Hepatoprotective effect of Fermented Water Kefir on Sprague-Dawley rats (*Rattus norvegicus*) induced with sublethal dose of Acetaminophen

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Abstract

This study evaluated the hepatoprotective effect of water kefir in sprague-dawley rats. Four treatment groups were administered with sugar solution and varying doses of water kefir (Sugar solution, 1, 2 and 3 ml respectively) for seven days. After which, sub-lethal dose of acetaminophen (640 mg/kg) was given to induce hepatocellular injury. The effects of water kefir were measured based on the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. According to the results, water kefir promotes hepatoprotective effect by significantly decreasing (p<0.05) the levels of AST and ALT enzymes beyond the normal values. Furthermore, there is a significant difference between the different dosages administered among the treatment groups. The dosage of fermented water kefir which provided the greatest hepatoprotective effect is 3 ml and 2 ml for AST and ALT respectively. The results of the study show that as the concentration of water kefir increases, the AST level of blood samples decreases. On the other hand, the results for ALT assay tests yielded an optimum concentration of 2 ml. The more prominent effects of water kefir in AST levels suggest that its mechanism of action is to protect the mitochondria from oxidative stress induced by acetaminophen toxicity, hence maintaining its integrity inside the mitochondrial membrane.

Keywords: Acetaminophen, Aspartate aminotransferase (AST), Alanine transaminase (ALT), Fermented Water Kefir, Hepatoprotective

Introduction

According to statistics, liver disease is a prevalent issue affecting almost 360 million people worldwide. Modern studies continue to indicate the yearly increase of mortality and morbidity due to hepatotoxicity. According to Nallamilli et al. (2013), nearly 20,000 deaths are recorded and over 250,000 new cases are reported each year. Furthermore, studies show that fifty percent of such cases can be attributed to drugs (Ozcelik et al., 2014). The liver is known to be the largest organ of vertebrates, weighing 3-4 pounds and is situated in the right upper quadrant of the abdominal cavity. The liver is known to contain 10 percent of the blood in the body hence; its primary function is to filter the blood and for biotransformation and excretion of foreign substances. (Funk and Wagnalls New World Encyclopedia, 2014) Furthermore, its relationship with the gastrointestinal
tract and portal location exposes it to numerous amounts of substances which could induce hepatotoxicity (Jaeschke et al., 2001).

As modernization progresses, researchers have discovered drugs as a useful tool in treating or preventing diseases (Funk & Wagnalls New World Encyclopedia, 2014) However, even though drugs are beneficial for the health, it poses some risks when taken in amounts more than the recommended.

Acetaminophen, also known by its chemical name, N-acetyl-p-aminophenol (APAP), is a drug used as an analgesic and anti-pyretic since the late 19th century (Sheen et al., 2002). It is found in many over-the-counter and prescription products, including cough-and-cold remedies and narcotic pain relievers. However, acetaminophen has been one of the common causes of acute liver failure in the U.S., hence, prompting the Americans to mandate the dosage of acetaminophen (Schilling et al., 2010).

According to the study of Sheen et al. (2002), acetaminophen has been the most common drug used in deliberate self-poisoning since it is readily available to the public. Meanwhile unintentional intoxication is often caused by multiple medications that contain acetaminophen, and due to the impulsive behaviour involving the lack of understanding of individuals taking these medications (Schilling et al., 2010). In addition to these, therapeutic doses of the said drug can react with substances like alcohol and could cause damage to the liver and gastrointestinal tract (Dart et al., 2010).

These cases have pushed the researchers to open a new avenue on discovering the potential of a new hepatoprotective agent. Since these types of diseases are commonly caused by drugs, it would be highly questionable if drugs would also be utilized in preventing the progression of drug-induced liver disease in a human person’s body. Thus, the researchers intend to seek an alternative way to avoid overdependence on drugs and start more avenues for further researches on other products.

Probiotic supplements have caught the attention of the researchers due to its potential to detoxify the gastrointestinal tract. A study done by Imani Fooladi et al. (2013) states that the healthy status of the gastrointestinal tract is in a symbiotic relationship with that of the liver. This is due to the significant role played by the microflora resident of the intestinal lumen to the function of the hepatocytes, wherein alterations to such may cause liver dysfunctions (Imani Fooladi et al., 2013).

Water kefir or tibicos, the probiotic compound used in the study is a water-sucrose-based beverage fermented by symbiosis of bacteria and yeast (Marsh et al., 2013). It is a homemade type of beverage which is cultured by adding the kefir grains to a water-sugar solution and incubated at room temperature for 48 hours (Anfiteatro, 2009). The potential health improving and health maintaining effects of the compound may be attributed to the microorganisms present and the important molecules such as polypeptide, polysaccharide, organic acid etc. (Alsayadi et al., 2013).

