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Identification of Plant Extracts that Inhibit the Formation of Diabetes-Linked IAPP Amyloid

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Abstract

The extracts of 27 vegetables, spices and herbs were screened for their functional ability to inhibit the aggregation of islet amyloid polypeptide (IAPP, amylin) into toxic amyloid aggregates. The aggregation of IAPP has been directly linked to the death of pancreatic β -islet cells in type 2 diabetes. Inhibiting the aggregation of IAPP is believed to have the potential to slow, if not prevent entirely, the progression of this disease. As vegetables, spices and herbs are known to possess many different positive health effects, the extracts of 27 plants (abundant within the United States and spanning several plant families) were screened for their ability to inhibit the formation of toxic IAPP aggregates. Their anti-amyloid activities were assessed through (1) thioflavin T binding assays, (2) visualization of amyloid fibers using atomic force microscopy and (3) cell rescue studies. From this research, mint, peppermint, red bell pepper and thyme emerged as possessing the greatest anti-amyloid activity.

Keywords

Amyloid Inhibition; Diabetes; Natural Product Therapeutics

1. Introduction

The natural molecular components of plants are known to possess a wide range of functional and medicinally beneficial activities. Extracts of vegetables, spices and herbs have been identified as having beneficial health activities. The major organic compounds in herbs, spices and vegetables have been studied for their antioxidant, anti-tumour and anti-aging properties (Bai et al., 2011; Deng et al., 2013; Manchali et al., 2012). The American

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Diabetes Association lists many non-starchy vegetables, spices and herbs on their list of best choices for foods for people with diabetes. We screened and assessed extracts of edible plants and herbs for their ability to inhibit the formation of toxic aggregates of the diabetes-linked protein islet amyloid polypeptide (IAPP). To the best of our knowledge, this is the first study to directly assess the ability of these plant products to inhibit IAPP aggregation.

Type 2 diabetes afflicts approximately 25.8 million Americans (approximately 8.3% of the population (Bai et al., 2011)), with nearly 2 million new cases each year. While the underlying causes of type 2 diabetes remain unclear, one of the factors contributing to the progression of this disease is the formation of protein aggregates of the amyloid protein IAPP. These aggregates have been shown to be highly toxic to mammalian cells, especially the insulin-producing β -cells of the pancreas (Montane et al., 2012). It is believed that preventing the formation of IAPP amyloid could slow, if not prevent, the progression of type 2 diabetes (Bram et al., 2014).

The exact structure of the IAPP aggregates is not fully understood. It is known that IAPP can aggregate into a variety of amyloidogenic states (Abedini & Schmidt, 2013; Apostolidou et al., 2008; Hull et al., 2004; Kahn et al., 1999). It is apparent that some of these aggregates, termed small soluble oligomers, are highly toxic to mammalian cells (Andrews et al., 2009; Cao et al., 2013; Nanga et al., 2008). Work by Eisenberg and coworkers and Ramamoorthy and coworkers suggested that the most toxic form of IAPP may be a cylindrin structure (Laganowsky et al., 2012; Liu et al., 2012; Patel et al., 2014). Regardless of the exact structure, IAPP aggregates play a role in the loss of pancreatic β -cells and the progression of type 2 diabetes (Ritzel et al., 2007).

The extracts of 27 vegetables, spices and herbs were screened for their ability to inhibit IAPP aggregation (Table 1). These plant products were chosen because of their relative abundance within the United States and because of their distribution across different families of plant products (Table 1). The extraction method, described below, produced active extracts while removing over 99% of the carbohydrates. As people afflicted with type 2 diabetes must limit their intake of carbohydrates, this extraction method would be suitable for yielding bioactive products with very low carbohydrate content.

2. Materials and Methods

2.1 Preparation of the Extracts

The vegetable extracts: 100g of each individual fresh vegetable was ground using a mortar and pestle mixed with 100 mL of ethyl acetate. The resulting slurry was filtered and the ethyl acetate layer separated. The ethyl acetate fraction for each vegetable was separated into 15 equal aliquots and speed vacuumed to dryness. These dehydrated extracts were stored at -20°C . Extracts were rehydrated by re-suspending each aliquot in 150 μL of 20 mM tris buffer (pH 7.4).

