Activation of Nrf2 Pathway by Natural Products

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Abstract

Oxidative stress is a cellular condition that has been linked to various medical conditions such as Alzheimer's disease, Parkinson's disease, chronic obstructive pulmonary disease (COPD), cancer, and many more. The primary cellular pathway responsible for mitigating the harmful effects of oxidative stress is the Nrf2-dependent antioxidant system as shown in Figure 1. Nrf2 is a transcription factor that is translocated to the nucleus in response to oxidative stress, resulting in its binding to the response elements of target genes, causing wide-scale induction. These target genes generally code for enzymes that play a role in neutralizing the damaging chemical species. Classical antioxidant products act by facilitating this translocation, which is described as the "early response". Termination of the response (the "delayed response") is mediated by Nrf2 export from the nucleus, which is controlled, in part, by the enzyme Glycogen Synthase Kinase-3b (GSK-3b). Although many activators of the pathway have been identified, very few natural compounds have been found that effect the NRF2 pathway via the delayed response, and even less is know regarding the combined effects of both. The objective of this study was to examine a broad range of natural products in an attempt to observe enhanced NRF2 activation due to synergistic action of multiple compounds. Several combinations have been identified, which will be highlighted in this research presentation.

Background

Certain fruits and vegetables have been found to contain many antioxidant properties, which significantly destroy the reactive oxygen species that cause oxidative stress. In this study, garlic, sage, and acai berry have all been identified as having antioxidant properties. After identifying these products as antioxidants, they are investigated for potential synergistic compounds.

Garlic: The valuable health effects of garlic in treating various diseases and illnesses including cancer and cardiovascular disease have been known for centuries. In fact, in the Egyptian Cordex Ebers, a document written about 3500 years ago, it is written that garlic was used to treat heart diseases, tumors, and headaches. More recently, several clinical trials have found that garlic contains antioxidant compounds that reduce oxidative stress. For example, a study published in the *Journal of Nutrition*[1] found that garlic contains various organosulfur compounds, which showed radical scavenging activity in multiple assay types. Throughout this research presentation, the antioxidant activity and synergistic effects of garlic will be discussed.

Sage: Previous studies have found that sage is one of the most potent spices as a natural antioxidant. It has been used throughout history to lower cholesterol and build up the immune system, but its antioxidant properties have just recently been researched. In a study by Cuvelier, Berset, and Richard, it was found that sage is one of the more antioxidant-rich spices and showed more radical scavenging activity than other herbs, such as rosemary, ginger, and cilantro.[2] It is currently thought that the main antioxidant components in sage are carnosol and carnosic acid.[3]

Acai berry: Studies have found that the components of the acai berry can stimulate the Nrf2 pathway, leading to increased antioxidant transcription. It is thought to be due to the bioactive phytochemical components found in the berry.[4]

Kenpaullone: This compound is an inhibitor of cyclin-dependent kinases 1,2,5 and GSK-3B.[5] Due to the inhibitory effects on GSK-3B, it allows the Nrf2 pathway to continue, activating it more. This is because GSK-3B is the kinase that phosphorylates Nrf2, terminating the pathway.

Methods

Preparation of Samples

0.1g of garlic was dissolved in 200 uL of water. A 1:5 serial dilution in water was then performed to make 4 more samples.

0.1g of sage was dissolved in 200 uL of water. A 1:5 serial dilution in water was then performed to make 4 more samples.

1.0g of pure acai berry powder was dissolved in 3 mL of ethanol and centrifuged. 500 uL of the supernatant was drawn out and combined with 500 uL of water. 500 uL was taken from that mixture and combined with 500 uL of water to create a 1:2 ratio. 500 uL of that was taken and combined with 500 uL of water to create a 1:4 ratio, and again to create a 1:16 ratio.

5 mM Kenpaullone was diluted 1:10 in water.

Cell Culture

Genetically modified human liver cells, HepLuc, were plated in a T75 culture plate using 12 mL of complete EMEM media with 144 uL of geneticine. Plate was placed in incubator for about 2 days, until cells showed 70-80% confluency as seen in Figure 2. Once cells reached this confluency, media was drawn out. 3 mL of trypsin was measured into the T75 and the plate was placed in the incubator for 5 minutes. The 3 mL of trypsin and cells were drawn out and combined with 22 mL of complete media and geneticine. 10 mL was used to put in a 96 well plate, with 100 uL in each well, and the remaining 12 mL were placed in another T75 to continue the cell line. The cells were allowed to grow in the incubator for 3 more days. When the T75 showed confluency, the 96 well plate was treated with the samples.

Treatment

In a 48 well plate, 500 uL of complete media was placed in each well. 20 uL of each dilution sample was pipetted into separate wells. The media of the confluent 96 well plate was vacuumed out, and 100 uL of each sample was pipetted in groups of 4 into the 96 well plate as shown in Figure 3. The plate was incubated for 20 hours.

Luminescence Assay

After 20 hours, the media was drawn out of the 96 well plate. 40 uL of plain EMEM was added to each well, followed by 40 uL of luciferase glo reagent. The plate was allowed to shake on a microplate shaker for 12 minutes at 300 speed. The plate was then placed in the plate reader, as shown in Figure 4, and the luminescence program was used to analyze the activity.

