Metabolic Study of Ginsenoside Rb_1 in Brine Shrimp

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Introduction

Ginseng, an herb with Chinese, Korean, and American variants, has been used as a supplement and a multipurpose medicine by many people (Sivula, 2012). The herb is considered highly medicinally beneficial and is often implemented in tea commercially. The Chinese variant, *Panax ginseng*, was discovered in Manchuria in China five-thousand years ago and now holds a valuable place in traditional Chinese medicine (Hemmerly, 1977). The genus "*Panax*" is derived from a Greek word meaning "universal health" (Court, 2000), indicating the nature of the herbs it encapsulates. Chinese ginseng, according to traditional Chinese medicine, has a warming effect on the body, while American ginseng (*Panax quinquefolius*) has a cooling effect (Sivula, 2012). The nature of this "cooling" and "warming" effect is rather ambiguous, however, and prompts further study.

According to Shin *et al.*, ginseng's active ingredients include saponins, polyacetylenes, polysaccharides, phenols, sesquiterpenes, alkaloids, oligosaccharides, oligopeptides, aminoglycosides, and flavonoids (2000). Ginsenosides fall under the category of saponins; the saponins found in ginseng are categorized as ginsenosides, notoginsenosides, and gypenosides (Hong *et al.*, 2009). Ginsenosides are derivatives of a thirty-carbon molecule called triterpenoid dammarane (Nah, 2014) and they are divided into three categories: protopanaxadiol ginsenosides (PPD), including Ra_1 to Ra_3, Rb_1 to Rb_3, etc.; protopanaxatriol ginsenosides (PPT), including Re, Rg_1, Rg_2, etc.; and oleanolic acid ginsenosides such as ginsenoside Ro (Fatmawati *et al.*, 2014). The main difference between protopanaxadiol and protopanaxadiol ginsenosides is that protopanaxatriol ginsenosides have a hydroxyl group at the sixth carbon. Oleanolic acid ginsenosides have a pentacylic triterpene skeleton (Nag *et al.*, 2012). These different ginsenoside

types have various different properties that may be the cause of the multi-purpose nature of the herb.

Ginsenoside Rh_2, for example, has shown to be an aldose reductase inhibitor (Fatmawati *et al.*, 2014), which may allow it to prevent complications involving diabetes by slowing down the NADPH-dependent reduction of glucose to sorbitol in the rate determining step of the polyol pathway. Ginsenoside Rd has been shown to slow tau protein phosphorylation through the PI3K/AKT/GSK-3b pathway (Zhang *et al.*, 2014), protect SH-SY5Y cells from injury caused by 1-methyl-4-phenylpyridinium (Liu, Y *et al.*, 2015), and improve learning and memory in amyloid β-protein precursor (APP) transgenic mice (Liu, J. *et al.*, 2015). Ginsenosides are also capable of causing various anti-cancer properties: ginsenoside Rb_1 and Rb_2 have anti-angiogenic and slightly anti-proliferative properties, compound K (a metabolite of ginsenoside Rb_1) can inhibit cancer cell growth, ginsenoside Rg_1 is anti-proliferative, ginsenoside Rg_2 is anti-genotoxic, ginsenoside Rg_2 is anti-proliferative and apoptotic, ginsenoside Rh_1 is strongly antiproliferative, and ginsenoside Rh_2 is strongly apoptotic (Nag *et al.*, 2012). Ginsenoside Rg_1 has also been found to attenuate obesity in zebrafish (Koh *et al.*, 2015). The multiple medicinal benefits of ginsenosides imply that the compound could potentially have more medicinal benefits, as researchers continue to discover more medical uses of the compound.

