Identification of Inhibitory Compounds in Medicinal Mushrooms Against Pathogenic Bacteria and Spoilage Yeasts

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ABSTRACT

Extracts from medicinal mushrooms were prepared and tested for anti-microbial activity against food pathogens and food spoilage microorganisms. The inhibitory activity was measured using a disk diffusion assay and with optical density (OD). For OD, 7 fractions were collected using HPLC for 4 (*A. blazei* Murrill, *G. lucidum, G. frondosa, I. obiquus*) medicinal mushrooms and 6 fractions from *L. edodes* and 8 fractions from *P. linteus*.

The results from disk diffusion assay showed that most mushrooms displayed significant inhibition compared to the ethanol. The exceptions were: *A. blazei* Murrill, *I. obliquus,* and *L. edodes* against *E. coli* O157:H7; *I. obliquus* against *L. monocytogenes* V7; *I. obliquus* against *S. cerevisiae* Y99*; L. edodes* against *Z. bailii* Y03*;* and *I. obliquus* against *Z. bailii/bisporus* Y108. Inhibition was more effective in yeasts than bacteria. The result from Bioscreen C showed that against *L. monocytogenes* V7, fraction 7 in *A. blazei* Murrill; fraction 1, 4 and 5 in *G. lucidum*; fraction 4 in *G. frondosa;* and fraction 4 and 5 in *I. obliquus* significantly inhibited the growth compared to ethanol. Against *Z. bailii* Y03, fraction 7 in *A. blazei* Murrill; all fractions from *G. lucidum, G. frondosa,* and *P. linteus*; fraction 1, 2, 3, and 6 from *I. obliquus*; and fraction 4 and 6 from *L. edodes* significantly inhibited growth compare to ethanol. The results indicated that there is significant antimicrobial activity against food pathogens and spoilage organisms in the medicinal mushrooms studied.

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Chapter 1

Introduction

Mushrooms have been used in different parts of the world for centuries as medicines against many diseases. In Eastern Asia, people use mushrooms as a multi-purpose medicine, and they are regarded as having high potency against many disease infections. Anticancer compounds have been found in shiitake mushroom, antitumor compound lentinan from reishi mushroom, and anti-viral compounds from psilocybin-containing mushrooms (Stamets and Chilton, 1983). Until recently anti-microbial activities in mushrooms were mostly considered to be mythical; yet, many ancient histories have shown that the people used these mushrooms for health benefits. Examples of natural medicines are quite common. Quinine, also commonly found in mushrooms, was used in first century for the treatment of tuberculosis and more recently for malaria; penicillin was produced from mold fungi in early 20^{th} century; recently a frozen mummy found in the Apls since three thousand B.C. was carrying two species of polypore mushrooms that are known to have antimicrobial effects (Stamets, 2002). Mushrooms vary in size, shape, nutrition content, and bioactive compounds. According to *Dictionary of the Fungi*, there are five hundred thousand to one million species of fungi and out of this, over fourteen thousand are considered to be mushrooms (Wasser, 2011). This also means that only ten percent have been identified so far out of an estimated one hundred and fifty thousand species of mushrooms (Wasser, 2011).

Recently, the significance of the medicinal mushrooms has been reported. High levels of antioxidant compounds such as ascorbic acid, phenols, and tocopherols were found in *Agrocybe cylindracea* using hot water extraction methods (Tsai et al., 2006). *Trichloma giganteum* was also explored for antitumor activity and it was reported that β-D-glucans, after chemical modification, yielded high antitumor activities (Mizuno et al., 1996). And yet, with the exception of well-known mushrooms such as *G. lucidum* or *A. bisporus*, the ability of mushrooms to inhibit the growth of microorganisms has not been fully researched. *G. lucidum* has been identified to contain compounds such as ganoderic acids and triterpenoids that are known to have antimicrobial activities (Cole and Schweikert, 2003). Identification of novel compounds is extremely difficult for many reasons. Much of the research looking at antimicrobial activities is often rudimentary; and work becomes difficult due to the varieties of different antimicrobials activities that can be seen (Wasser and Weis, 1999; Wasser, 2011; Lindequist et al., 2005; Rosa et al., 2003). The limitations include the lack of standardized method of cultivation as well as lack of proper classification and uniformity of taxonomy; even within each species, nutritional contents and composition can vary due to the different cultivation method or soil make up (Singh and Singh, 2011).

The demand for more natural products with minimum additives has become an important attribute for consumers (Vigil et al., 2005). Consumers are becoming more aware of additives in the foods they eat. One way to replace artificial food additives is to discover naturally occurring compounds with similar activity. There are many naturally sources of antimicrobial preservatives, including plants and animals as well as microorganisms themselves (Vigil et al., 2005). Often many natural antimicrobial compounds have wide spectrums that are especially helpful against bacteria, fungi, parasites, and viruses (Stopforth et al., 2005).

Research Objectives

The objectives of this research are: to quantify the inhibitory effect of ethanolic extracts of six medicinal mushrooms against pathogenic bacteria and spoilage yeasts, and then to determine whether the specific fractions of the extracts in medicinal mushrooms possessed more antimicrobial capacity than the others against *L. monocytogenes* and *Z. bailii*. The results will show the effectiveness of ethanolic medicinal mushroom extracts against food related microorganisms.

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Chapter 2

Review of Literature

1. Mushrooms

Definition of Mushroom

Mushrooms are classified under kingdom Fungi. The mushroom is a fruiting body of macro-fungus that is visible to human eyes. Majority of species that forms fruiting body in its life cycle are classified under phylum Ascomycota and Basidiomycota. The differences between these two phyla are that Ascomycota goes through asexual sporulation in favorable conditions but could go through sexual reproduction resulting in fruiting bodies, called ascocarps, while species in Basidiomycota goes through sexual reproduction, in favorable conditions, resulting in fruiting body called basidiocarps (Carlile et al., 2001; Miles and Chang, 1997).

Typically considered mushrooms, macro-fungi are classified under Basiodomycetes, a subdivision of Badiomycota that includes 16,000 species (Carlile et al., 2001; Miles and Chang, 1997). The fruiting body of fungi is composed of the volva, stem, cap, and gills (Roberts and Evans, 2011). Mushrooms are unique part of kingdom Fungi and have significant culinary value due to their favorable textures and flavors. Beyond the nutritional values, they have been used for their medicinal, hallucinogenic, and poisonous effects as well. Basidiomycetes go through sexual reproduction to produce fruiting bodies or basidiocarps. Basidiocarps have gills, formed by the collection of basidium with increase in surface area, which functions as factory for

basidiospore production through meiosis (Carlile et al., 2001). Basidiospores then become the primary mycelium that goes through plasmogamy and dikaryotization to create secondary mycelium that develops into fruiting body (Carlile et al., 2001). Although not all mushrooms go through same life cycle, this represents the general life cycle of Order Agaricales (Carlile et al., 2001).