The herb extracts: Samples were prepared as described above for vegetable extracts, except that 25 g of each individual herb/spice was ground using a mortar and pestle mixed with 100

mL of ethyl acetate. 2-mL aliquots were speed vacuumed to dryness and stored at -20°C . These samples were rehydrated by re-suspension in 150 mL of 20 mM tris buffer (pH 7.4).

2.2 Preparation of IAPP stock solutions

IAPP stock solutions were prepared by dissolving 1 mg of synthetic amylin (Anaspec Corp. Fremont, CA, USA) in 8 mL of hexafluoroisopropanol (HFIP, Sigma Aldrich, St. Louis, MO, USA). This IAPP solution was fully disaggregated by sonicating in a 25°C water bath for 5 min. The resulting IAPP stock solution was stored at -80°C .

2.3 Thioflavin T binding assays

Aliquots of the IAPP stock solution were placed in glass tubes and placed under speed vacuum to remove the HFIP solvent. The resulting dry IAPP samples were re-suspended in 20 mM tris buffer (pH 7.4) containing each individual food extract. The final in-solution concentration of IAPP was $106\ \mu\text{M}$. To initiate protein aggregation the samples were vigorously shaken (200 rpm) at 37°C . $17\text{-}\mu\text{L}$ aliquots of each sample were removed after 25 min and mixed with $663\ \mu\text{L}$ of $50.0\ \mu\text{M}$ thioflavin T (ThT) in 20 mM Tris buffer (pH 7.4). The IAPP/ThT mixture was incubated at room temperature in the dark for 2 min before recording the ThT fluorescence emission spectrum ($\text{Ex}_{450\text{nm}}$) using a Hitachi F-7000 fluorescence spectrophotometer.

2.4 Atomic Force Microscopy

Synthetic IAPP, prepared as described above, was incubated with shaking at 37°C in the presence of each individual plant extract. After 40 min of incubation, $17\ \mu\text{L}$ of each IAPP/food sample was deposited onto freshly cleaved mica. The mica was incubated at room temperature for 5 min before washing with $200\ \mu\text{L}$ sterile-filtered water. The sample was allowed to air dry. Samples were scanned using an MFP-3D atomic force microscope (Asylum Research) set on A/C mode and a $240\text{-}\mu\text{m}$ silicon cantilever (Olympus). All images are shown as the raw data with no flattening.

2.5 MTT Assay

An equal number of HeLa cells were plated in triplicate in 96-well plates. Each plate was incubated for 22–24 hours at 37°C . After incubation, F-12K media with phenol red was replaced with Dulbecco's Modified Eagle Medium (DMEM) without phenol red. $30\ \mu\text{M}$ of IAPP re-suspended in HFIP was dried using Thermo Scientific Savant ISS110 Speedvac Concentrator (Asheville, NC) at low temperature. The dried IAPP was re-suspended in a 1:1 ratio of DMEM. The stock solutions of extracts were prepared by vortexing for 2 min followed by centrifuging at 14,000 rpm for 2 min. $20\ \mu\text{L}$ of each extract and $30\ \mu\text{M}$ of IAPP were added into corresponding wells in the 96-well plate. The plate was incubated at 37°C for 22–24 hours. Post-incubation, the wells were washed with DMEM in order to remove colouring from the extracts. Thiazolyl blue tetrazolium bromide (MTT) was added to each well. The plate was incubated for 2 hours at 37°C . The formazan crystals that formed at the bottom of the wells were re-suspended in a 1:1 solubilization buffer (20% SDS and 50% dimethylformamide). The absorbance was read at 570 nm using Thermo Scientific Multiskan FC (Fisher Scientific, Waltham MA).