Current research is more focused on studying zebrafish. The organism is native to streams in India, where it is commonly kept as a pet (Wixon, 2000). The males are slender, torpedo-shaped and have black, longitudinal stripes, and have a gold coloration on their bellies and fins. Females appear similar, but do not have the gold markings that the males do, and are larger when carrying eggs (Wixon, 2000). Zebrafish have a relatively short generation period of three months, allowing relatively fast study, with a great amount of data available for

experiments across multiple generations. They can be used for tumor and cancer research, as well as various other large-scale physiological ailments, such as wounds or illness. For example, Hong *et al.* have discovered through research on zebrafish that while ginsenosides can have an anti-angiogenic effect against cancerous cells, the compound may actually stimulate angiogenesis in normal, healthy cells (2009), causing it to be significant for wound healing purposes. This is confirmed by Kim *et al.*, who studied ginsenoside Rd's wound healing effect. Zebrafish are also useful as an organism of study because they have been researched thoroughly in terms of growth markers and other physiological indicators; Ulloa *et al.* studied how RNA sequencing affected gene expression, Dupret *et al.* studied the protein Pcgf1 in zebrafish and its effects on aging (2016), and Kettleborough *et al.* examined and analyzed the mechanism of zebrafish protein coding genes and how they operate (2013). Due to the existing research on zebrafish, testing ginsenosides on zebrafish is useful when studying ailments on a larger scale.

However, if brine shrimp are able to metabolize ginsenosides they may be a qualified organism for ginsenoside study. Brine shrimp are brachiopods that develop dormant cysts in unfavorable growing conditions that can remain viable for up to 25 years. They are aquatic organisms that are first-level consumers in the ocean ecosystem. Brine shrimp could be a better organism for study because they are easier to grow and maintain, and they generate less animal cruelty concerns than zebrafish. They also are easy to observe since their transparency allows their organs to be easily studied under a microscope (Boman, K. and Shmaefsky, B., 1997). Experimentation can also be conducted using smaller amounts of the treatment compound, allowing expensive compounds such as ginsenosides to be used in smaller quantities, which leads to more inexpensive research, which would be beneficial to both universities and research corporations alike. They grow extremely quickly, with nauplii ready for experimentation after

only two days of incubation. Brine shrimp are also useful in applications in the real world, as they are cultivated as fish food and are first-level consumers in the ocean ecosystem (De Los Ríos, P., & Gajardo, G., 2004) meaning that there could potentially be a direct application of a study done on brine shrimp, though perhaps not in this particular case, as brine shrimp do not come in contact with ginsenosides naturally, and the expensive compound does not pose a threat in terms of exposure due to pollution. Studies conducted on brine shrimp would involve the toxicity test, and tests to see if brine shrimp were more resistant to a compound after exposure to ginsenosides, similar to existing toxicity tests involving other compounds (Hisem *et al.*, 2011). Assuming the ginsenoside is metabolized, brine shrimp vitality after exposure to the compound could be gauged to determine if ginsenosides have positive effects on survivability and life span of brine shrimp.

Many metabolism studies on ginsenosides have already been conducted. The metabolization of ginsenoside Rg_1 was analyzed in *Escherchia coli*, a prokaryotic bacteria species (Wang *et al.*, 2000). Ginsenosides Rk_3, Rh_4, Rb_1, and Rg_1 were analyzed in zebrafish (Wei *et al.*, 2011). In both experiments, the ginsenoside was found to follow a metabolic pathway where each ginsenoside progresses to a different ginsenoside. For example, ginsenoside Rb_1 was found to follow the metabolic pathway seen in figure 1. After the current experiment, which uses one ginsenoside, more ginsenosides will need to be tested in brine shrimp and measured to accurately understand the metabolic pathways utilized by brine shrimp.

In order to test the metabolization of the compound, one needs both the ginsenoside and at least one metabolite of said ginsenoside in order to detect progress through the metabolic pathway. The metabolite ginsenosides allow one to create standard solutions and subsequently recognize that the metabolite is present in experimental samples. In this experiment, ginsenoside

Rb_1 was used. Ginsenoside Rb_1 has chemical formula $C_{54}H_{92}O_{23}$, has a molecular weight of 1109.307 grams per mole, and has metabolites ginsenoside Rd and Rg_3, progressing through the metabolic pathway shown in figure 1.

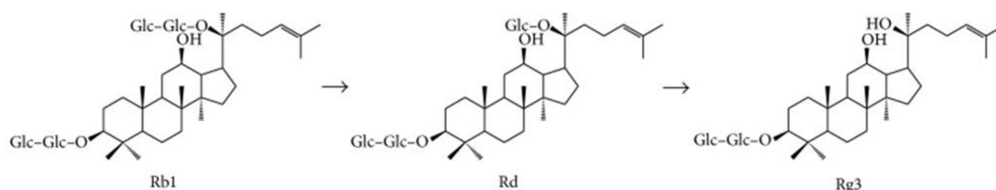

Figure 1: Metabolic pathway of ginsenoside Rb_1 Chen et al., 2016.