Although mushrooms vary in shape, size and functions, they are all composed of protein, fat, fibers, carbohydrates and mostly water (Chang and Miles, 2004). Generally, mushrooms are composed of 16-85% carbohydrates, 0.2-87% lipids, 14-44% proteins, 1-10% RNA, 0.15-0.3% DNA and 1-29% ash (Chang and Miles, 2004). Mushrooms are considered a healthy food because the average fat content is ~4.0% and is composed of about 72% unsaturated fatty acids in edible mushrooms (Chang and Miles, 2004).

β-Glucan

One of the most important active compounds found in mushroom is β-glucan. It is primarily created from sugars. It is also not a novel compound found only in fungi, it is found in algae, lichens and plants (Rahar et al., 2011). β-Glucan provides structural integrity to the fungus, and functions much like chitin, chitosan, and cellulose do in other species (Carlile, 2001). Structural rigidity comes from its β-(1,3)-glucan structure; however, β-(1,6)-glucan allows for many side chains and functional group to attach to the backbone structure. This results in a variety of β-glucans with different bioactivities (Rahar et al., 2011). β-Glucan chains can have varying degree of homo- or heteropolysaccharide in their structure that affect bioactivity, which is enhanced further by the addition of protein complexes (Wasser and Weis, 1999).

The uniqueness of the compounds structures provides mushrooms with different functions in medical fields. β-Glucan is important in its functional activities in human health.

Examples include lentinan, which is formed with five β-(1,3)-glucan monomers and two β-(1,6)-glucopyranosides as side branches, to create triple-helix fibers having antitumor and immunostimulatory activities (Wasser and Diduhk, 2005; Mantovani et al., 2008). Of the 660 known species of mushrooms in different taxa, 77.2% of them were found to have bioactive polysaccharides that are antitumor or immunostimulating; notably, *G. lucidum*, *L. edodes*, and *A. blazei* had bioactive compounds present in fruiting body, mycelial biomass, and liquid broth (Chang and Miles, 2004). Most research examined oral administration as the best mode of β-glucan administration, the way traditional oriental medicinal mushrooms are. Antihyperglycemic, antihypertriglyceridaemic, antihypercholesterolemic, and antiarteriosclerotic activities have been observed, in conjunction with lowering blood glucose concentration in diabetic rats (Rahar et al., 2011). The structures of β-glucans in some medicinal mushrooms features glucan-protein complexed such as αβ-glucan in *A. blazei*; homopolysaccharides such as β-(1-3)-D-glucan, (1-6)-glucan with (1-4)-branches at O-4-α-(1-4)-D-glucopyranosyl, and heteropolysaccharides such as β-(1-3)-D-galacto pyranosyl with glucose and galactose branches in *G. lucidum*; homopolysaccharides such as (1-3),(1-6)-D-polysacchrides and heteropolysaccharides such as fuctomannogalactan of (1-6) with α-D-galactopyranose braches in *L. edodes*; and homopolysacchride such as β-(1-3)-D-glucan in *P. linteus* (Villares et al., 2012). These structures are different from each other and provide unique bioactivities.

2. **Composition of Medicinal Mushrooms**

***Agaricus blazei* Murrill** is classified under order *Agariales* having large brown, white, and cream colored cap that is round convex with pileus that can grow up to 25 cm (Chang and Miles, 2004). It is commonly known as Almond Portobello, ABM, Cogmelo de Deus,

Himemtatsutake, Kawariharatake and Royal Sun Agaricus (Stamets, 2000). On average, it is composed of 86% water on wet basis; 4% protein, 5% fat, 5% fiber, and 5% ash on dry basis on average (Firenzuoli et al., 2008; Fermor et al., 1985). *Agaricus blazei* Murrill can be found in wooded forests or grassland that are rich in lignicolous debris and are commonly found in the southeastern U.S. and in Brazil (Stamets, 2000). ABM contains around 227 mg/g of total free amino acids, 22 mg/g of essential amino acids, and 30 mg/g of total carbohydrates (Kim et al., 2009). In a different study, *A. blazei* reportedly contained 702 μg/g of phenolic compounds in 2 g of mushroom (dry basis); it had 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of 67% at reaction time of 30 minutes and superoxide dismutase (SOD) activities of 48% respectively (Kim et al., 2008). Out of five edible and five medicinal mushrooms tested by Kim et al. (2008), 326 μg/g of average total concentration of phenolic compounds with 477μg of average total concentration of phenolic compounds within medicinal mushrooms; 5% to 78% range of DPPH scavenging activities at reaction time of 30 minutes; along with average of 28% of SOD activity (Kim et al., 2009). *A. blazei* Murrill was discovered to contain higher amounts of total polyphenols and antioxidant activities than the average of tested mushrooms.

Recent research has focused on the value of *Agaricus blazei* Murrill for its medicinal properties. *Agaricus blazei* Murrill has been studied and shown to have anti-tumor, immunostimulating, and antiviral activities. *Agaricus blazei* Murrill contains 70% of β-1,2-linked mannose units that can be used to build glucomannan (Mizuno et al. 1999). The specificity of the structural make up is important for its biological activities including anti-tumor effects. Extensive research involving antitumor activity has shown that the main component of proteoglucan HM3-G involved β-(1,6)- backbone (Wasser, S., 2002). The lipid fraction containing ergosetrol was found to suppress angiogenesis (Takaku et al., 2001). ABM was also

found to have side chains in the β-(1,6)-glucan backbone of β-(1,3)- that shows immunomodulatory activity; oral consumption increased IgG and T-cell populations (Chan et al., 2006; Firenzouli et al., 2008). It is also noted to be rich in agaritine, a compound reported to be antiviral, genotoxic, and carcinogenic; it is found in mushrooms at concentrations around 112-731 mg/kg (dry basis) (Nagaoka et al., 2006). *A. blazei* Murrill has also been noted to inhibit virus attachment to HSV-1 and BoHV-1 in HEp-2 cell cultures (Bruggemann et al., 2006).

Ganoderma lucidum is classified under family *Ganodermataceae* and is praised for its medicinal properties in East Asia (Roberts and Evans, 2011). It is found in woodlands near oak, chestnut and plum trees around the world (Roberts and Evans, 2011). It has a reddish-brown colored, irregular shape with cap diameter of 300 mm (Roberts and Evans, 2011). It is considered a phytopathogenic fungus due to its biological functions that works as parasite against some plant species (Chang and Miles, 2004). The nutritional value can fluctuate from different sources, but *G. lucidum* from Serbia reportedly has 814.8 mg/g carbohydrates, 43.3 mg/g fat, 113.4 mg/g proteins, 28.0 mg/g ash, 4,109.3 kcal/g energy, 3.7 μg/g total phenolic compounds, and 1.05 μg/g total tocopherol on dry weight (Stojkovic et al., 2013). In other research, *G. lucidum* was found to contain 162 μg/g of total phenolic compounds in 2 g (dry basis), and had 74% DPPH scavenging activity at reaction time of 30 minutes and 8% SOD activity (Kim et al., 2008). In the preceding work, *G. lucidum* was found to contain 6.06 mg/g of total free amino acids and 8.58 mg/g of total carbohydrates on dry weight (Kim et al., 2009). It is important to note the significance of growth conditions as it adds to the variability in nutritional and medicinal compositions of mushrooms. But generally, β-glucan is found at unusually high concentrations (40.6% on dry basis) in *G. lucidum* (Stamets, 2000).

