-(2-nitrovinyl) benzene (20)



Molecular formula: $C_{10}H_{11}NO_4$; MM: 209.07 g/mol; Yield: 63%, yellow crystals. ^{1}H NMR (300 MHz, d_6-$CDCl_3$): δ 8.46 (1H, d, *J*=14 Hz, -CH), δ 8.02 (1H, d, *J*=14 Hz, -CH), δ 7.42 (1H, d, *J*=8 Hz, ArH), δ 6.92 (1H, s, ArH), δ 6.76 (2H, d, *J*=8 Hz, ArH), δ 3.85 (3H, s, -OCH_3), δ 3.72 (3H, s, -OCH_3). ^{13}C NMR (150 MHz, d_6- $CDCl_3$): 152.62, 152.01, 137.32, 136.43, 124.43, 123.55, 118.56, 117.92, 112.43, 55.55. GC-MS: m/z (%) 209 M+, 210 M+1.

(E)-4-(2-nitrovinyl) benzene-1,2-diol (16)



Compound **16** was prepared by demethylating compound **20** according to the procedure described by Park et al. (2003). Methoxy product **20** (1 equiv.) was dissolved in dry CH_2Cl_2 and the solution was cooled using a dry ice acetone mixture. A solution of BBr_3 in CH_2Cl_2 (1.0 M, 3 equiv. per methoxy group) was added to the mixture and stirred for 1 hour. Then, the mixture was allowed to cool at room temperature and stirred for 24 hours.

Methanol was added after that to quench the reaction and then the solvent was evaporated. The product was purified by column chromatography to give a pure product with high yield (66%).

Molecular formula: $C_8H_7NO_4$; MM: 181.04 g/mol; Yield: 66%, brown solid. ^{1}H NMR (300 MHz, d_6-DMSO): δ 10.05 (1H, s, OH), δ 9.29 (1H, s, OH), δ 7.95 (2H, s, -CH), δ 7.20 (2H, s, ArH), δ 6.83 (1H, d, *J*=9 Hz, ArH). ^{13}C NMR (150 MHz, d_6- DMSO): 162.97, 139.04, 135.06, 131.18, 122.57, 114.95, 55.55. GC-MS:m/z (%) 181 M+.

6. ThT fluorescence assay to examine amyloid aggregation

6.1 Materials and methods

6.1.1 Proteins and reagents

Hen egg white lysozyme (MW 14.3 kDa) was obtained from Sigma-Aldrich, ThT and all phenolic compounds were purchased from Sigma-Aldrich and AK-scientefic, namely, Caffeic acid, 2,4-dihydroxycinnamic acid, L-dopa, 3-hydroxyphenyl acetic acid, vanillin, homovanilic acid, ferulic acid, 3,4-dihydroxybenzoic acid, vanillic acid, cinnamaldehyde, 4-hydroxyphenyl acetic acid. All materials were used without further purification.

6.1.2 Preparation and characterization of lysozyme fibrils

Lysozyme fibrils were prepared by dissolving 120 mg hen egg white lysozyme in 6 ml buffer (pH 2.0) with a concentration of 20 mg/ml. In this study, the buffer was prepared first and it was a mixture of 0.1M K_2HPO_4 and 0.1M NaOH to reach to pH7, and after adding HEWL protein, with or without samples, the pH was adjusted to be 2.0. The concentrations of phenolic compounds were 0–2 mg/ml. The mixture was incubated for 3–6 days at 70 °C. The growth of lysozyme fibril was monitored by ThT fluorescence, and the fluorescence emission spectra measured in (λex 440 nm, λem 450–700 nm).

6.1.3 ThT assay

ThT fluorescence assay was performed with a Horiba scientific fluoromax X-4. An excitation wavelength of 440 nm and an emission wavelength of 486 nm were used. A stock solution of ThT was prepared in 0.1 M phosphate buffer (pH 7). This stock solution (1 mL) was diluted with 50 mL of phosphate buffer and then used as a working solution (50 μM).

To measure fibrillation kinetics HEWL, incubation solutions without or with phenolic compounds were taken out of the incubation vial at different times (0 h, 2 h, 7 h, 24 h, 48h, 72h and 144 h) and were subjected to the assay immediately. This assay has been performed in triplicate, and for each measurement 10 μL of incubation solution was added into 4 mL of ThT solution in a quartz cuvette. The solution in the cuvette was shaken before running each acquisition.