As seen above, ginsenoside Rb_1 progresses to ginsenoside Rd by hydrolysis of one glycoside. Ginsenoside Rd then progresses to ginsenoside Rg_3 by hydrolysis of another glycoside.

Having two metabolic derivatives of ginsenoside Rb_1 to determine the metabolization of the compound allows the process to be more accurately determined, as the compound could potentially progress past ginsenoside Rd to Rg_3 before the compound could be detected due to various factors, such as a fast metabolism or an easily metabolized compound. With only the metabolite ginsenoside Rd, if the metabolic pathway were to progress past Rd, the metabolite after ginsenoside Rd would be unknown and unverifiable until the same analysis process was conducted with the metabolite ginsenoside Rg_3. Likewise, with only the metabolite ginsenoside Rg_3, if the metabolic pathway did not progress to Rg_3, the metabolization would not be noticed until ginsenoside Rd was examined.

A process called high performance liquid chromatography was used to evaluate whether or not the ginsenoside was metabolized. High performance liquid chromatography runs a mobile phase solvent over a stationary phase column. The solvent can be water, acetonitrile, or various different solvents. The stationary phase used in this experiment was a Zorbax C-18 column. Compounds that are more similar in intermolecular forces with the column than the solvent will

bind to the column and have a longer retention time. Compounds that are different from the column will leave the column earlier. A diode array detector at the end of the column detects the absorbance of the compounds leaving the column. This process is especially useful for separating samples into peaks with different retention times. The particular type of high performance liquid chromatography used in this experiment was reversed phase high performance liquid chromatography. Reversed phase high performance liquid chromatography involves a column that is covalent with solvents that are polar and nonpolar on a gradient.

Experimental procedures in the experiment were similar to an experiment conducted by Wei *et al.*, who conducted their experiment on zebrafish rather than brine shrimp (2011). Procedures were modified for brine shrimp metabolization rather than zebrafish metabolization. Concentrations were increased to maximize ginsenoside intake of the brine shrimp.

Purpose

The metabolization of ginsenoside Rb_1 in brine shrimp was analyzed in order to determine whether or not ginsenosides were metabolized. The purpose of this experiment is to evaluate the viability of brine shrimp as an organism for ginsenoside study. Brine shrimp would aid in experimentation on anti-toxicity of ginsenosides. In addition, brine shrimp are an easier organism to study because they are smaller organisms that do not require as large an amount of a compound for treatments, they are easy to care for in a laboratory setting, they generate less animal cruelty concerns, and they may have direct applications to the ocean environment.

Research Question

Are brine shrimp capable of metabolizing ginsenoside Rb_1?

Hypothesis

Ginsenoside Rb_1 will be metabolized by brine shrimp. Approximately 10% of ginsenoside administered will be metabolized. As a result of this, brine shrimp will be a viable organism for ginsenoside study regarding potential anti-toxic effects of the compound. Metabolization will be detected through peaks for the metabolite ginsenosides in the chromatogram in high performance liquid chromatography.

Materials

- 50 mg ginsenoside Rb_1 (Cayman Chemical)
- .1 mg ginsenoside Rd (Cayman Chemical)
- .1 mg ginsenoside Rg_3 (Cayman Chemical)
- 1 L high performance liquid chromatography grade acetonitrile (Merck Millipore)
- 10 mL formic acid (Sigma-Aldrich)
- 6 L deionized water (from laboratory
- 2.5 g Sodium chloride
- 50 mL 90% methanol (from laboratory)
- P-1000 pipet (Labnet)
- P-1000 pipet tips (Art Molecular Bioproducts)
- Fluorescent light (from laboratory)
- Air pump (Whisper40 Air Pump)
- 4 100 mL Erlenmeyer flasks (from laboratory)
- Brine shrimp hatchery

