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# Safety assessment of potential food ingredients in canine hepatocytes

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ABSTRACT

This research aimed to develop *in vitro* methods to assess hazard of canine food ingredients. Canine hepatocytes were harvested and cell viability of clove-leaf oil (CLO), eugenol (EUG), lemongrass oil (LGO), guanosine monophosphate (GMP), inosine monophosphate (IMP), sorbose, ginger-root extract (GRE), cinnamon-bark oil (CBO), cinnamaldehyde (CINA), thymol oil (TO), thymol (THYM), and citric acid were assessed with positive controls: acetaminophen (APAP), aflatoxin B1 and xylitol. Molecular Toxicology PathwayFinder array (MTPF) analyzed toxicity mechanisms for LGO. $LC_{50}$ for APAP was similar among human (3.45), rat (2.35), dog (4.26 mg/ml). Aflatoxin B1 had an $LC_{50}$ of 4.43 (human), 5.78 (rat) and 6.05 (dog) μg/ml; xylitol did not decrease viability. $LC_{50}$ of CLO (0.185 ± 0.075(SD)), EUG (0.165 ± 0.112), LGO (0.220 ± 0.012), GRE (1.54 ± 0.31) mg/ml; GMP (166.03 ± 41.83), GMP + IMP (208.67 ± 15.27) mM; CBO (0.08 ± 0.03), CINA (0.11 ± 0.01), TO (0.21 ± 0.03), THYM (0.05 ± 0.01), citric acid (1.58 ± 0.08) mg/ml, while sorbose was non-toxic. LGO induced upregulation of 16 and down-regulation of 24 genes, which CYP and heat shock most affected. These results suggest that *in vitro* assays such as this may be useful for hazard assessment of food ingredients for altered hepatic function.



## 1. Introduction

The development of an *in vitro* canine model would be a useful tool for safety screening of novel raw materials and ingredients in pet food. The purpose of this research is to develop an *in vitro* method to assess the relative safety of novel pet food ingredients for dogs in a defined model system and provide a bridge to compare to data available in other species and humans as suggested by a recent NRC report on assessing safety of dietary supplements in companion animals (NRC, 2009).

We developed and assessed the toxicity of traditional toxicants such as acetaminophen (APAP) and aflatoxin B1 as positive controls in canine cell cultures and compared the results to human and rodent data. Then we assessed the toxicity of a wide variety of potential dietary compounds including clove leave oil (CLO) and its primary component eugenol (EUG), lemon grass oil (LGO), guanosine monophosphate (GMP), inosine monophosphate (IMP), sorbose, xylitol and ginger root extract (GRE), cinnamon bark oil (CBO) and its component cinnamaldehyde (CINA), thymol oil (TO) and its component thymol (THYM), and citric acid for their potential toxicity in isolated canine hepatocyte cells as an *in vitro* toxicity model. The goal is to begin developing an alternative humane model for initial ingredient screening and start collecting comparative safety data on use of these additives in dog food and to determine which mechanistic pathways are involved if effects are detected.

Pet foods may contain additives, impurities, bacteria or toxins that are unhealthy or even toxic to dogs. For example, renal failure has occurred in dogs after the ingestion of grapes or raisins (Eubig et al., 2005). Evaluation of the hepatotoxic potential through isolation and culturing of canine hepatocytes is a potential tool for screening the safety of candidate feed ingredients. This is especially true since the dog is susceptible to a number of hepatic diseases.

To accomplish this goal, we optimized a protocol for isolation and culturing of well-characterized and functional primary canine hepatocytes. APAP, and aflatoxin B1 are well-known liver toxicants that were selected to serve as positive controls in the hepatotoxicity studies since canine mortality cases have been reported after exposure and their level of toxicity and mechanisms/pathways have been established in human and rat. When comparing the acetaminophen toxicity in rat, dog and monkey cells, the canine hepatocytes exhibited the highest level of acetaminophen-induced cytotoxicity. The sensitivity of dog hepatocytes to acetaminophen may be due to their low conjugating enzyme activity (Smolarek et al., 1990). Acetaminophen induced necrosis and apoptosis in mouse hepatocytes via mitochondrial permeability transition (MPT), a phenomenon characterized by mitochondrial swelling, uncoupling, and inner membrane permeabilization (mitochondrial

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membrane potential) (Kon et al., 2004). In addition, recent studies have shown that APAP alters the permeability of the membrane and affects the absorption in intestinal epithelial cell membranes (Schäfer et al., 2013). Aflatoxin B1 is a well-studied food contaminant and a liver toxicant in multiple species including humans (Zhang et al., 1990). Additionally, xylitol, a very popular sweetener used in candy, baked food, and medical products was assessed. This sweetener has been reported to result in severe hypoglycemia and liver failure in dogs with liver enzyme elevations that is reversible after a few days (Todd and Powell, 2007). Therefore, xylitol was exposed to canine hepatocytes to determine its potential direct toxicity.