7. Conclusion

We investigated whether the anti-AD effects of the human dietary intake of polyphenols may hinge on confirming the efficacy of the major or highest median levels of urinary excretion of phenolic acids to inhibit amyloid fibrils. Therefore, thioflavin T (ThT) assays were performed on a library of simple phenolic acids and related compounds to study the efficacy of their prevention of amyloid fibril generation. Three of the analyzed polyphenolic acids, namely 3-hydroxyphenyl acetic acid, caffeic acid, 2,4-dihydroxycinnamic acid including *L*-dopa, showed significant amyloid fibril inhibition, an indication of potential anti-amyloidogenic activity. Also, a library of related compounds was prepared based on the previous results and most showed improved ThT assay fluorescence inhibition. In addition, combinations of caffeic acid with natural flavonoids were similarly analyzed using the inhibition of HEWL fibril formation by the ThT dye protocol. The fluorescent intensity of the flavonoids before mixing with caffeic acid showed low activity. However, after mixing, the mixture of naringenin or hesperetin and caffeic acid demonstrated better activity in reducing the fluorescent intensity. The mixture that contains naringenin and caffeic acid exhibited better activity compared to the mixture of hesperetin and caffeic acid and that may be related to the chemical structure of both flavonoids. In other words, naringenin and caffeic acid may interact more strongly with the amyloid beta structure and that may be because of the number and position of the hydroxyl groups in the structures. The study of the effects of combining inhibitors is a new topic and there are no prior studies that we are aware of on this. Further studies and biological tests are required to understand the effects of combining inhibitors of fibril formation.

References

Allegretta, G. W., Empting, M. & Hartmann, R. W. (2015). Catechol-based substrates of chalcone synthase as a scaffold for novel inhibitors of PqsD. *Eur. J. Med. Chem., 90*, 351-359.

Almeida, A. F., Santos, C. N. & Ventura, M. R. (2017). Synthesis of new sulfated and glucuronated metabolites of dietary phenolic compounds identified in human biological samples. *J. Agr. Food Chem., 65* (31), 6460-6466.

Chaari, A. F., C., Chevillot-Biraud, A. & Rholam, M. (2015). Insights into kinetics of agitation-induced aggregation of hen lysozyme under heat and acidic conditions from various spectroscopic methods. *PLoS ONE*, 1-25.

Figueira, I., Garcia, G., Pimpão, R. C., Terrasso, A. P., Costa, I., Almeida, A. F., Tavares, L., Pais, T. F., Pinto, P., Ventura, M. R., Filipe, A., McDougall, G. J., Stewart, D., Kim, K. S., Palmela, I., Brites, D., Brito, M. A., Brito, C. & Santos, C. N. (2017). Polyphenols journey through blood-brain barrier towards neuronal protection. *Sci. Rep., 7* (1), 11456.

Gaire, B. P., Kwon, O. W., Park, S. H., Chun, K. H., Kim, S. Y., Shin, D. Y. & Choi, J. W. (2015). Neuroprotective effect of 6-paradol in focal cerebral ischemia involves the attenuation of neuroinflammatory responses in activated microglia. *PLoS One, 10* (3), e0120203.

Gallagher, R., Shimmon, R. & McDonagh, A. M., (2012). Synthesis and impurity profiling of MDMA prepared from commonly available starting materials. *Forensic Sci. Int., 223* (1), 306-313.

Gasperotti, M., Passamonti, S., Tramer, F., Masuero, D., Guella, G., Mattivi, F. & Vrhovsek, U. (2015). Fate of microbial metabolites of dietary polyphenols in rats: Is the brain their target destination? *ACS Chem. Neurosci., 6* (8), 1341-1352.

Gazova, Z., Siposova, K., Kurin, E., Mucaji, P. & Nagy, M. (2013). Amyloid aggregation of lysozyme: the synergy study of red wine polyphenols. *Proteins, 81* (6), 994-1004.

Graeber, M. B., Kösel, S., Egensperger, R., Banati, R. B., Müller, U., Bise, K., Hoff, P., Möller, H. J., Fujisawa, K. & Mehraein, P. (1997)Rediscovery of the case described by Alois Alzheimer in 1911: historical, histological and molecular genetic analysis. *Neurogenetics, 1* (1), 73-80.

Hudson, S. A., Ecroyd, H., Kee, T. W. & Carver, J. A. (2009). The thioflavin T fluorescence assay for amyloid fibril detection can be biased by the presence of exogenous compounds. *FEBS J., 276* (20), 5960-5972.