3. Results

The ethyl-acetate extracts of 27 vegetables, spices and herbs were screened to identify those possessing significant amyloid inhibiting activity. A variety of tests are commonly used to assess the ability of a given substance to prevent the formation of amyloid. Each extract was analyzed to determine its ability to prevent ThT binding to IAPP aggregates (Figure 1). ThT binding is a common and facile method for detecting and quantifying amyloid formation. Alone in aqueous solution, ThT shows little fluorescence emission. However, when bound by amyloidogenic aggregates of IAPP, ThT fluorescence increases in a concentration-dependent manner (LeVine, 1993). Therefore, the more amyloid aggregates that form, the greater the amount of ThT fluorescence. In Figure 1, IAPP was incubated alone (IAPP sample) or in the presence of each of the 27 extracts under conditions known to promote amyloid aggregation. After 25 min, each sample was assayed to determine the amount of ThT fluorescence. Many of the extracts showed significant ability to prevent ThT binding.

While ThT binding is commonly used for identifying aggregation inhibitors, it is considered an indirect method. To directly identify the ability of the extracts to prevent amyloid fibril formation, atomic force microscopy (AFM) was performed. AFM allows for the direct detection of amyloidogenic species, such as fibrils, in the presence and absence of each extract. Figure 2 shows the AFM results of the 11 extracts found to prevent IAPP fibril formation. These 11 extracts were found to prevent fibril formation under all conditions tested. The remaining 16 extracts showed little ability to prevent fibril formation (supplemental figure).

To identify extracts capable of protecting mammalian cells from IAPP amyloid toxicity, the MTT assay was performed using HeLa cells (Figure 3). The MTT test quantitates cell viability in the presence of IAPP alone and with each extract. The test also quantitates cell viability in the presence of each extract, without IAPP. The MTT levels of HeLa cells alone were set as 100%. Addition of IAPP to the HeLa cells decreased the cell viability by over 60% in less than 24 h. To ensure that there was no conflict with cell viability, the 25 mM Tris buffer was also tested.

4. Discussion

4.1 Thioflavin T binding

ThT binding to IAPP amyloid (Figure 1) indicated that 18 of the 27 extracts were capable of slowing and/or preventing the aggregation of IAPP under the rigorous conditions known to produce amyloid. The nine extracts from kale, onion, parsley, cumin, crushed red pepper, eggplant, cauliflower, potato and garlic showed little to no ability to inhibit amyloid formation as assessed through ThT binding. The remaining extracts showed significant inhibitory potential. ThT binding by amyloid is a useful diagnostic, routinely used to gauge the ability of a potential therapeutic to prevent amyloid formation. However, many examples exist where the lack of ThT fluorescence was actually due to inhibition of ThT binding to the amyloid, rather than preventing the formation of amyloid (Suzuki et al., 2012). For this reason, additional experiments must be performed to confirm ThT results.

4.2 Atomic Force Microscopy

AFM, which allows the direct visualization of amyloidogenic fibrils (Figure 2), indicated that some of the extracts that successfully prevented ThT binding (Figure 1) were not capable of preventing amyloid fibril formation. Due to the fact that significant amounts of fibrils were observed, 11 additional extracts (yellow bell pepper, broccoli, brussel sprout, carrot, green bean, jalapeno, bok choy, huacatay, cilantro, dill and turmeric) were identified as being non-active (Figure 2). Cauliflower, cumin, and parsley, all of which appeared to fail the ThT test (Figure 1), were found to inhibit fibril formation according to the AFM results (Figure 2). All three of these extracts appeared to have non-amyloidogenic aggregates on the AFM plates, which likely caused the high ThT fluorescence. The remaining extracts in Figure 2 showed significant ability to inhibit fibril formation compared with IAPP alone.

4.3 Cell Rescue

To identify the extracts with the greatest potential to protect living cells from IAPP amyloid, the MTT assay was performed using HeLa cells (Figure 3). The MTT assay is used to quantitate the oxidative capacity of HeLa cells after treatment with toxic IAPP and/or each extract. IAPP added to the cells decreased cellular viability (only about 38% viability after 24 hours of incubation – Figure 3). Of the extracts found to inhibit aggregation *in vitro* (Figures 1 and 2), only mint, peppermint, thyme, and red bell pepper were found to significantly protect HeLa cells from amyloid toxicity. These four extracts were found to be the strongest inhibitors of IAPP aggregation in all tests performed and appeared to have the greatest inhibitory activity of the 27 plants tested.