According to Schneedorf (2012), some of the bacteria present in kefir include: Lactobacillus brevis, Lactobacillus hilgardii, Lactobacillus lactis cremoris, Lactobacillus casei subsp. Casei, Acetobacter aceti, Lactobacillus kefiri, etc.

The aim of this study is to determine the hepatoprotective effect of fermented water kefir on
acetaminophen-induced liver toxicity on sprague-dawley rats. It seeks to determine the potential of water kefir as a hepatoprotective agent through the use of AST and ALT liver assays in which, significant increase or decrease on the liver enzymes would indicate whether or not, the fermented water kefir has the potential to protect the liver from acetaminophen toxicity.

This study had seen the potential of the fermented water kefir in treating acute liver injury. Its trust is to provide valuable information to the medical field, hepatically challenged people and future researchers. The findings of the study would benefit the medical field by providing useful information that would be valuable to numerous hepatotoxicity cases. This study may be used to uncover the full potential of fermented water kefir in treating hepatic diseases prevalent in today’s society. This study would enable hepatically challenged people to afford highly effective hepatoprotective supplements with low financial demands. This study would also provide a significant amount of information about the detrimental effects of drugs when taken excessively, as well as other potential health benefits of fermented water kefir. It would benefit future researchers as it may serve as a foundation for studies to be conducted in the future. In addition, more use for probiotics and other cures for hepatotoxicity may be discovered in the years to come. Moreover, this study should not convey a message that people can abuse medication thinking that their liver can be protected by probiotics. This study only seeks to help those who are unintentionally affected by drug-induced liver injury.

Materials and Methods

The Experimental Method was used in determining the hepatoprotective effect of the fermented water kefir on sprague-dawley rats administered with sublethal dose of acetaminophen. Randomized Complete Block Design (RCBD) was employed. Furthermore, four treatment groups were used in the study. Two replicates were conducted to assert the continuity and accuracy of data gathered.

\[ T_0 = \text{Sugar Solution + Acetaminophen (7 Days Post-Probiotic)} \]

\[ T_1 = 1 \text{ ml Fermented Water Kefir + Acetaminophen (7 Days Post-Probiotic)} \]

\[ T_2 = 2 \text{ ml Fermented Water Kefir + Acetaminophen (7 Days Post-Probiotic)} \]

\[ T_3 = 3 \text{ ml Fermented Water Kefir + Acetaminophen (7 Days Post-Probiotic)} \]

Procurement of Materials and Samples

A total of twenty-eight sprague-dawley rats of the same gender, aging 6-8 weeks old, were purchased at the Food and Drug Administration, Civic Drive, Filinvest Corporate City, Alabang, Muntinlupa, Philippines. The rats weighed approximately 150-200 g. Acclimatization and treatment of the rats was conducted on the Rat Facility of the Environmental Resource Management and Campus Development Office of De La Salle University-Dasmariñas, Cavite (DLSU-D). Five thousand milligrams of acetaminophen (Tylenol™) was obtained from Mercury Drug, Walter Mart Dasmariñas, Cavite. Meanwhile, the kefir grains were acquired from Elises residence at Tayabas, Quezon which were then cultured at the Aspiras’ residence at GUV Village Sto. Niño San Pascual Batangas, Philippines.

Preparation of Fermented Water Kefir

The compound was prepared by washing the water kefir grains with distilled water. One part of the kefir grains was inoculated in sugar solution which was
prepared using the following measurements: 1/2 kg sugar preferably *sangkaka* (cooked, processed and hardened molasses from sugarcane) in 3 litres mineral drinking water (May 2014 personal interview with R. Elises). The mixture was placed in a plastic container with screen cloth as a cover and incubated at room temperature for 48 hrs. A plastic container is used because the acidity of fermented water kefir may degrade metals such as aluminum and iron which could mix with the drink thereby causing harmful effects to the body (Yemoos Nourishing Cultures, 2012). After fermentation, kefir grains was sieved by filtration through a plastic sieve and washed for another process. The water kefir drink was stored at 4°C for 24 hrs prior to inducement on the test rats.

*Acclimatization of animals*

Among the total number of rats, 24 healthy rats were selected for the experiment. The animals were randomly grouped and confined in a 6x12x6 inches cage made of aluminum screen and small lumber. The test species were allowed to acclimatize for 7 days. Throughout the acclimation period, the test animals were given standard commercial pellets and water *ad libitum*. The researchers regularly removed left-over food and water and also collected feces at the bottom of the cages to maintain proper sanitation (Caravana et al., 2003).