Interest in *G. lucidum*'s active compounds is expanding due to its medicinal functions; it is commonly known as the 'mushroom of immortality'. Few secondary metabolites have been isolated from *G. lucidum*: Ganoderic acid derivatives (A, B, C1… Y, and Z), Ganodermic acid derivatives (R, and S), Ganoderiol derivatives (A, B, C, D, E, F, G, H, and I), Lucidenic Acids derivatives (A, B, C, D1, D2, E1, E2, F, G, H, I, J, K, L, and M), and Lucidone derivatives (A, B, and C); where each letter indicates unique derivative of each compound (Cole and Schweikert, 2003). Liquid chromatography and mass spectrometry allowed for identification of 26 known triterpenoids and 6 novel triterpenoids (Yang et al., 2007). High concentrations of these compounds, mostly terpenoids, give strong bitterness to the mushroom. This unique complex of bioactive compounds was reported to have cytotoxic effect against cancer cells, and have antiviral and hepatoprotective activities (Russell and Paterson, 2001). It also contains polysaccharides that can boost phagocytosis activity and improve humoral and cellular immunity; synergistically, it can also improve production of H_2O_2 through production of interleukin-1 by lipopolysaccharides (Lin and Zhang, 2004). It is also reported to increase natural killer cell production by promoting mixed lymphocyte reaction (Lin and Zhang, 2004). The ability to affect multiple biological reactions allows *G. lucidum* to be highly effective as an anti-tumor and immunomodulating medicinal mushroom.

Grifola frondosa, a soft fleshed polypore, is highly regarded mushroom for its medicinal properties and culinary values. It is commonly known as Hen of the Woods or Maitake. It is taxonomically classified under family *Meripilaceae*, distributed mostly in woodlands of upper hemisphere (Roberts and Evans, 2011). *G. frondosa* is a saprophyte living by the base of trees, but sometimes may acts as parasites (Stamets, 2000). The fruiting body can grow up to 600 mm

in height and 600 in diameter; it does not look like conventional mushrooms, rather like spoon-shaped caps overlapping one another to form a mound (Roberts and Evans, 2011). It is called polypore due to its morphological differences by having pores instead of gills (Chang and Miles 2004). Although different in shape than most mushrooms, it contains 91% moisture on a wet basis; 19.7% protein (13.1% pure protein), 61.06% carbohydrates, 9.7% fiber, and 3.2% crude lipid on a dry basis (Chang and Miles, 2004). It contains many organic and inorganic compounds, but interestingly contained neither Vitamins A nor C (Mizuno and Zhuang, 1995). It contains a 1:2 ratio of essential to nonessential amino acids (Chang and Miles, 2004), and also contains 14.5% β-glucan on a dry basis (Stamets, 2000).

Grifola frondosa contains many bioactive compounds and have widely been researched for its medicinal properties. A novel compound found in the mushroom is grifolan. Its structure is a (1-3)-β-D-glucan backbone with β-D-glucopyranosyl attached as side chain (Bohn and BeMiller, 1995). The side chain allows for its anti-tumor functionality. From mycelia extract, grifolan showed 95% anti-tumor activity in an allogeneic tumor system (Suzuki et al., 1987). Grifolan was found to contain receptors at the end of side chains that are important for biological activities such as phagocytosis and TNF-inducing activities (Ishibashi et al., 2001). Another report showed that it's not just the β-glucan polysaccharide structure, but rather the polysaccharide-protein complex that is responsible for antitumor activity on MH-46 carcinoma and ICM carcinoma (Mizuno and Zhuang, 1995). *G. frondosa* is reported to contain lipids and peptidoglycan that control hypoglycemic activity, although actual compounds responsible are unknown (Kubo et al., 1994).

Inonotus obliquus, commonly known as chaga, is classified under family *Hymenochaetaceae*, and is found in woodlands of northern hemisphere (Roberts and Evans, 2011). It is also known as the Clinker Polypore for its parasitic ability to canker rots of host trees (Roberts and Evans, 2011). It appears that it has no caps or stem and can grow irregular shaped dark brown conk with diameter of 300 mm and thickness of 10 mm (Roberts and Evans, 2011). Although it is considered nonedible, it has been consumed for medicinal purpose as tea in Russia and Oriental countries for cancer and tuberculosis (Roberts and Evans, 2011). The nutritional composition is 13.2% water (wet basis), and (on a dry basis) 2.4% protein, 2.4% lipids, 71.9% carbohydrate (12% β-glucan), 10.1% ash, and 159 kcal total energy per 100 grams of fruiting body (Koyama et al., 2008). It is also reported to contain 547 μg/g of total phenolic compounds in 2 g of dry weight, 56% DPPH scavenging activity at reaction time of 30 minutes, and 13% SOD activity (Kim et al., 2008). Further research showed that the fruiting body of *Inonotus obliquus* contains 2 mg/g of total free amino acids with miniscule 0.09 mg/g of essential amino acid dry basis; it contains 5.03 mg/g of mono- and disaccharides dry basis (Kim et al., 2009).

The culinary value of the fruiting body is limited as a tea infusion and is more used as medicine than for pleasure of aroma or taste. It has been reported that the fruiting body contains secondary metabolites that are effective in diabetes control, such as lanosterol, 3β-hydroxy-lanosta-8,24-diene-21-al, inotodiol, ergosterol peroxide, and trametenolic acid (Lu et al., 2009). Inotodiol and trametenolic acid also have DPPH scavenging activity (Lu et al., 2009). A different study showed there were inhibitions of endogenous and Fe^{2+}-Cys-induced lipid peroxidation using mycelia of *I. obliquus* (Song et al., 2008). Although it has high levels of antioxidant activity, the extract is also known to have cytotoxic effects and prevents proliferation of cells (Cui et al., 2005). The mechanism of the cytotoxicity is still unknown. However, it has also been

reported to inhibit the rate of growth of cancer cells (Song et al., 2008). Other secondary metabolites from the sclerotia of *I. obliquus* include powerful antioxidants such as inoscavins, interfunginns, phelligridins, and hispolon, antiviral compounds such as betulinic acid, and a range of antitumor metabolites (Zheng et al., 2010).