5. Conclusion

We recently discovered that many fruit extracts possess the potential to inhibit IAPP aggregation, but only a few have significant inhibitory activity (Kao et al., 2015). Likewise, this study indicated that many vegetables, spices and herbs possess some ability to inhibit IAPP aggregation, yet only a few displayed significant therapeutic potential. The results from this comparative study indicated that mint, peppermint, thyme and red bell pepper displayed the greatest amyloid inhibiting functionality of the 27 samples tested.

As described previously, the use of ethyl acetate as the extraction solvent has the benefit of removing over 99% of the total carbohydrate of each extract while retaining (and concentrating) functional natural products such as polyhydroxylated phenols and catechins (Kao et al., 2015). As individuals suffering from type 2 diabetes must control their sugar intake, this extraction process could be used to produce a therapeutically viable form from each plant. More work will be required to identify the active substance(s) from these extracts. Knowing whether it is a single component, or a mixture of many, could be useful for future attempts at herbal formulations.

We believe this is the first study to compare the anti-amyloidogenic properties of a wide collection of vegetables and herbs. These results indicate that many natural product sources have the potential to inhibit amyloid aggregation, but only a small subset are capable of significant activity. While future tests will be needed to determine the physiological effects

of these extracts on patients suffering from type 2 diabetes, we believe these results will help to narrow the search for substances that can slow the aggregation of amyloidogenic IAPP and possibly slow, if not prevent altogether, the progression of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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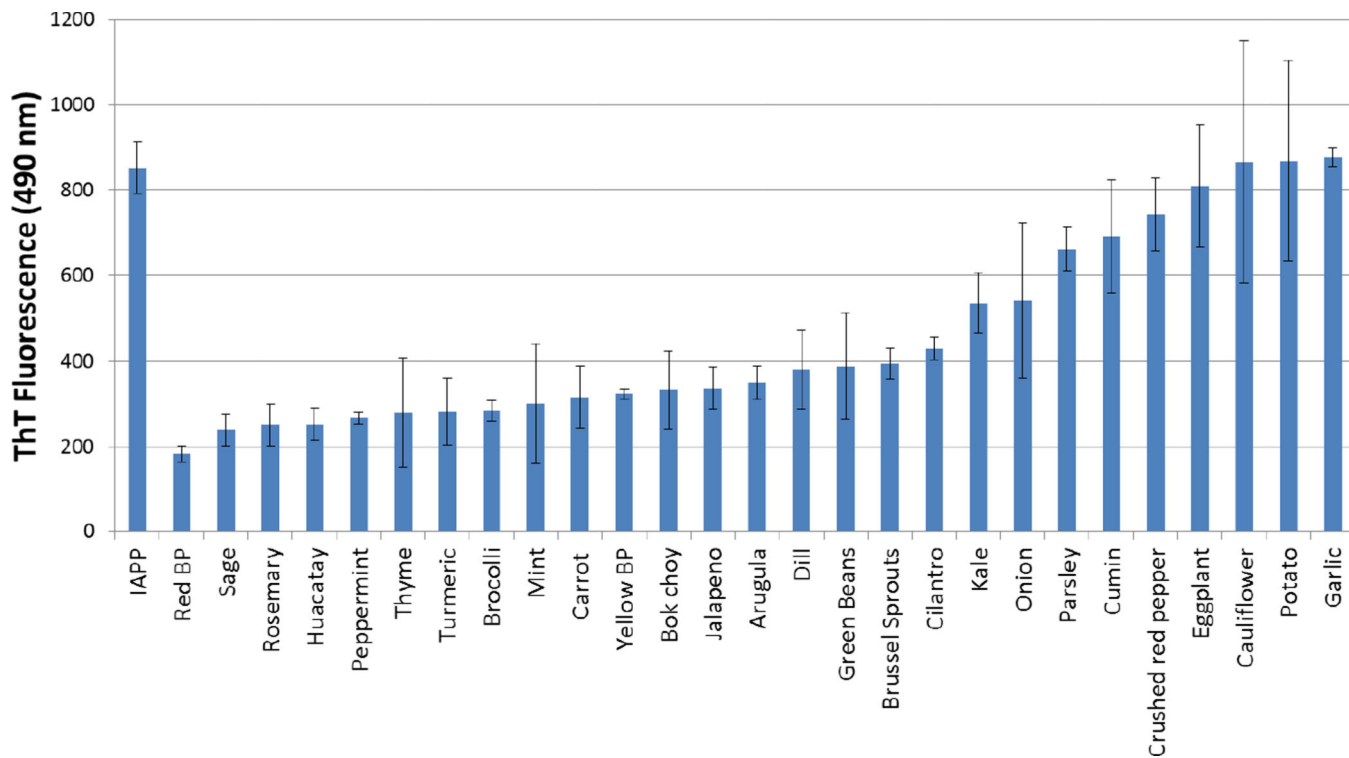