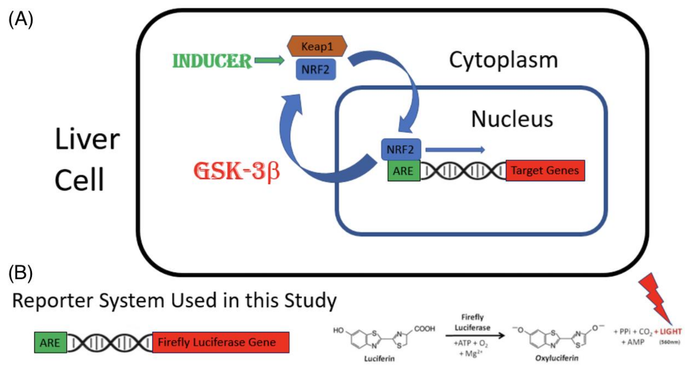

Figure 1. Antioxidant response pathway. (A) The nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that is activated by oxidative stress. It responds by binding to the antioxidant response element (ARE) which is located in the promotor of genes that code for antioxidant enzymes. This causes a wide-scale induction of the target antioxidant genes. When there is no oxidative stress, Keap1 is formed and acts as a regulator that targets Nrf2 for degradation. Glycogen synthase kinase (GSK-3B) is responsible for the termination of the antioxidant response. GSK-3B phosphorylates Nrf2 which results in the degradation of Nrf2. **(B)** The system used in this study to measure the activity of the Nrf2 pathway is the firefly luciferase gene. The human liver cells were genetically modified so the target gene of the Nrf2 pathway was the firefly luciferase gene. Then, when the cells were treated with samples and the firefly luciferase reagent, the activity could be measured by the luminescence emitted from the firefly luciferase gene being activated through the pathway.

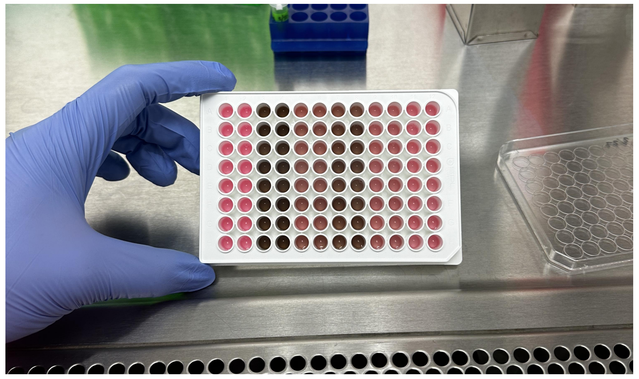

Figure 3. Cell treatment of acai berry. The 96 well plate is shown immediately after treatment of acai berry and kenpaullone sample dilutions.

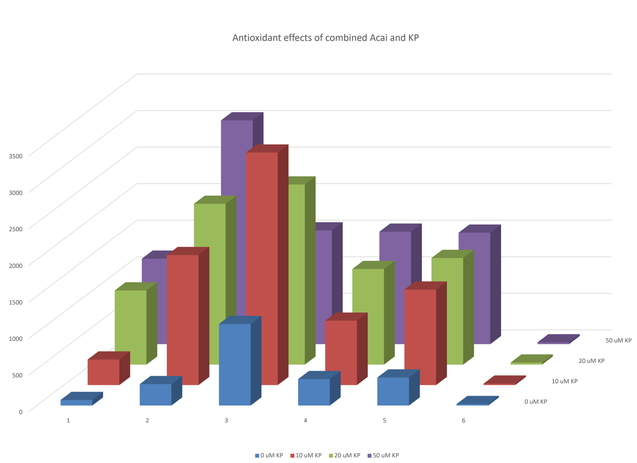

Figure 5. Antioxidant effects of combined Acai and KP. This figure shows the highest activity in the cells treated with 50 uM KP combined with the 1:2 dilution of acai berry powder.

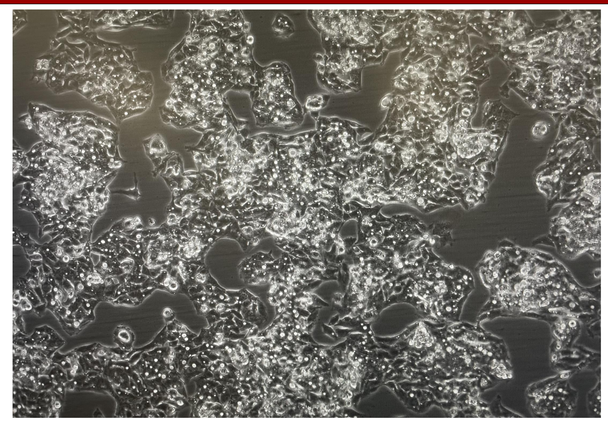

Figure 2. Confluency of cells on culture plate. The cells are shown at 80% confluency under a microscope on a T75 culture plate. At this confluency, they are ready for treatment.