Clove oil is an essential oil and contains EUG as is its major component. This oil can be extracted from clove leaves, consisting of 82–88% EUG (Lawless, 1995), with small amount of eugenyl acetate, β-caryophyllene, α-humulene as well as a trace amount of methyleugenol present (Kegley et al., 2008). It is widely used as a food flavoring substance or for dental pain relief in humans, although there is evidence that clove oil causes liver failure in humans after ingestion as little as 5–10 ml of clove oil (Eisen et al., 2004; Janes et al., 2005). Its main ingredient EUG functions as an antioxidant (Ogata et al., 2000) and is widely used in the food industry and dentistry as a cement and sedative agent (Markowitz et al., 1992). It has been shown to induce chromosomal aberrations and endoreduplication *in vitro* (Maralhas et al., 2006). Other research suggests that methyleugenol may cause DNA damage in rat liver (Ding et al., 2011). However, it is unclear if CLO or one of its components could cause hepatotoxicity in dogs.

Lemon grass oil (LGO) is widely used in traditional human medicine, and is employed as a pesticide and preservative. However, it was found to cause functional damage to the rat liver when given orally for 14 consecutive days at 1.5 g/kg body weight (Fandohan et al., 2008). The $LD_{50}$ for acute oral toxicity in Swiss mice is 3.5 g/kg (Costa et al., 2011). It seems that β-myrcene, an acyclic monoterpene found in LGO, has the ability to inhibit CYP2B1 monooxygenase (De-Oliveira et al., 1997). However, there is no information available for the LGO hepatotoxicity in dogs.

Ginger has been used as a flavoring and herb for thousands of years and has been employed in the food and drink industry and as a fragrance in cosmetics and soaps. Ginger extracts have also exhibited a hepatoprotective effect in bromobenzene or carbon tetrachloride treated rats (Atta et al., 2010; El-Sharaky et al., 2009). However, there is no information on the adverse effects of ginger extract on canine liver cells.

L-sorbose is a type of monosaccharide that may induce hemolysis in canine erythrocytes due to the inhibition of hexokinase by sorbose-1-phosphate, while L-sorbose is not phosphorylated in human erythrocytes (Goto et al., 1994). Therefore, it is important to investigate if L-sorbose can introduce adverse effects in dog liver due to a similar mechanism.

Natural remedies such as CBO are used for its antimicrobial and anticarcinogenic properties (Unlu et al., 2010). CBO is an antioxidant and may have a protective effect through an oxidative stresss mechanism on the male reproductive system (Yüce et al., 2014). CINA is a major constituent of CBO (~70%) which is an active antifungal compound (Singh et al., 1995) that can inhibit NF-kB through signal tansduction pathways NIK/IKK, ERK, and p38 MAPK, thereby showing good antioxidant properties (Kim et al., 2007).

Similarly, TO has been attributed with antitumoric effects both *in vivo* and *in vitro* (Ait et al., 2007). Since THYM is a major constituent of TO (~40%), it has antioxidant and antimicrobial properties that can inhibit bacterial growth at 0.5% (Bagamboula et al., 2004; Yanishlieva et al., 1999). THYM can induce calcium release from the endoplasmic reticulum and apoptosis via generation of reactive oxygen species (Chang et al., 2014). Citric acid is also used in human and veterinary periodontal procedures as a root canal irrigant, as well as a topical antibacterial agent for wound infections in animals and humans (Nagoba et al., 2014; Ririe et al., 1980; Yabanoglu et al., 2013; Zaccaro et al., 2001) but its hepatotoxicity in the canine species is unknown.

In this study, we have evaluated CLO with its primary component EUG, LGO, GMP, IMP, sorbose, and GRE, CBO and its major component CINA, TO and its primary component THYM, and citric acid as examples of pet food ingredients using an isolated canine hepatocyte culture as an *in vitro* model to begin to define possible hazard and toxicity of these compounds in a defined model system, and to determine mechanistic pathways involved in LGO toxicity.