Kang, Y. J., Seo, D. G. & Park, S. Y. (2016**).** Phenylpropanoids from cinnamon bark reduced beta-amyloid production by the inhibition of beta-secretase in Chinese hamster ovarian cells stably expressing amyloid precursor protein. Nutr. Res., 36 (11), 1277-1284.

Letort, S., Lejeune, M., Kardos, N., Metay, E., Popowycz, F., Lemaire, M. & Draye, M. (2017). New insights into the catalytic reduction of aliphatic nitro compounds with hypophosphites under ultrasonic irradiation. *Green Chem., 19* (19), 4583-4590.

Lima, R. N. & Porto, A. L. M. (2017). Facile synthesis of new quinoxalines from ethyl gallate by green chemistry protocol. *Tetrahedron Lett., 58* (9), 825-828.

Lin, W., Yang, H., Wang, D. & Ling, W. (2016). Influence of intestinal microbiota on the catabolism of flavonoids in mice. *J. Food Sci., 81*, H3027.

Liu, Y., Pukala, T. L., Musgrave, I. F., Williams, D. M., Dehle, F. C. & Carver, J. A., (2013). Gallic acid is the major component of grape seed extract that inhibits amyloid fibril formation. *Bioorg. Med. Chem. Lett., 23* (23), 6336-6340.

Luzzio, F. A. (2001). The Henry reaction: recent examples. *Tetrahedron, 57*, 915±945.

Manach, C. S., A., Morand, C., Rémésy, C. & Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr., 79*, 727-747.

O'Keefe, J. H. B., S. K., Bajwa, A., DiNicolantonio, J. J., PharmD & Lavie, C. J. (2014). Alcohol and cardiovascular health: The dose makes the poison.Or the remedy. *Mayo. Clin. Proc., 89,* 382-393.

Park, S.-H., Kang, S.-H., Lim, S.-H., Oh, H.-S. & Lee, K.-H. (2003). Design and synthesis of small chemical inhibitors containing different scaffolds for lck SH2 domain. *Bioorg. Med. Chem. Lett., 13* (20), 3455-3459.

Pimpão, R. C., Ventura, M. R., Ferreira, R. B., Williamson, G. & Santos, C. N. (2015). Phenolic sulfates as new and highly abundant metabolites in human plasma after ingestion of a mixed berry fruit purée. *Br. J. Nutr., 113* (3), 454-463.

Porzoor, A. A., B., Hügel, H., Grando, M. D., Caine, J. & Macreadie, I. (2015) Anti-amyloidogenic properties of some phenolic compounds. *Biomolecules, 5,* 505-527.

Rio, D., Spencer, J., Tognolini, M., Borges, G. & Crozier, A. (2013). Dietary (poly) phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox sign, 18,* 1818-1892.

Sasot, G., Martínez-Huélamo, M., Vallverdú-Queralt, A., Mercader-Martí, M., Estruch, R. a. & Lamuela-Raventós, R. M. (2017). Identification of phenolic metabolites in human urine after the intake of a functional food made from grape extract by a high resolution LTQ-Orbitrap-MS approach. *Food Res. Int.*, 1-10.

Selma, M. V., Espín, J. C. & Tomás-Barberán, F. A. (2009) Interaction between phenolics and gut microbiota: Role in human health. *J. Agr. Food Chem., 57* (15), 6485-6501.

Shi, Z.-H., Li, N.-G., Tang, Y.-P., Shi, Q.-P., Zhang, W., Zhang, P.-X., Dong, Z.-X., Li, W. & Duan, J.-A. (2015). Design and synthesis of novel aspirin-caffeic acid ester hybrids for cardioprotection with reduced risk of hemorrhagic stroke. *Asian J. Chem., 27* (4), 1342-1346.

Takahama, U. & Murata, H. (2002). The presence of 4-hydroxyphenylacetic acid in human saliva and the possibility of its nitration by salivary nitrite in the stomach. *FEBS Lett., 518*, 116-118.

Urpi-Sarda, M., Queipo-Ortuno M., Tulipani, S., Corella, D., Estruch, R., Tinahones, F. J. & Andres-Lacueva C., (2015). Phenolic and microbial-targeted metabolomics to discovering and evaluating wine intake biomarkers in human urine and plasma. *Electrophoresis, 36*, 2259-2268.

Varma, R. S., Dahiya, R. & Kumar, S. (1997). Microwave-assisted Henry reaction: Solventless synthesis of conjugated nitroalkenes. *Tetrahedron Lett., 38* (29), 5131-5134.