- Phosphate-buffered saline (created using laboratory chemicals and chemical from Cayman Chemical)
- Tissue homogenizer (Tissue Tearor Model 985 370)
- Centrifuge (Eppendorf Centrifuge 5418)
- High performance liquid chromatography machine (Hewlett Packard series 1100 high performance liquid chromatograph)
- 1 g brine shrimp cysts (Amazon)
- 1 Zorbax C-18 column, 5 μm, 250 mm × 4.6 mm (Agilent)
- 1% aqueous DMSO (Sigma-Aldrich)
- Analytical balance (Sargent-Welch)
- 30 2 mL Eppendorf tubes
- 10 10 mL falcon tubes
- Vortexer (Vortex-Genie 2)
- Agilent Chemstation Rev. B.04.03-SP2[108]
- 25 high performance liquid chromatography vials
- 5 L high performance liquid chromatography solvent bottle
- 2 L high performance liquid chromatography solvent bottle

Methods

High performance liquid chromatography solvent preparation

High performance liquid chromatography solvents were prepared by adding 0.5 mL formic acid to water and acetonitrile in 1 L volumetric flasks and filling the volumetric flasks with the respective solvents to the 1 L mark. A higher amount of water was needed than the

acetonitrile, so 5 L of the water was prepared, and 2 L of the acetonitrile was prepared. Solvents were filtered using a vacuum pump, sidearm flask, filtration funnel, and 0.45 µm filter to prevent any contaminants from affecting the experiment.

Brine shrimp growth media preparation

2.5 grams of sodium chloride was dissolved in approximately 1 L of deionized water to create a solution containing 2.5 percent sodium chloride, or 25 parts per thousand, which is the optimal salinity for brine shrimp hatching. The aqueous sodium chloride was then added to a brine shrimp hatchery. Sodium hydroxide was used to increase the pH to 8.0, the optimal pH for brine shrimp hatching.

Brine shrimp incubation

The brine shrimp were incubated at 28 ± 5° in the prepared salt solution. They were aerated with an air pump for hatching. A fluorescent lamp was placed above the brine shrimp as part of the hatching process. Brine shrimp were given two days to hatch. After hatching, 5 x 0.5 mL of approximately 1500 brine shrimp/mL salt water (15 brine shrimp in 10 µL) was obtained from the hatchery and placed in five 2 mL Eppendorf tubes for treatment.

Brine shrimp treatment

Ginsenoside Rb_1 was dissolved in 1% dimethyl sulfoxide at concentrations of 50, 100, 200, and 400 parts per million. 0.5 mL of each concentration were added to brine shrimp cultures in different Eppendorf tubes, resulting in a total volume of 1 mL in the 2 mL Eppendorf tubes. The brine shrimp were then treated on a bench-top shaker shaking at approximately 2 Hz.

Sample preparation

After 1 day of treatment, the samples were centrifuged at 16670 rpm. As much supernatant as possible was removed with a P-1000 pipet and transferred to an Eppendorf tube and filtered into high performance liquid chromatography vials to use as samples. The remaining 3 mL brine shrimp were then transferred to small falcon tubes with 7 mL phosphate buffered saline. The solutions of brine shrimp and phosphate-buffered saline were then homogenized with a Tissue Tearor 985 370. The resulting solutions were filtered through a 5 µm filter into high performance liquid chromatography vials.

High performance liquid chromatography

First, the high performance liquid chromatography machine ran water through the machine at a rate of 0.01 mL/min overnight to purge the column. The samples were then run through high performance liquid chromatography with 0.05% aqueous formic acid as solvent A and 0.05% acetonitrile formic acid as solvent B on a step gradient from 0% solvent B to 80%

Table 1: High performance liquid chromatography method. Time is shown in minutes on the left. % of solvent B (acetonitrile formic acid, .05%) is shown on the right.

Time (min)	**Gradient (% B)**
0–5	0–7
5–15	7–17
15–17	17–20
17–21	20–21
21–37	21
37–45	21–29
45–60	29–35
60–70	35–65
70–85	65–80
85–90	80
90–95	80–0
95–100	0

solvent B at a flow rate of 0.7 mL/min. The full gradient is shown in Table 1. The analysis was

conducted on Agilent Chemstation Rev. B.04.03-SP2[108]. After the chromatography was finished, a solution containing 70% water and 30% acetonitrile was run through the column to prepare it for storage.