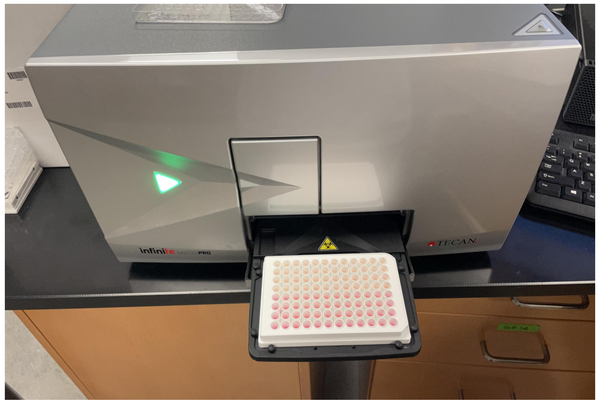

Figure 4. Plate reader. The treated 96 well plate is shown going into the plate reader.

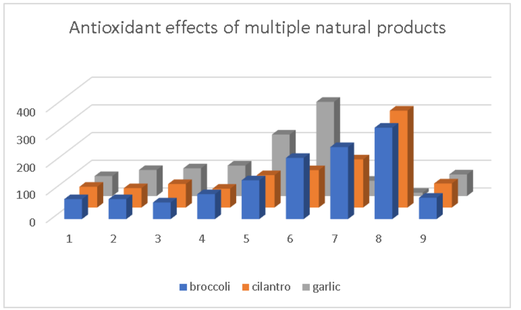

Figure 6. Antioxidant effects of multiple natural products. This graph shows the increasing concentrations of broccoli, cilantro, and garlic and their effects on the Nrf2 pathway activation. 1 shows a 1:128 dilution, 2 is 1:64, 3 is 1:32, 4 is 1:16, 5 is 1:8, 6 is 1:4, 7 is 1:2 and 8 is crude

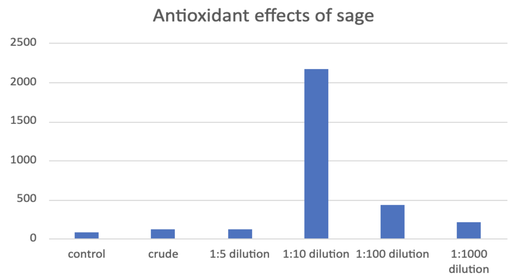

Figure 7. Antioxidant effects of sage. This figure shows the effects of different dilutions of sage on the Nrf2 pathway.

Results & Discussion

Results and Discussion

Garlic, acai, sage, broccoli, and cilantro all showed dose-dependent activation of the NRF2 anti-oxidant pathway in cultured human hepatoma cells. This was demonstrated using a luciferase-based transcriptional model shown in figure 1, where the firefly luciferase gene could be produced in response to the presence of antioxidant compounds. Figure 2 shows an image of the Hepatoma cells used in the experiment growing on a clear plastic surface, and figure 3 shows the 96-well plates that were used when measuring the induction of the luciferase gene in response to extracts of the acai berry. Figure 4 illustrates the instrument used to detect light coming off from each sample, which is how activation of the luciferase gene was quantified. More light means more luciferase was produced, which means more activation of the anti-oxidant response in the cells.

Figure 5 shows the combined antioxidant effects of acai and KP, where the highest antioxidant activity is seen with 20 uM KP and a 1:2 dilution of a crude 50% ethanol extract from acai berry powder. What is significant in this image is that KP alone or the acai sample alone could produce a signal of the same magnitude, suggesting some type of molecular synergy. This is the type of synergy predicted based on the model shown in figure 1 where we propose that acai extract activates the early response, while KP inhibits the delayed response. In other words, a chemical in the acai extract causes the release of Nrf2 to the nucleus of the cell where it induces genes controlled by an ARE. KP, on the other hand, inhibits the mechanism for export of the Nrf2 from the nucleus, resulting in extended time in the nucleus, and thus enhanced activity. Additional plant samples were screened for their ability to activate this pathway and several crude extracts showed prominent activities. Figure 6 shows the activity of broccoli, cilantro, and garlic. The activity seems to increase as the concentration gets higher for all three of these products. More specifically, broccoli at its peak showed a 4-fold increase in activity. The cilantro also showed a 4-fold increase in activity at the highest concentration. Both broccoli and cilantro showed the highest activity in the crude sample. Garlic peaked at a 1:4 dilution with a 5-fold increase compared to the control group. In figure 7, the antioxidant effects of sage are shown. This figure shows a 26-fold spike in activity at a 1:10 dilution. Overall, the selected plant extracts increased the activation of the Nrf2 pathway when compared to no added plant extracts. Experiments to probe potential synergistic action with any of these crude plant extracts are planned, along with studies intended to provide more detailed mechanistic information concerning the synergy observed with KP. Ultimately, an understanding of this synergy may provide opportunities to maintain or improve human health through nutritional and or nutraceutical strategies targeting the Nrf2 antioxidant pathway.

Summary

- Several plant extracts were found to contain antioxidant properties.
- The selected plant extracts used in coordination with Kenpaullone have generated a synergistic induction in NRF2 activity.

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