Lentinula edodes, the Shiitake, has large, brown hemispherical caps and is classified under the family *Marasmiacea.* It grows up to 75 mm in height and 150mm in cap diameter (Roberts and Evans, 2011). *L. edodes* grows on logs and dead branches found in woodlands of East Asia, Australia, and New Zealand (Roberts and Evans, 2011). It is the most cultivated mushroom second to *Agaricus bisporus* (Roberts and Evans, 2011). It is composed of 90% moisture of fresh weight; 13.4-17.5% protein, 4.9-8.0% fat, 65.5-78.0% carbohydrate, 7.5-8.7% fiber, 3.7-7.0% ash of dry weight; and 387 kcal per 100 g dry weight (Chang and Miles, 2004). It contains 1.77 μg/g of phenolic compounds along with 31% DPPH radical scavenging activity at reaction time of 30 minutes and 49% SOD activity (Kim et al., 2008). It is interesting to note that out of thirty phenolic compounds analyzed, only three were present in *L. edodes*: gallic acid, protocatechuic acid, and (+)-catechin (Kim et al., 2008). In similar research, 55.69 mg/g of amino acids, 5.28 mg/g of essential amino acids, and 74.64 mg/g of carbohydrates on dry basis were reported (Kim et al., 2009).

L. edodes fruiting body have been consumed and utilized in East Asia as functional food and medicine for many years. It is also one of the highly researched mushrooms. An interesting component of *L. edodes* is lentinan. It is a β-glucan base structured polysaccharide with the ability to suppress tumor cells (Chihara et al., 1970). Lentinan is soluble only in hot water, dimethyl sulfoxide, aqueous alkali, or formic acid (Chihara et al., 1970). It has infrared spectra

and NMR spectra of 890 cm^{-1} and τ 5.4 (Chihara et al., 1970). The antitumor activity *in vivo* in mice was observed in high molecular weight molecules with triple helix structure without cytotoxity even in 60 mg/kg (Zhang et al., 2005). Eritadenine, 2(R),3(R)-dihydroxy-4-(9-adenyl), is found to lower blood cholesterol, and was first identified in *L. edodes* (Jong and Birmingham, 1993). Antioxidant activity has also been researched, and the total phenolic concentration was reportedly 5% w/w using an ethyl acetate extraction method (Kitzberger et al., 2007). *Lentinula edodes* mycelia, not the fruiting body, have immunostimulating effects by increasing the macrophages and TNF-α production (Kojima et al., 2010).

Phellinus linteus is a rare fruiting body that is still unknown to western cultures, and yet it is well established in oriental medicine. Infusion such as tea is consumed as functional food for its medicinal properties. It is classified under family *Hymenochaetaceae*. Difficulty in cultivation, lack of standardized cultivation method, and inconsistency in batch to batch create difficulties to obtain quality samples (Zhu et al., 2008). It contains total phenolic compounds of 213 μg/g in 2 g of dry weight, showed 36% DPPH scavenging activity at reaction time of 30 minutes, and shown 24% of SOD activities (Kim et al., 2008). Also another study has shown that *P. linteus* strain No. CL-01 has 4.55 mg/g free amino acids composed entirely of nonessential amino acids, and 55.28 mg/g mono- and disaccharides on dry basis (Kim et al., 2009).

In oriental medicine, it is known to treat liver toxicity, lymphatic disease, diabetes, gastroenteritis, and improve hepatoprotection (Zhu et al., 2008). Also, recent research reported many novel compounds discovered in fractions of *P. linteus* fruiting body. Phellinone is a furanone derivative from *P. linteus*. Extracted compound exhibit a white needle like structure with absorption characteristics of λ_{max} 254 nm; the structure of Phellinone contains 12-dimethyl,

8S-methyl, and 7S-vinyl furanone substituted cyclohexanone system (Yeo et al., 2007). It was also shown to have antimicrobial activities against *B. subtilis* IAM 1069, but had limited activity in other microorganisms (Yeo et al., 2007). Another novel compound identified in *P. linteus* is phellinstatin, a trimeric hispidin derivative. It is reported to have enoyl-ACP reductase inhibition (Cho et al., 2011). Furthermore, two novel furan derivatives were reported but not named. The phellinusfuran had the confirmed structure of 5α(6,7-dihydroexyethyl)-4-(5'-hydroxymethyl-furan-2-ylmethylene)-2α-methoxy-dihydrofuran-3-one and 5β-(6,7-dihydroxyehtyl)-4-(5'-hydroxymethyl-furan-2-ylmethylene)-2α-methoxy-dihydrofuran-3-one with unknown bioactivities from both compounds (Min et al., 2006).

Besides novel compounds, it is also known to contain high levels of bioactive secondary metabolites with anti-inflammatory activities. It is report to contain inotilone that suppresses LPS-induced MMP-9 expression by inhibiting different chemical pathways, while allowing the increased activity of enzymes for antioxidant production resulting improved anti-inflammatory activities (Huang et al., 2012). Yet, due to the lack of availability, *P. linteus* still needs to be further researched in its medicinal properties hailed by the oriental culture.

3. **Microorganisms**

It is suspected that foodborne diseases affect 48 million Americans and had $77 billion of economic damage (Schariff, 2012). And this trend only seems to increase every year. In 2009 and 2010, there were reports of 1,527 cases and 23 deaths related to foodborne diseases; out of 23 deaths, 3 were related to *E. coli* O157, 2 were related to *Salmonella spp.*, and 2 were related to *Listeria* (CDC, 2013). *Salmonella* infection rates are same in 2011 and 1998; also there has been increase in public health surveillance, outbreak detection, investigation in prevention

of outbreaks (Braden and Tauxe, 2013). The level of foodborne illness is still persistent in food products due to main reasons compared to previous decades. This is due to the fact that there is the increase in mass production, availability of food products, and the increase in detection of outbreaks related to foods. There are also increases in microorganisms that are multiple drug and antibiotic resistant (Banerjee and Sarkar, 2004). The difficulty in controlling bacterial contamination results from microorganisms' structure such as Gram-negative verses Gram-positive; spore formation; different levels of heat, acid, chemical resistance; biofilm formation; as well as antibiotic resistance buildup (Montville and Matthews, 2008).

Escherichia coli is a facultative, anaerobic Gram-negative that has serological and genetical different *E. coli* isolates (Meng et al., 2013). There are six isolates of pathogenic *E. coli* depending on its virulence, pathogenicity, toxicity, symptoms, and O:H serotypes (Montville and Matthews, 2008). *E. coli* O157:H7 is important serotype due to its cytotoxicity and wide spread outbreaks related to the microorganism. It is Gram-negative, Shiga toxin producing, motile, acid tolerant down to pH 4.0, but not heat labile (Montville and Matthews, 2008). *E. coli* O157:H7 has immediate reaction that lasts from two to three week since the onset and can remain in body for 62 days after the onset (Meng, et al., 2013). It is known to have low infectious dosage as few as 10 CFU (Meng, et al., 2013).