Results

It was observed that brine shrimp treated with ginsenosides were qualitatively more active than the ones that were not treated. Brine shrimp treated with more ginsenoside (100, 200 parts per million) were significantly more active than the brine shrimp control.

The methanol blank had a high number of peaks after 80 minutes, and the expected noise could be observed in the chromatograms of the standard solutions. Peak area for ginsenoside Rb_1 at 68.3 minutes was 37.7 AU. Ginsenoside Rd at 1000 parts per million was detected at 68.77 minutes, but peak area could not be obtained due to sample deterioration and saving of incorrect frequency in Agilent Chemstation for analysis. The program does not support the selection of frequency after high performance liquid chromatography runs. Ginsenoside Rg_3 at 1000 parts per million was detected in standard solution at 70.73 minutes, but peak area was also not detected.

Ginsenoside Rb_1 was the only peak found in the supernatant, and a peak was detected in the homogenized brine shrimp. Peaks for metabolite ginsenosides could not be found in both the homogenized brine shrimp or the supernatant.

Discussion

As expected, the average retention time for ginsenoside Rb_1 was 69.98 minutes with the Zorbax C-18 column. This follows closely the time presented in Wei *et al.*'s experiment, which was 48.83 minutes at a higher flow rate of 1 mL per minute (2011). The flow rate and retention

time for identical methods are inversely related, so the theoretical retention time expected would be 69.76 minutes, indicating a 0.3 percent error in comparison to the previous experiment.

Though the data regarding brine shrimp activity was qualitative, it could indicate that further research may be necessary to verify if ginsenosides may allow brine shrimp to be more resistant to chemicals such as dimethyl sulfoxide, though dimethyl sulfoxide should not be toxic to brine shrimp until concentration reaches eight percent.

The concentration of ginsenoside Rb_1 in the brine shrimp samples was likely too low for proper detection in high performance liquid chromatography, as maximum concentration in the supernatant was 400 parts per million, which provided a peak in high performance liquid chromatography, but because the homogenate was further diluted with phosphate buffered saline, the homogenate had a lower concentration of ginsenosides and a peak for ginsenosides was not detected in the homogenate. Sample deterioration most likely posed an issue, as it is recommended to store ginsenosides in solution for no more than a day. Ginsenoside was verified as being present in standard and experimental samples, but peaks for possible metabolites were too small to identify as peaks rather than noise. Data indicates that the ginsenoside may not have been metabolized, as peaks for metabolic derivatives of ginsenoside Rb_1 in the samples did not appear.

The data obtained from this experiment indicates that original hypothesis was not correct. However, it is possible that the ginsenoside concentration was not great enough for it to be metabolized, or that the compound was metabolized but the samples deteriorated. Thus, it is still possible that brine shrimp may metabolize ginsenosides. However, no ginsenosides were detected in the sample compounds, indicating that the concentration had decreased so that the

peak area was small enough to be mistaken as noise. As a result, no hard conclusion can be drawn, since data cannot support or reject the null hypothesis.

The qualitative observation of the brine shrimp's activeness and motion is not substantial enough to show that ginsenosides were indeed metabolized, as it is possible that this was either an observational error or that it was a coincidence that this occurred.

Sources of Error

The ginsenosides could have deteriorated over time, resulting in less ginsenosides being detected. Some of the brine shrimp may not have been healthy, which would result in the organism metabolizing the compound differently than it would had the shrimp been healthy. The high performance liquid chromatography machine was also experimenting power failures for an unknown reason during overnight runs. The power failure could potentially be attributed to the power source in the laboratory being shut down overnight, causing the computers connected to the high performance liquid chromatography machine and the machine itself to lose power and fail. In addition, measuring milligrams of ginsenoside was extremely difficult, as the quantity of ginsenoside had to be measured by hand. It is also possible that the metabolites were indeed present, but that they combined with ginsenoside Rb_1 in the supernatant samples to create one large peak. Another source of error presents itself in the high performance liquid chromatography column. The column was a used column, as the columns are expensive and cannot be easily obtained. As a result, residue from previous runs may have affected the experiment, despite the conducting of purging procedures.