## 2. Materials and methods

### 2.1. Chemicals and cells

EUG, LGO, GMP, IMP, sorbose, GRE, CBO and CINA, TO and its component THYM, citric acid, APAP, aflatoxin B1 and xylitol as positive controls were purchased from Sigma-Aldrich (St. Louis, MO). Canine hepatocytes for comparison with cells isolated from our study were purchased from Triangle Research Labs (TRL; Research Triangle Park, NC). Human and rat hepatocytes were purchased from Lonza (Portsmouth, NH).

### 2.2. Animals

Dogs used in this study were of mixed breeds and ranging in ages from 1.5 to 9 years (n = 3). Dogs were housed individually and provided with food and water *ad libitum* and housed under normal environmental conditions. The liver was obtained from dogs that were euthanized for clinical issues after owner consent. Prior to euthanasia, the dogs were first anesthetized with 1:1 mixture of ketamine and xylazine (i.v., 55 to 74 μl/kg) and then euthanized with sodium pentobarbital (i.v., 222 μl/kg). The liver was removed immediately. The use of the tissue for research purposes was approved by the university's Institutional Animal Care and Use Committee protocol 3214.

### 2.3. Liver collection and hepatocyte isolation

The liver was perfused with University of Wisconsin (UW) solution containing 10 U/ml heparin via the portal vein. The liver was brought to the lab and perfused with Hank's Balanced Salt Solution (HBSS) containing **ethylene glycol tetraacetic acid (**EGTA), followed by liver perfusion with collagenase type XI buffer (Sigma-Aldrich, St. Louis, MO). After 15 min of liver digestion, the cells were released into the culture medium, centrifuged, washed, and filtered through cheesecloth layers and a 100 μm cell strainer. If the viability for isolated cells was lower than 70% when assayed by trypan blue, then the hepatocytes were separated with Percoll. Cell suspension was centrifuged and Percoll was aspirated. Cells were plated or cryopreserved in the UW solution with 10% dimethylsulfoxide (DMSO). Rat, human and canine hepatocytes were recovered from liquid nitrogen and the cells were placed in a water bath for 1 min followed by centrifugation to remove any residual DMSO. Cells were cultured in Clonetics™ HCM™ hepatocyte culture medium (HCM; Lonza, Walkersville, MD) containing 5% fetal bovine serum (FBS), and were positively stained by anti-hepatocyte antibody (Dako, Carpinteria, CA). The staining pattern and color intensity is similar to human hepatocytes.

### 2.4. Cell culture and alamar blue viability assay

The alamar blue (aB) (Invitrogen, CA) viability assay was used to assess for cytotoxicity in canine hepatocytes. Acetaminophen (APAP) was used as a positive hepatotoxic control. Canine hepatocytes were recovered from liquid nitrogen with greater than 50% viability and seeded in 96 well plates at 50,000 cells/well in HCM with 5% FBS. Four hours later, the culture medium was replaced with fresh medium without FBS. Hepatocytes (n = 6/well) were incubated overnight and then treated with the test compounds for 24 h. A serial dilution was conducted for each of the test compounds. Cell viability was evaluated by adding 10 μl aB reagent to 100 μl of cell medium followed by 3 h of incubation. The fluorescence was detected using a synergy H1 hybrid multi-mode microplate reader at Ex555/Em585nm (BioTek Instruments, Inc., Winooski, VT). Viability was calculated by subtracting the non-cell control value and normalized to the control cells (without chemical treatment). The $LC_{50}$ (n = 3 dogs) with a fitting curve was calculated using Origin 8.0 (Northampton, MA) software.

### 2.5. Molecular toxicology PathwayFinder array

Two million hepatocytes, treated with LGO for 24h at 80% viability, were harvested for real time PCR array. Cell lysates were preserved in Ribozol (Amresco, Solon, OH), and RNA was isolated or purified by Total RNA Kit I (Omega bio-tek, Norcross, GA). The concentrations of isolated RNA were measured by Nanodrop (Nanodrop, Wilmington, DE) and quality of RAN by BioAnalyzer 2100 (Santa Clara, CA). RNA Integrity Number (RIN) was assigned automatically by the software based on the ratio