Figure 1. Thioflavin T fluorescence of IAPP alone and IAPP in the presence of each food extract. Fluorescence was measured after 25 min incubation under conditions known to promote IAPP aggregation. IAPP concentration was 106 μ M for each sample. Data is the average of at least 3 data sets. Error bars show the standard deviation from the average of three or more data sets.

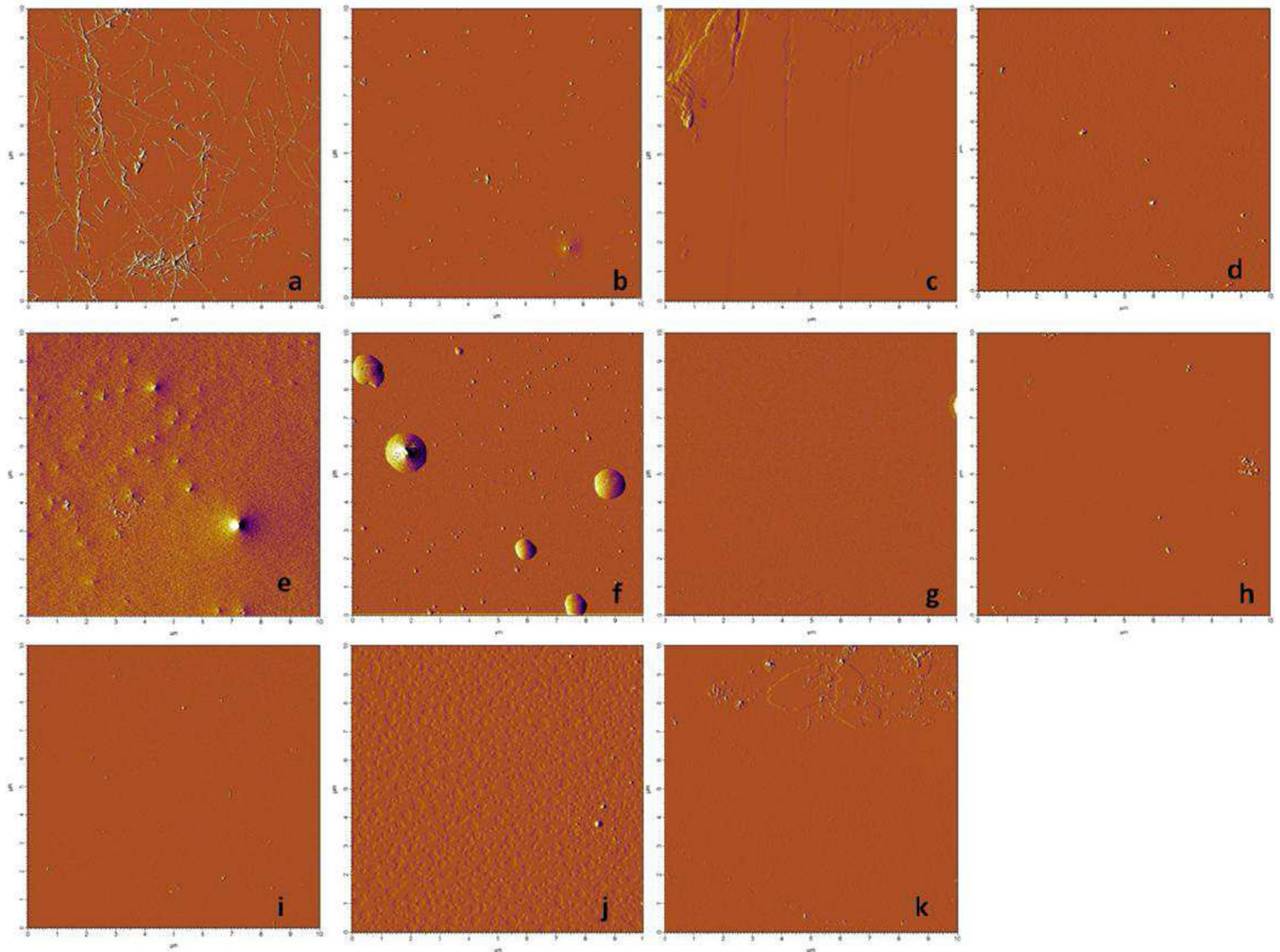


Figure 2.

Atomic force microscope images of plant extracts showing significant ability to inhibit IAPP fibril formation. a) IAPP alone b) arugala c) cauliflower d) peppermint e) red bell pepper f) cumin g) mint h) thyme i) rosemary j) sage k) parsley. Scale is $10\ \mu\text{m} \times 10\ \mu\text{m}$ for each amplitude image.