*Preparation of Acetaminophen*

The tablets were powdered using a mortar and pestle and then weighed using the analytical balance according to the amount needed. The amount needed was determined in accordance to the weight of the rats. The dosage of acetaminophen used, 640 mg/kg, was based on the studies of Janbaz and Gilani (2002) which showed favourable effects in inducing hepatotoxicity in rats. Prior to oral administration, the weighed acetaminophen was diluted in 1 mL distilled water.

*Multiple Dose treatment*

The sprague-dawley rats were divided into four treatment groups. Two rats comprised each treatment group. The fermented water kefir was administered through the oral route via gavage method. The period of treatment followed the modified procedure stated in the study of Ramirez (2008). In the study, the rats were treated with probiotics seven days before and seven days after administration of acetaminophen. For this study, the rats were treated only for seven days and on the seventh day, acetaminophen was administered in order to induce acute liver toxicity to the laboratory test rats. T0 received 1 ml of the sugar solution used in the preparation of the fermented water kefir. This was done every twenty-four hours for a period of seven days. Meanwhile, the rest of the treatment groups were given fermented water kefir which was conducted also every twenty-four hours for a period of 7 days. Specifically, T1, T2 and T3 were given fermented water kefir concentrations of 1, 2 and 3 ml respectively.

*Administration of Acetaminophen*

The test animals were subjected to 24 hrs fasting prior to dosing. At the seventh day of the multiple dose treatment, the test animals were administered with sub-lethal dose of acetaminophen, 640mg/kg, by means of oral gavage method.

*Blood collection*

To optimize results, all rats were not fed for 24 hrs prior to blood collection. The rats were exposed to ether, one by one, in a close container, for a few minutes. The use of ether may be questioned since it exhibits toxicity to the liver. However, several hepatoprotective studies
utilize the inhalation agent in order to draw blood successfully from the laboratory rats without hemolysis, hence its use in the study (Ramirez, 2008; Guadana et al., 2014). Using a 27 gauge needle and a 5 ml syringe, cardiac puncture was employed to extract two to three ml of blood (Ramirez, 2008). The obtained blood samples were then placed in red top bottles prior to laboratory submission.

**AST and ALT assay testing**

To evaluate the hepatoprotective effect of the fermented water kefir, the serum levels of different marker enzymes, particularly alanine aminotransferase (AST) and alanine transaminase (ALT), were determined. After blood collection, the blood samples were submitted to a laboratory in Alabang Medical Center for enzyme level determination. This was done under the supervision of a registered medical technologist.

**Statistical analysis**

The AST and ALT levels obtained from the assay tests were used for statistical analysis. The biochemical result was expressed as mean ± Standard Error Mean (S.E.M.). The data were assessed using one-way ANOVA, in which the P value of <0.05 was considered statistically significant (Guadana et al., 2014).

**Results**

**Mean Values of AST Levels**

The data derived from the assay tests were used in the statistical analysis. Figure 1 shows the mean levels of AST obtained from the treatment groups. Due to the limited number of animals approved by the Ethics Committee, the researchers obtained the AST and ALT values of the control group from the study of Casimiro et al. (2010), which also utilized sprague-dawley rats from the Food and Drug Administration, Alabang Muntinlupa, Philippines. This was done to determine the potency of water kefir in not just lowering but also in normalizing the AST and ALT values.

![Fig. 1. Mean values of AST Levels](image)

According to the statistical results, water kefir was able to normalize the AST levels and actually lower it beyond the control values. AST levels of Treatment 2, Treatment 3 and Treatment 4 are significantly different (p<.05) from Treatment 1. This shows that increasing concentrations of water kefir, up to 3 ml, significantly lowers the levels of AST. Furthermore, it signifies the efficacy of water kefir in lowering and normalizing the AST values. Moreover, the significant difference found between the control and T1 indicates that acetaminophen can promote hepatotoxicity as shown by the increase of
AST values. Meanwhile, a decreasing trend on AST levels was also observed simultaneous with the increasing concentration of water kefir. This shows that as the concentration of water kefir increases, the AST level decreases. The results also indicate that water kefir was able to normalize the AST levels; hence it is effective in protecting the liver from hepatotoxicity.

**Mean values of ALT Levels**

The data for ALT levels were obtained using the same procedure conducted in acquiring the AST levels. The ALT value of the control group also came from the study of Casimiro et al. (2010). Figure 2 shows the mean values of ALT obtained from the treatment groups.