In 1982 outbreaks, it was first identified as human pathogen (Meng et al., 2013). Since 1982 to 1998, the reported number of outbreaks totaled 196 and cases totaled 4,085 (Montville and Matthews, 2008). 264 outbreaks with 5,875 cases were reported from 2000 to 2010with average of 4000 cases per year in U.S. (Meng et al., 2013). Recently, in 2009 there was an outbreak with ready-to-bake cookie dough resulting hospitalization of 77 patients (Neil et al.,

2012). From 2000 to 2010, 31.74% were related to ground beef with 11% related to produce (Meng et al., 2013). Since it was first identified, cattle and cattle farms have also been closely associated with *E. coli* O157:H7 outbreaks. Individual cattle may shed up to 10^6 CFU/g and contamination of meat can come from cattle with 10^3 CFU/g of *E. coli* O157:H7 (Meng et al., 2013). Although much has improved in the beef industry, there are random events with high level of *E. coli* O157:H7; it was found that 80% of contamination was coming from beef hides, thus adding importance of hygiene practice regarding removal of hides from carcass (Arthur et al., 2013).

***Salmonella enterica* serovar Typhimurium** is Gram-negative, facultative anaerobe, and rod shaped microorganism that is classified under the family *Enterobacteriaceae* along with 2,442 other *S. enterica* serovars (Montville and Matthews, 2008). *Salmonella spp.* are unique in that the organisms will utilize glucose, but are unable to metabolize lactose or sucrose (Montville and Matthews, 2008). It is also known to survive from freezing temperatures up to 54 ˚C, pH 4.5 to 9.5, $A_w > 0.93$, and 3 to 4% NaCl brine solution at 10 to 30 ˚C (Montville and Matthews, 2008).

Salmonellosis exists in two forms: typhoidal and nontyphoidal. Typhoidal *Salmonella* species can be obtained through contaminated water, animal products or direct contact with contaminated individuals (Coburn et al., 2007). *Salmonella* species causing foodborne illnesses are often related nontyphoidal *Salmonella* species (Hohmann, 2001). Salmonellosis is collective term for illness caused by *Salmonella* species resulting gastroenteritis, bacteremia, and focal infections (Hohmann, 2001). Symptoms include headache, myalgia, nausea, anorexia, and constipation (Coburn et al., 2007). Healthy individuals are not critically sickened from the

infection with an infectious dose around 10^5 CFU (Kothary and Babu, 2001). However, infants, elderly, and immunocompromised individuals are more likely to be critically ill. Also there is discrepancy of infectious dose depending on the food products, where chocolate and hamburger is known to least infectious dose at 10^1 CFU (Montville and Matthews, 2008). Symptoms are visible in 8 to 72 hours of infection (Montville and Matthews, 2008).

Salmonella was responsible for the highest number of foodborne illnesses in 2009 and 2010 in U.S., with 234 confirmed outbreaks (CDC, 2013). With the increased use of wide spectrum antimicrobial agents, more multi drug resistant bacteria are seen: recently 68.3% of sampled *Salmonella* species from meat products was shown to be resistant to antimicrobial compounds (Maka et al., 2014). Within a couple of decades, there has been an emergence of *Salmonella* species that shows level of resistance to nalidixic acid, cephalosporin and other extended-spectrum cephalosporins (Hohmann, 2001). These resistance mechanisms are done by enzymatic degradation, chemical modification, drug-binding site gene mutation, and activation of the efflux pump; and because genes responsible for these mechanisms are in mobile genetic element, *Salmonella* is able to transfer them effectively (Li et al., 2013).

Listeria monocytogenes is a Gram positive microorganism. It is a facultative anaerobe, human pathogen, and has unique characteristics include D-xylose, L-rhamnose, α-methyl-D-mannoside production (Montville and Matthews, 2008). *L. monocytogenes* lives in the soil, but can activate pathogenic gene in favorable conditions. It was first identified in 1926 in guinea pigs and rabbits and it wasn't until 1986 that *L. moncytogenes* was associated with food pathogens in humans (Cossart, 2011). It is also known to have pH and brine resistance as well as to temperatures down to 4 ˚C in food products (Cossart, 2011). *Listeria monocytogenes* has ability

to control the pathogenicity through transcriptional regulator called PrfA (Freitag et al., 2009). PrfA protein is hyperinvasive and enhances the virulence of the microorganisms when activated (Freitag et al., 2009). Duality in mode of existence makes this microorganism robust in harsh environment as well as problematic in processing facilities once the facility has been contaminated (Ratani et al., 2012).

Infection from *L. monocytogenes* causes listerosis in human body which can possibly results in low-grade flu, headaches, psychosis, encephalitis, infectious mononucleosis, and septicemia, with 20-30% lethality (Montville and Matthews, 2008). Infection is caused by proteins InlA and InlB which cause changes to the phospholipid arrangements of cell membranes, making *L. monocytogenes* an effective pathogen once consumed (Cossart, 2011). Food sources of outbreaks often come from contaminated meat, cheese, raw milks and many other dairy products. There have been recent reports of gastroenteritis associated with listeriosis outbreak, but it may have up to 10 weeks of hibernating period makes it difficult to accurately account all cases (Ryser and Buchanan, 2013). Globally, in 2005 to 2008, there were 5 reported outbreaks with 42 deaths (Todd and Notermans, 2011). In U.S., listeria outbreak has been decreased to 2.4 cases per million population in 2010 but it has been increasing in European countries from 3.9 to 10.3 cases per million population in 2006 (Ryser and Buchanan, 2013).

Saccharomyces cerevisiae, Zygosaccharomyces bailii,* and *Z. bisporus are yeasts often related to the spoiled foods, but also are major contributors to the fermentation of foods. It can grow in 55-60% in sucrose, acidic, and 18% ethanol environment (Jay et al., 2005). Compared to the pathogenic bacteria, spoilage yeasts are often detected before consumed for its characteristic odor and physical change of food matrix. A spoilage is important factor in food industry because

it limits the shelf life and product quality, which causes enormous economic losses to the producers. Therefore good management of spoilage microorganism controls is important.

Yeasts are classified under same kingdom as the mushrooms. However, yeast are unicellular verses the multicellular structure of the fruiting body of mushrooms. Yeasts reproduce asexually through meiosis and sporulation using simple sugar as the energy source (Carlile et al., 2001). *S. cerevisiae* is commonly used in beer, wine, cider, and bread production and is perhaps most common yeast that humans have used extensively (Carlile et al., 2001). It is an anaerobic organism that utilizes glucose, even under aerobic condition can double in number every 90 minutes (Carlile et al., 2001). Although it is used in many different applications, uncontrolled or undesired contamination from *S. cerevisiae* can spoil food products. In wine, careful control of added nutrition is needed to control hydrogen sulphide production (Malfeito-Ferrerira, 2011). Yeast is also responsible for haze, off-flavors, and sedimentation resulting in poor quality wine (Malfeito-Ferrerira, 2011).