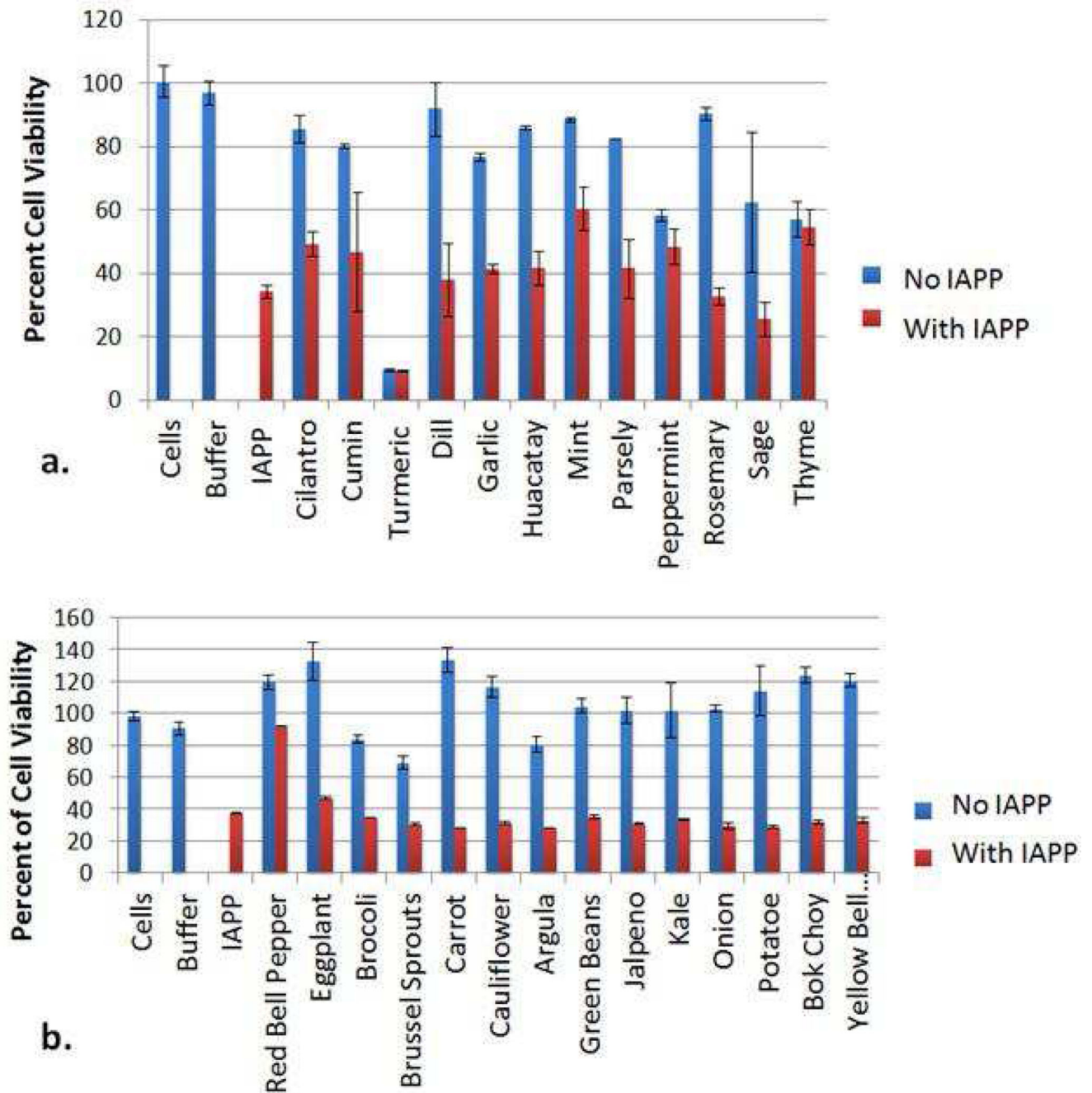


Figure 3.

MTT assay to test HeLa cell viability with the addition of IAPP (red bars) with or without (a) spice and herb extracts and (b) vegetable extracts. Blue bars show the effect of each extract on HeLa cells in the absence of IAPP. Error bars represent the standard deviation from three separate trials. Viability $>100\%$ suggest higher levels of cellular respiration under that condition.

Table 1

Plant extracts screened for their ability to inhibit IAPP aggregation.

Spices/Herbs	Genus	Species	Family
Red Pepper	<i>Capsicum</i>	<i>annuum</i>	Solanaceae
Mint	<i>Mentha</i>	<i>sachalinensis</i>	Lamiaceae
Cumin	<i>Cuminum</i>	<i>cuminum</i>	Apiaceae
Turmeric	<i>Curcuma</i>	<i>longa</i>	Zingiberaceae
Cilantro	<i>Coriandrum</i>	<i>sativum</i>	Apiaceae
Parsley	<i>Petroselinum</i>	<i>crispum</i>	Apiaceae
Sage	<i>Salvia</i>	<i>officinalis</i>	Lamiaceae
Thyme	<i>Thymus</i>	<i>vulgaris</i>	Lamiaceae
Dill	<i>Anethum</i>	<i>graveolens</i>	Apiaceae