Based on the results, water kefir was able to lower the values of ALT beyond the normal level. The results of the statistical treatment indicated that the ALT levels of Treatment 2, Treatment 3 and Treatment 4 are significantly different from the control. In addition, Treatment 2 and Treatment 3 are significantly different from Treatment 1. This indicates the potency of fermented water kefir in significantly lowering the levels of ALT. However, it can be noted that Treatment 3 (2 ml Water Kefir) yielded the lowest amount of ALT levels, hence the most hepatoprotective concentration among the treatment groups. The increase of ALT level at Treatment 4 indicates that hepatocellular injury occurs at higher doses, hence an optimum concentration of 2 ml. In comparison with the results of the two enzymes, it can be noted that water kefir elicit more effects on AST that in ALT due to decreasing trend observed in the AST levels.

**Discussion**

This study focuses on two liver enzymes. First is serum aspartate aminotransferase or AST. It is a cytosolic and mitochondrial isoenzyme found in high concentrations in the heart (Limdi and Hyde, 2003). In addition, it is also found in the liver, cardiac muscle, skeletal muscle, kidneys brain, pancreas, lungs, leukocytes and red cells in lower concentrations (Limdi and Hyde, 2003; Gowda et al., 2009). Second is serum alanine aminotransferase or ALT. Unlike AST, this enzyme is a purely cytosolic enzyme found in high concentrations in the liver and in lower concentrations in the other tissues of the body such as the kidney, heart and muscles (Gowda et al., 2009). Due to the many direct and indirect mechanisms of drug-induced cellular damage, hepatotoxicity remains a major cause for drug withdrawal during medication. This urged the researchers to discover a new hepatoprotective agent. In this study, fermented water kefir is the substance used to protect the liver from toxicity.

Water Kefir is a home-made fermented beverage based on a sucrose solution, which consists of a gelatinous and irregular grains formed by a consortium of yeasts and lactic acid bacteria (Schneendorf, 2012). There are two known types of kefir, water kefir and milk kefir. According to Schneendorf (2012), “the structure, associated microorganisms and products formed during fermentation process” of milk kefir grains are very similar to water kefir or sugary kefir grains. This enabled the researchers to utilize literatures and studies which made use of milk kefir in order to support the findings of the study.

Based on the results gathered, the researchers had observed that the effect of water kefir on hepatocellular damage is based on its concentration. The results of the experiment indicate that as the dose of water kefir increases, the AST levels decreases. Meanwhile on ALT,
Treatment 3 (2 ml Water Kefir) yielded the lowest ALT level. It can be concluded that water kefir has an optimum concentration for reducing ALT levels and any dose higher than the optimum concentration would not encourage a healthier liver but rather increase cellular damage. This is supported by Pereira et al. (2013) in his study, in which he stated, kefir reduces total lipids in serum in a dose-dependent way. This may be related to other components of the blood serum such as ALT which increases at higher doses of water kefir and meanwhile decreases until an optimum concentration is achieved.

Rosa et al. (2014) emphasizes the mechanism of how water kefir affects the gastrointestinal tract, particularly the intestinal mucosa, compared to a control group treated with normal saline. According to their study, the animals treated with kefir manifested well defined mucosa, villi and intestinal crypts. The villi were greater in height and wider in width. The animals also exhibited a thicker mucosal layer indicating a healthy gastrointestinal tract. Rosa et al. (2014) also explained in their study that bacterial translocation is the “passage of viable bacteria from the gastrointestinal tract through mucosal epithelium to other tissues”. If such phenomenon transpires, the liver is the first organ to be compromised due to its direct connection through the portal blood. Further results of their study stated that normal or high doses of kefir prevent translocation of bacteria, thereby protecting the liver organ from damage.

One of the major antioxidants in the liver that protects it from toxicity if glutathione (Hussain et al., 2014). According to the hepatoprotective study of Padmanabhan and Jangle (2014), decrease in amount of total glutathione is due to the protection acts against oxidative stress. The glutathione acts as a free radical scavenger and plays a central role in coordinating the antioxidant defense process of the body. In relation with water kefir, Can et al. (2012) stated that the counteracting antioxidant system of kefir enables the synthesis other antioxidant molecules and enables the gene expression of protective enzymes. Because of this, free radicals caused by toxic amounts of drugs, particularly acetaminophen may be reduced by the antioxidant role played by water kefir. Hence, the utilization of probiotic may prevent the consumption of the cell’s natural storage of antioxidant systems (Castex et al. 2009 as mentioned by Can et al., 2012). Aside from this, kefir also competes with pathogenic bacteria for adhesion sites and further strengthens the immunological barrier function of the intestine (Rosa et al., 2014). Because of this, toxic metabolites such as drugs may be prevented from entering the liver due to the greater immune response exhibited by the healthy intestinal mucosal barrier.