Zygosaccharomyces is very similar to *Saccharomyces*, but it only has six identified species including *bisporus* and *bailii* species (Zuehlke et al., 2013). Much like *Saccharomyces,* it has a cylindrical/ellipsoidal shape that grows up to 9 by 13 μm (Zuehlke et al., 2013). *Zygosaccharomyces* will utilize fructose as the main energy source (Zuehlke et al., 2013). Thus, *Zygosaccharomyces* are important in the wine industry as it can spoil the grapes used in the process yielding poor quality wine (Barata et al., 2007). It has also been reported to carbonate excessively in still wine, resulting explosions. *Zygosaccharomyces* can also grow after bottling, causing changes in flavor, color, or texture (Zuehlke et al., 2012). The persistence of *Zygosaccharomyces bailii* have been observed even in acidic environment of pH of 1.8 (Jay et al., 2005). There has been an increased detection of *Zygosaccharomyces* in processed foods. It has

been reported that it is highly resistant to lipophillic weak organic acids and have been adapted well to external stresses (Smits and Brul, 2005).

4. Natural Antimicrobials

The ability to inhibit microbial growth in processed food products is of utmost importance in the food industry. It has direct consequences in consumer's end and thus there are many regulations and control set by government to oversee the food safety. Thus industry employs a variety of methods to disinfect or preserve products from microbial contamination. There are many different ways to maintain the safety of foods such as lowering pH, decreasing moisture level, heat treatment, or adding preservatives. There are many different types of antimicrobial compound such as plant based biocides, essential oils, bacteriophage cocktail, weak acids, and many more available for use depending on the food products (Acharya et al., 2011).

Some of the common chemical preservatives include sodium benzoate, sorbic acid, sulfite, and nitrite. Benzoic acid is one of the oldest chemical preservatives and it is widely used due to its availability, cost, lack of color, and low toxicity (Davidson, 2001). It is often used as sodium benzoate in order to increase the solubility in water, and is effective at concentrations as low as 0.1% (in acid media) and 0.2% (in neutral media) against a wide spectrum of bacteria, fungi, and yeasts (Chipley, 2005). Although it is effective against wide spectrum of microorganisms, there is a concern for resistance build up in microorganisms in carbonated and noncarbonated citrus drinks and fruit juices (Chipley, 2005). Sorbic acid and different forms of salts are collectively termed sorbates, and are widely used in food products as antimicrobials and preservatives (Stopforth et al., 2005). Due to its ability to inhibit yeasts and mold growth, it is

widely used in dairy products, baked goods, and confectioneries directly by addition to formulation, spraying, immersion, dusting, or coating at the concentration of 0.02% to 0.3% (Stopforth et al., 2005). Sulfur is an important ingredient in wine and fermentation of juice, but sulfites are used as "an antioxidant, to inhibit enzymatic browning and the Maillard reaction, as a dough conditioner, and to prevent black spot on crustaceans;" (Papazian, 1996; Gould, 2000) and sulfur dioxide is also used as a microbial inhibitor (Cornelius and Lilian, 2005). Sulfur is used as sulfurous acid to inhibit growth of a number of *Saccharomyces* and *Zygosaccharomyces* spp., at concentrations of 0.1-20.2 and 7.2-8.7 mg/L, respectively, and had some antibacterial activity against a broad spectrum of gram-negative bacteria (Cornelius and Lilian, 2005).

In order to extend the shelf life, additives have been chosen to minimize spoilage. Preservation, nutritional additives, flavoring agents, coloring agents, texturizing agents, and miscellaneous additives are primary functions of food additives (Branen and Haggerty, 2002). As for the preservation additives, there are three types of additives: antimicrobial, antioxidant, and anti-browning (Branen and Haggerty, 2002). The FDA closely regulates any chemicals added to food products. Under FDA 21 CFR 101.22(a)(5), antimicrobial compounds used in foods are "any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties" (FDA, 2009).

5. Antimicrobial activities in mushrooms

All living organisms have ways to defend themselves from harm from the natural environment. There are simple physical defense such as protective layers but there are also

complicated immune systems and biocides for chemical defense. Although mushrooms aren't as complicated organism as mammals or plants, many different modes of antimicrobial activities have been observed in a variety of mushrooms. Mushrooms are good sources of natural antimicrobial compounds. Mushrooms have many different compounds of polyphenols or polysaccharides that shows antimicrobial compounds isolated from fruiting bodies of basidiomycetes. Armillaric acid isolated from *Armillariella mellea* had activities against many gram-positive bacteria and yeasts; centinamycin A and B isolated from *Lentinula edodes* had anti-infective activity, schizophyllan isolated from *Schizophyllumi commune* was effective against *P. aeruginosa, S. aureus, E. coli,* and *K. pneumoniae* in mice; coriolin isolated from *Trichomonas vaginalis* was effective against gram-positive bacteria; campestrin and psalliotin isolated from *A. campestis* and *A. xanthoderma,* respectively, had inhibition against gram-positive bacteria; ganomycin A and B, derivatives of sesquiterpenoid hydroquinones, isolated from *G. pfeifferi* and *G. lucidum* had inhibition against gram-positive, gram-negative bacteria, and multiresistant bacteria are only few compounds that were isolated and identified from fungi sources (Wasser and Weis, 1999; Gao, et al., 2003; Lindequist et al., 2005).

Mushrooms contain a vast array of compounds and secondary metabolites such as flavonoids, terpenoids, polyphenols. Benzaldehyde, a precursor to the benzoic acid, can be found in *A. braziliensis* (Lindequist et al., 2005). Also compounds such as ascorbic acid, a natural preservative and antioxidant, has been reported in *Lycoperdon, perlatum, Cantharellus cibarius, Clavaria vermiculris, Ramaria Formosa, Marasmius oreades,* and *Pleurotus pulmonarius* (Ramesh and Pattar, 2010). Some antimicrobial compound such as cinnabarin, phlebia kauranol aldehyde, and scorodonin were investigated and isolated from *Coriolus*

sanguineus, *Punctularia atropurpurascens*, and *Marasmius scorodonius*, respectively (Chung et al., 1978; Anke et al., 1980, 1986).

There are numerous researches conducted looking at the antimicrobial and antifungal activities of varieties of mushrooms. Not all have been able to identify the specific compound or the mode of action responsible for the inhibitory activities. *Ramaria flava*, a wild mushroom, showed inhibition of Gram-positive bacteria using an agar well diffusion assay (Gezer et al., 2006). In similar research, *Clitocybe alexandri* and *Rhizopogon roseolus* mushrooms were found to have antimicrobial effects against certain microorganisms such as *E. coli* (ATCC 11230) (Solak et al., 2006). *Agaricus bisporus*, a popular culinary mushroom, showed strong inhibition of gram-positive bacteria (Jagadish et al., 2009). Although simple research methods using agar-well diffusion or disk diffusion assays hint at the existence of the inhibitory compounds, it is important to study and identify these compounds in order to uncover the novel compounds hiding in the matrix of bioactive components in the mushrooms complex system.