Rosemary	<i>Rosmarinus</i>	<i>officinalis</i>	Lamiaceae
Garlic	<i>Allium</i>	<i>sativum</i>	Amaryllidaceae
Huacatay	<i>Tagetes</i>	<i>minuta</i>	Asteraceae
Peppermint	<i>Mentha</i>	<i>piperita</i>	Lamiaceae
Vegetables			
Eggplant	<i>Solanum</i>	<i>melongena</i>	Solanaceae
Potato	<i>Solanum</i>	<i>tuberosum</i>	Solanaceae
Onion	<i>Allium</i>	<i>cepa</i>	Amaryllidaceae
Kale	<i>Brassica</i>	<i>oleracea</i>	Brassicaceae
Red Bell Pepper	<i>Capsicum</i>	<i>annuum</i>	Solanaceae
Brussel Sprouts	<i>Brassica</i>	<i>oleracea</i>	Brassicaceae
Green Bean	<i>Phaseolus</i>	<i>vulgaris</i>	Fabaceae
Arugula	<i>Eruca</i>	<i>sativa</i>	Brassicaceae
Carrot	<i>Daucus</i>	<i>carota</i>	Apiaceae
Bok Choy	<i>Brassica</i>	<i>rapa</i>	Brassicaceae
Yellow Bell Pepper	<i>Capsicum</i>	<i>annuum</i>	Solanaceae
Jalapeno	<i>Capsicum</i>	<i>annuum</i>	Solanaceae
Broccoli	<i>Brassica</i>	<i>oleracea</i>	Brassicaceae
Cauliflower	<i>Brassica</i>	<i>oleracea</i>	Brassicaceae