Conclusion

Data indicates brine shrimp may not be able to metabolize ginsenosides, but the ginsenoside concentration was not enough to definitively determine whether or not it was metabolized. Data failed to both confirm and deny the null hypothesis, but supports the null hypothesis more than it denies it. However, the understanding that the ginsenoside concentration was not sufficient will aid in future experiments to help future experimentation on brine shrimp metabolization to become successful, knowing that the concentration must be increased to higher amounts. Brine shrimp are capable of surviving dimethyl sulfoxide concentrations of up to 8 percent, allowing the ginsenoside concentration used to be 800 percent greater. Reconducting the experiment may allow experiments on ginsenoside anti-toxicity if the resulting conclusion is that brine shrimp can metabolize ginsenosides. The experiment should be conducted with a high performance liquid chromatography machine that is working properly. A mass spectrometer could also be used.

Further Work

To determine definitively whether or not the brine shrimp may metabolize ginsenosides, the experiment should be repeated using higher concentrations of ginsenosides. Brine shrimp can survive in water with up to eight percent dimethyl sulfoxide, so the ginsenoside concentration can be eight times greater. Brine shrimp extraction method could also be improved by filtering out the brine shrimp rather than manually using a pipet to remove the supernatant. A properly operating high-performance liquid chromatography machine would also aid in the experiment. Mass spectrometry could also be utilized to examine the compound in greater detail. Mass spectrometry is an analytical process involving launching charged, ionized particles through a

magnetic field. The process allows the exact molar mass of a compound to be detected, allowing for easier verification of what compound was present. Metabolites of ginsenosides would be easier to detect using this method, since one would be able to detect the exact molar mass of the ginsenoside and simply match the molar masses, rather than looking for similar retention times.

A different ginsenoside could also be tested in order to determine if brine shrimp metabolize some ginsenosides but not others. If this were the case, then brine shrimp would be suitable for studying the compounds it could metabolize, and further research would be necessary to determine why some ginsenosides are not able to be metabolized.

After the metabolization of ginsenosides in brine shrimp is confirmed, studies could be conducted to determine if ginsenosides may cause increased survivability for brine shrimp exposed to toxic chemicals. This would aid in determining if ginsenosides have anti-toxin properties, similar to other Chinese medicines. If ginsenosides are found to have anti-toxin properties, they will be even more significant in medicine, as they may be suitable treatments for poisons and other toxic chemicals. Additionally, differing concentrations of ginsenosides can be used to treat the brine shrimp to create a function that relates the number of brine shrimp, the concentration used, and metabolite produced. The function would allow the effect of ginsenosides to be easily determined, as a relation between the variables could then be compared with brine shrimp survivability in a toxic environment if treated with ginsenosides.

However, if the null hypothesis holds true in this experiment, other organisms may also be tested for ginsenoside metabolization. Insects such as flies (*Drosophila melanogaster, Drosophila hydei*) are a potential organism for further experimentation with ginsenosides. Flies are useful in research due to their fast reproductive cycles and their high mutation rate (Ffrench-

Constant *et al.*, 1993). If the experiment with flies is successful, then toxicity and survivability research could also be conducted on flies.

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References

Boman, K., & Shmaefsky, B. (1997). The brine shrimp as a model organism for biology: Arthropods useful in demonstrating properties of organisms. *Journal of College Science Teaching*, 26(5), 358-359.

Chen, B., Wei, Y., Wang, D., & Jia, X. (2015). Metabolism of ginsenosides rk 3 and rh 4 from steamed notoginseng in zebrafish by ultraperformance liquid chromatography/quadrupole-time-of-flight mass spectrometry. *Archives of Pharmacal Research*, *38*(8), 1468-1476.

Chen, X., Zhang, X., Shui, Y., Wan, J., & Gao, J. (2016). Anticancer Activities of Protopanaxadiol- and Protopanaxatriol-Type Ginsenosides and Their Metabolites, *Evidence-Based Complementary and Alternative Medicine, vol. 2016*, doi:10.1155/2016/5738694

Court W. (2000). Ginseng: The Genus Panax. *New York: Harwood Academic Publishers*.

De Los Ríos, P., & Gajardo, G. (2004). The brine shrimp artemia (crustacea; anostraca): A model organism to evaluate management policies in aquatic resources. *Revista Chilena De Historia Natural*, 77(1), 3-4.