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Chapter 3

Identification of Inhibitory Compounds in Medicinal Mushrooms Against Pathogenic Bacteria and Spoilage Yeasts

Abstract

Extracts from medicinal mushrooms were prepared and tested for anti-microbial activity against food pathogens and food spoilage microorganisms. The inhibitory activity was measured using a disk diffusion assay and with optical density (OD). For OD, 7 fractions were collected using HPLC for 4 (*A. blazei* Murrill, *G. lucidum, G. frondosa, I. obiquus*) medicinal mushrooms and 6 fractions from *L. edodes* and 8 fractions from *P. linteus*.

The results from disk diffusion assay showed that most mushrooms displayed significant inhibition compared to the ethanol. The exceptions were: *A. blazei* Murrill, *I. obliquus,* and *L. edodes* against *E. coli* O157:H7; *I. obliquus* against *L. monocytogenes* V7; *I. obliquus* against *S. cerevisiae* Y99*; L. edodes* against *Z. bailii* Y03*;* and *I. obliquus* against *Z. bailii/bisporus* Y108. Inhibition was more effective in yeasts than bacteria. The result from Bioscreen C showed that against *L. monocytogenes* V7, fraction 7 in *A. blazei* Murrill; fraction 1, 4 and 5 in *G. lucidum*; fraction 4 in *G. frondosa;* and fraction 4 and 5 in *I. obliquus* significantly inhibited the growth compared to ethanol. Against *Z. bailii* Y03, fraction 7 in *A. blazei* Murrill; all fractions from *G. lucidum, G. frondosa,* and *P. linteus*; fraction 1, 2, 3, and 6 from *I. obliquus*; and fraction 4 and 6 from *L. edodes* significantly inhibited growth compare to ethanol. The results indicated that there is significant antimicrobial activity against food pathogens and spoilage organisms in the medicinal mushrooms studied.

Keyword: Inhibition, antimicrobial, spoilage, yeast, pathogen, bacteria, fraction, medicinal mushrooms

1. **Introduction**

Mushrooms have been used in different parts of the world for centuries as a medicine for many diseases. In Eastern Asia, mushrooms are regarded to have high potency against many diseases and infections. Medicinal mushrooms (MM) contain high level of secondary metabolites such as polyphenols and triterpenoids naturally created as a defense mechanism to survive in the natural environment. A few popular mushrooms such as *Ganoderma lucidum* and *Lentinula edodes* have been widely researched and found to contain many novel compound such as ganomycin, ganoderic acids, and episterol (Cole and Schweikert, 2003). Only in recent years has the medicinal value of mushrooms been researched. Some mushrooms are reported to contain antitumor, antioxidant, immunomodulating, antiviral, antimicrobial, antidiabetic, and/or hepatoprotective activities (Wasser, 2011). However, the majority of secondary metabolites in mushrooms species haven't been identified due to lack of clear taxonomical classification of species, undiscovered species, understanding of cultivation techniques (Wasser, 2011).

Preservatives are used by the food industry to prolong the shelf life of products and eliminate the growth of pathogenic bacteria and spoilage yeasts. FDA closely regulates any chemicals added into food products for consumption. Commonly used antimicrobial compounds include benzoic acid, potassium sorbate, sulfite, and nitrite (Jay, 1978). The trend in foodborne illnesses and outbreaks is never subsiding, and even with stringent regulations, there was over 650 cases of Salmonellosis from bean sprouts in 2005 (Monteville and Matthews, 2008). CDC reports that 380 people have been reported to have been infected with *Salmonella* Heidelberg in 2013 alone (CDC, 2013). In the U.S., there are an estimated 9.4 million illnesses from 31 foodborne pathogens (Scallan, et al., 2011). Control of foodborne bacteria results to be

problematic as microorganisms are resistance to antibiotic and preservatives; for example, *E. coli* O157:H7, *S. enterica*, and *Campylobacter spp.* have been reported to show resistance to antimicrobial compounds in developed countries (Threlfall et al., 2000). Therefore, it is important to continue searching for effective antimicrobial compounds from natural sources.

Microorganisms are able to adapt and evolve based on the environmental and physical stresses. Identification of novel antimicrobial compounds is important task in order to fight foodborne pathogens and spoilage yeasts. In order to practically identify novel compounds, observation of inhibitory activity and understanding of chemical matrix is required. Then, high performance liquid chromatography can be used to fractionate medicinal mushroom extracts to help identify compounds or groups of compounds of interest. Testing inhibition of these fractions by looking OD of liquid broth can help identify which compounds have activity.

The objectives of this research are: to quantify the inhibitory effect of ethanolic extracts of six medicinal mushrooms against pathogenic bacteria and spoilage yeasts, and then to determine whether the specific fractions of the extracts in medicinal mushrooms possessed more antimicrobial capacity than the others against *L. monocytogenes* and *Z. bailii*. The results will show the effectiveness of ethanolic medicinal mushroom extracts against food related microorganisms.

2. Materials and Methods

2.1 Samples

Six MMs evaluated in this study were chosen for their significance in medical fields. *Agaricus blazei* Murrill (2010, sourced from China), *Ganoderma lucidum* (2010, sourced from China), *Grifola frondosa* (2010, sourced from China), *Inonotus obliquus* (2010, sourced from USA and Russia), and *Lentinula edodes* (2010, sourced from China) were obtained from commercial source of Mountain rose herbs™. *Phellinus linteus* (2010, sourced from S. Korea) was obtained from a commercial source in S. Korea. All MMs were received in dried conditions. Prior to grinding, MMs were stored in 4 ˚C refrigerator. MMs were taken out of the refrigerator and ground, using Waring blender, then sieved to a fine powder. Powders were then stored at -20 ^{o}C until extraction. Potassium sorbate (Sigma Aldrich; St. Louis, MO), *trans*-cinnamaldehyde (Aldrich, St. Louis, MO), and ethanol (95.5% USP grade, Ricca Chemical, Arlington, Texas) were used as positive and negative controls for confirmation of inhibition of growth of *L. monocytogenes* and *Z. bailii*.

For measuring OD, Bioscreen C Microbiology Reader (Bioscreen C; Piscataway, NJ) was utilized. Also only *L. monocytogenes* was chosen out of three pathogenic bacteria because it had most consistent results in disk diffusion assay. Also *E. coli* O157:H7 and *S.* Typhimurium are Gram positive and yet had large discrepancy in results. For similar reason, only *Z. bailii* was tested out of three spoilage yeasts.