If the toxic metabolites such as toxic doses of acetaminophen were counteracted by the mechanism of the water kefir inside the gastrointestinal tract, then the hepatocytes remain stable and free of damage. Because of this, it could be concluded that enzymes such as AST and ALT would remain inside the cell, hence their low levels in the blood stream. In comparison with the results of AST and ALT, it can be noted that the results yielded from AST provided a more significant difference than the results yielded from ALT. It is possible that during hepatocellular injury, the internal organelles such as the mitochondria get damaged first, hence the enzymes are released outside the cell. This is supported by Trump et al. (1997) in his study by stating that there are series of reactions in the cell that change or alter the cell’s shape.
and volume. Through electron microscopy, necrotic cells show several modifications particularly condensation of mitochondria following swelling, mitochondrial densities and/or calcification.

Kaplowitz (2004) stated that rise in AST levels is a manifestation of acute liver disease caused by drugs. According to the study, the drug metabolites which went through a series of chemical reactions have a direct effect on organelles such as the mitochondria, endoplasmic reticulum, etc. In her study, Ramirez (2008) emphasized that 80% of the total activity of AST is mitochondrial in location. The mitochondrion is an organelle full of oxidizers due to the Electron Transport Chain (SciTechnol, 2013). It is possible that when mitochondrial membrane disruption happens, such oxidizers would be released outside the cell causing oxidative stress. This explains the effects of drugs to the mitochondria leading to the release of liver enzymes. In addition, Jaeschke et al. (2012) re-evaluated their findings and found out that the initial metabolism of a drug, acetaminophen in particular, forms mitochondrial adducts which covalently binds to proteins inhibiting the function of the mitochondria such as ATP synthesis leading to and a prominent decrease in GSH levels.

Studies also conducted by Can et al. (2012) on the species of Coruh Trout treated by water kefir supports the results of the study. It was stated that at a lower dose of water kefir, the values of the total antioxidant status (TAS) and total oxidative status (TOS) were rapidly reduced at the first 2 months of their experimentation. At the end of three months, a further decrease with the highest dosage was observed. This indicates that the affectivity of water kefir were dependent on the amount of the dosage of water kefir and the duration of observation.

According to the study of Alsayadi et al. (2013) water kefir possesses antioxidants making it a good scavenger of free radicals found in the body. Because of this, it is possible that water kefir can prevent the disruption of the mitochondrial membrane, thereby maintaining the integrity of AST inside the mitochondria. On the other hand, the enzyme ALT is known to be concentrated in the cytosol. However, the cytosol does not contain much oxidizer which may trigger oxidative stress (Lopez-Mirabal and Winther, 2007). Hence, the potency of water kefir is more effective in lowering AST levels than ALT levels.

**Conclusion**

Based on the results of the study, water kefir promotes hepatoprotective effect on rats administered with sub-lethal dose of acetaminophen. This was proven by the ability of water kefir to significantly reduce the levels of AST and ALT enzymes beyond the normal levels. Furthermore, there is significant difference between the different dosages administered among the treatment groups. The dosage of fermented water kefir which provided the greatest hepatoprotective effect is 3 ml and 2ml for AST and ALT, respectively. The results of the study show that as the concentration of water kefir increases, the AST level of blood samples decreases. On the otherhand, the results for ALT assay tests yielded an optimum concentration of 2 ml. More prominent effects of water kefir in AST levels suggest that its mechanism of action is to protect the mitochondria from oxidative stress induced by acetaminophen toxicity, hence maintaining its integrity inside mitochondrial membrane.
Acknowledgements

The researchers would like to express their profound gratitude to the Almighty God, who never failed to endow wisdom and knowledge to the researchers; to Mr. Marlon Pareja, for nourishing the researchers with knowledge and time to make this paper possible; to the Panelists, Dr. Johnny Ching, Dr. Arnold Fonollera and Mrs. Myra Lagat, for their recommendations that made this study truly significant; to Mr. Roy Elises, who introduced us fermented water kefir or tibicos, the independent variable of the study; to Ms. Mari Julianne Tesalona, for her willingness to share her resources and skills in this study; to the researchers’ family and friends, who have continuously supported throughout the making of this proposal. Their everlasting love and understanding made the journey a lot easier despite of the obstacles encountered.

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