Ding, Y., Wang, B., Wen, C., Sun, C., Lee, H., *et al.* (2015). Evaluation of the teratogenic effects of three traditional Chinese medicines, si jun zi tang, liu jun zi tang and shenling baizhu san, during zebrafish pronephros development. *Journal of Toxicologic Pathology*, *28*(3), 141-149.

Duan, C., Ding, J., Li, Q., Tsai, W., & Pozios, K. (1999). Insulin-like growth factor binding protein 2 is a growth inhibitory protein conserved in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America, 96*(26), 15274-15279.

Dupret, B., Völkel, P., Le Bourhis, X., & Angrand, P. (2016). The polycomb group protein pcgf1 is dispensable in zebrafish but involved in early growth and aging. *PloS One, 11*(7), e0158700.

Fatmawati, S., Ersam, T., Yu, H., Zhang, C., Jin, F., *et al.* (2014). 20(s)-ginsenoside rh2 as aldose reductase inhibitor from Panax ginseng. *Bioorganic & Medicinal Chemistry Letters*, *24*(18), 4407-4409.

Hemmerly T. E. (1977). A ginseng farm in Lawrence County, Tennessee. Econ. Bot. 31, 160–16210.1007/BF02866586.

Hisem, D., Hrouzek, P., Tomek, P., Tomšíčková, J., Zapomělová, E., *et al.* (2011). Cyanobacterial cytotoxicity versus toxicity to brine shrimp artemia salina. *Toxicon*, 57(1), 76-83.

Hong, S., Wan, J., Zhang, Y., Hu, G., Lin, H., *et al.* (2009). Angiogenic effect of saponin extract from Panax notoginseng on huvecs in vitro and zebrafish in vivo. *Phytotherapy Research, 23*(5), 677-686.

Kettleborough, R., Busch-Nentwich, E., Harvey, S., Dooley, C., Bruijn, E., *et al.* (2013). A systematic genome-wide analysis of zebrafish protein-coding gene function. *Nature, 496*(7446), 494.

Koh, E., Kim, K., Choi, J., Jeon, H., Seo, M., *et al.* (2015). Ginsenoside rg1 suppresses early stage of adipocyte development via activation of c/ebp homologous protein-10 in 3t3-l1 and attenuates fat accumulation in high fat diet-induced obese zebrafish. *Journal of Ginseng Research.*

Kim, W., Song, S., Oh, W., Kaewsuwan, S., Tran, T., *et al.* (2013). Wound-healing effect of ginsenoside rd from leaves of Panax ginseng via cyclic amp-dependent protein kinase pathway. *European Journal of Pharmacology*, *702*(1-3), 285-293.

Lam, S., Chua, H., Gong, Z., Lam, T., & Sin, Y. (2004). Development and maturation of the immune system in zebrafish, Danio rerio: A gene expression profiling, in situ hybridization and immunological study. *Developmental and Comparative Immunology*, 28(1), 9-28.

Lee, D. Y., Cha, B. J., Lee, Y. S., Kim, G. S., Noh, H. J., *et al.* (2015). The potential of minor ginsenosides isolated from the leaves of Panax ginseng as inhibitors of melanogenesis. *International journal of molecular sciences*, *16*(1), 1677-1690.

Lee, D., Jeong, S., Jeong, Y., Lee, M., Seo, K., *et al.* (2015). Antimelanogenic effects of picrionoside a isolated from the leaves of Korean ginseng. *Biological & Pharmaceutical Bulletin*, *38*(10), 1663.

Liu, J., Yan, X., Li, L., Li, Y., Zhou, L., *et al.* (2015). Ginsenoside rd improves learning and memory ability in app transgenic mice. *Journal of Molecular Neuroscience*, *57*(4), 522-528.

Li, S., Han, Q., Qiao, C., Song, J., Lung Cheng, C., *et al.* (2008). Chemical markers for the quality control of herbal medicines: An overview. *Chinese Medicine*, 3, 7.

Liu, Y., Zhang, R., Zhao, J., Dong, Z., Feng, D., *et al.* (2015). Ginsenoside rd protects sh-sy5y cells against 1-methyl-4-phenylpyridinium induced injury. *International Journal of Molecular Sciences*, *16*(7), 14395-14408.

Lu, D., Morishita, R., Xu, A., Mai, H., Zhao, J., *et al.* (2015). The synergistic effects of heat shock protein 70 and ginsenoside rg1 against tert-butyl hydroperoxide damage model in vitro. *Oxidative Medicine and Cellular Longevity*, *2015*.

McGraw, J., Lubbers, A., Van Der Voort, M., Mooney, E., Furedi, M., *et al.* (2013). Ecology and conservation of ginseng (Panax quinquefolius) in a changing world. *Annals of the New York Academy of Sciences*, *12861*(1), 62-91.

Nag, S. A., Qin, J., Wang, W., Wang, M., Wang, H., & Zhang, R. (2012). Ginsenosides as Anticancer Agents: *In vitro* and *in vivo* Activities, Structure–Activity Relationships, and Molecular Mechanisms of Action. Retrieved April 05, 2017.

Nah, S. (2014). Ginseng ginsenoside pharmacology in the nervous system: Involvement in the regulation of ion channels and receptors. *Frontiers in Physiology Front. Physiol., 5*. doi:10.3389/fphys.2014.00098

Raj, K. (2002). *U.S. Patent No. WO2002003813 A1*. Washington, DC: U.S. Patent and Trademark Office.

Shang, H., Li, Q., Feng, G., Cui, Z., & Lustig, A. (2011). Identification and characterization of alternative promoters, transcripts and protein isoforms of zebrafish r2 gene (regulation of r2 gene expression in zebrafish). *PLoS ONE*, *6*(8), e24089.

Shin, H.R., Kim, J.Y., Yun, T.K. *et al.* Cancer Causes Control (2000) 11: 565. doi:10.1023/A:1008980200583

Sivula, N. J. (2012). Research and Clinical Applications of Ren Shen (Ginseng). *American Journal Of Traditional Chinese Veterinary Medicine, 7*(2), 33-37.

Ulloa, P., Rincón, G., Islas-Trejo, A., Araneda, C., Iturra, P., *et al.* (2015). Rna sequencing to study gene expression and snp variations associated with growth in zebrafish fed a plant protein-based diet. *Marine Biotechnology*, 17(3), 353-363.

Wang, Y., Wang, B. X., Liu, T. H., Minami, M., Nagata, T., & Ikejima, T. (2000). Metabolism of ginsenoside Rg1 by intestinal bacteria. II. Immunological activity of ginsenoside Rg1 and Rh1. *Acta Pharmacologica Sinica*, 21(9), 792-796.

Wei, Y., Li, P., Fan, H., Peng, Y., Liu, W., *et al.* (2011). Metabolism study of notoginsenoside r1, ginsenoside rg1 and ginsenoside rb1 of radix Panax notoginseng in zebrafish. *Molecules (Basel, Switzerland)*, *16*(8), 6621.

Wei, Y., Li, P., Shu, B., Li, H., Peng, Y., *et al.* (2007). Analysis of chemical and metabolic components in traditional Chinese medicinal combined prescription containing radix salvia miltiorrhiza and radix Panax notoginseng by lc-esi-ms methods. *Biomedical Chromatography*, 21(8), 797-809.

Wixon, J. (2000). Featured organism: Danio rerio, the zebrafish. *Yeast*,*17*(3), 225-231.

Zhang Y., Li G., Jiang C. *et al.*, “Tissue-specific distribution of ginsenosides in different aged ginseng and antioxidant activity of ginseng leaf,” *Molecules*, vol. 19, no. 11, pp. 17381–17399, 2014.

Zhang, X., Shi, M., Ye, R., Wang, W., Liu, X., *et al.* (2014). Ginsenoside rd attenuates tau protein phosphorylation via the pi3k/akt/gsk-3[beta] pathway after transient forebrain ischemia. *Neurochemical Research*, *39*(7), 1363-1373.

Zhu, D., Liu, M., Yang, Y., Ma, L., Jiang, Y., *et al.* (2014). Ginsenoside rd ameliorates experimental autoimmune encephalomyelitis in c57bl/6 mice. *Journal of Neuroscience Research*, *92*(9), 1217-1226.