2.2 Culture and Growth Condition

Escherichia coli O157:H7 EHEC 994, *Salmonella enterica* serovar Typhimurium ATCC 14028, *Listeria monocytogenes* V7, *Saccharomyces cerevisiae* Y99, *Zygosaccharomyces bailii/bisporus* Y108, and *Zygosaccharomyces bailii* Y03 were obtained from The American Type Culture Collection. Microorganisms were used in this study. *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were prepared in Tryptic Soy broth (TSB; Bacto™; Becton,

Dickinson and Company; Spark, MD) for 48 hours in a 37˚C incubator prior to the experiments. Yeast strains of *S. cerevisia* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03 were prepared in Sabouraud Dextrose broth (SDB; Difco™; Becton, Dickinson and Company; Spark, MD) for 25˚C incubation prior to the experiment. Tryptic Soy agar (TSA; Difco™; Becton, Dickinson and Company; Spark, MD) was used in calculating culture populations for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* V7. Sabouraud Dextrose agar (SDA; BBL™; Becton, Dickinson and Company; Spark, MD) was used in calculating culture populations for *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03. Dilution factors were used to calculate the population size. Dilution blanks using peptone water was used at 0.1% to resist further microbial growth. 1mL broth with incubating microorganisms was transferred to 9 mL peptone water. From 9 mL peptone water + 1 mL broth, 1 mL is taken out and pipetted to another 9 mL peptone water. This process repeated up to 8 times. They were then inoculated on the nutrient plates (TSA for bacteria and SDA for yeasts). After incubation time, colonies were counted and original population is estimated. The three bacteria and three yeasts cultures were calculated to be at the level of 10^8 CFU/mL and 10^7 CFU/mL, respectively, when used.

The same nutrient agars were used for disk diffusion assay. TSA was used for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* in 37˚C incubator for 48 hours, and SDA was used for *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03 in 25˚C incubator for 72 hours. The same broths were used for OD measurements. TSB was used for *L. monocytogenes* in 37˚C for 48 hours, and SDB was used for *Z. bailii* in 25˚C for 72 hours.

2.3 Microorganism Identification Tests

Confirmation of microorganisms was conducted prior to the experiments. *E. coli* O157:H7 was isolated, from frozen culture, using Eosin Methylene Blue agar (Levine; Oxoid). *S.* Typhimurium was first isolated using Xylose Lysine Deoxycholate agar (Difco™;Becton, Dickison and Company; Spark, MD). *E. coli* O157:H7 and *S.* Typhimurium were confirmed using API-20E (BioMérieux; Marcy l'Etoile, France). *E. coli* O157:H7 was further tested using latex agglutination kit from RIM *E. coli* O157:H7 (Remel, Lenexa, KS). *L. monocytogenes* was isolated on modified Oxford Agar base (Remel, Lenexa, KS), black colonies with sunken centers. *L. monocytogenes* was confirmed using API-Listeria (BioMérieux; Marcy l'Etoile, France) before transferring the isolated colony to nutrient broth of TSB. SDA was used to isolate and SDB was used as nutrient for *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03.

2.4 Single Solvent Extraction

Powders of six medicinal mushrooms (*A. blazei* Murrill, *G. lucidum, G. frondosa, I. obliquus, L. edodes,* and *P. linteus*) were weighed (5.0 grams) individually and soaked in 200 mL of ethanol (95.5% v/v aq.) in 500 mL flasks. Then the flasks were sealed and placed in a water shaker bath (Precision Scientific 260 Orbital Shaker Bath) for 24 hours at shaker rate of 120 and water temperature of 36˚C. The liquid extracts were then filtered using Whitman No.4 filter paper (cellulose standard grade; Whatman Filters). Filtered extracts were evaporated with a Rotavap (R-3000, Buchi) at 75˚C to remove ethanol. The extracts were transferred to 10 mL centrifuge tubes (Thermo Scientific, Rochester, NY) and the remaining solvent was evaporated using a stream of N_2 gas. Percent yields of extracts were measured using an electronic balance.

2.5 Normal Phase HPLC Fractionation

The method was adapted from the report of Kim et al. (2008) to separate methanol extracted phenolic compounds. An Agilent 1260 (Palo Alto, CA) high performance liquid

chromatograph was used to fractionate the mushroom extracts using Nucleosil (25cm x 4.6 i.d., 5μm, 100Å pore size; Macherey-Nagel) porous silica column. The solvent, used with a flow rate of 1.0 mL/min, was 0.1% v/v aq. acetic acid (solvent A) and 0.1% v/v acetic acid in acetonitrile (solvent B). The solvent was used as follows: initial conditions 92% solvent A, then 92%-70% solvent A from 0 to 3 minutes, 70%-50% solvent A from 3 to 12 minutes, 50%-10% solvent A from 12 minutes to 38 minutes, 10%-0% solvent A from 38 to 50 minutes, hold 0% solvent A for 10 minutes, and then 0%-92% solvent A to regenerate the column. A diode array detector (DAD) was used with UV range signal collected from 190 to 400 nm. Signals at 254 nm, 280 nm, and 310 nm were plotted, but only the 280 nm wavelength was used in selection of fractions for collection. A column thermostat was set and maintained at 25˚C throughout the fractionation.

10 mg of dried extracts were dissolved in 1 mL of ethanol. This was fractionated by using high performance liquid chromatography (HPLC) prior to the OD measurement experiment. The extracts were injected into the system in volumes of 20 μL; a total of five injections were carried out. All injected extracted were fractionated, but using different times for each medicinal mushroom extract. Individual medicinal mushroom extracts were collected after each injection using specific time for each collection (Table. 7). Fractionated extracts were then combined in 50 mL conical centrifuge tubes. After all five sets of fractions were collected, the fractions were dried using N_2 gas in a heated bath at 45˚C until completely dried.

2.6 Disk Diffusion Assay

TSA and SDA were used as the growth medium for pathogenic bacteria and spoilage yeasts respectively. The extracts were reconstituted in ethanol (95.5% v/v aq.) at a concentration of 5 mg/mL. *Trans*-cinnamaldehyde and potassium sorbate were dissolved in ethanol at concentrations of 500 μg/mL. One hundred μL of 0.1% peptone water containing 10^5 CFU/mL

of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* were individually pipetted and spread using L-shaped spreader onto the TSA plates. The same process was repeated for the *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03 on SDA plates. Disks (Whatman No.4 filter paper, 7mm in diameter) were laid on the TSA plates with *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*. It was repeated on the SDA plates with *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03. Then, 15 μL of 5,000 ppm extracts were applied onto the disks using pipets. Two plates were prepared for each set of run with each plate had three different medicinal mushroom extracts with a disk of positive control, and a negative control. Positive controls were 500 ppm *trans*-cinnamaldehyde for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*, while 500 ppm potassium sorbate was positive control for *S. cerevisiae* Y99, *Z. bailii/bisporus* Y108, and *Z. bailii* Y03. Negative controls were ethanol (95.5% v/v aq.; Ricca Chemical; Arlington, Texas) for all microorganisms. Negative control was added to see the effective of extract solvent on microorganisms; no true negative, such as water, was included in this research. TSA was then incubated at 37 ˚C for 24 hours and SDA at 25 ˚C for 48 hours. The zone of inhibition was measured in millimeters using a micrometer. Statistical analysis was performed using two sample t-tests for significant differences compared to the negative control.

2.7 Determination of Optical Density

Only *L. monocytogenes* and *Z. bailii* Y03 were further studied to evaluate antimicrobial activities of fractionated extracts. *L. monocytogenes* was prepared in TSB for 48 hours in 37 ˚C incubator prior to the experiment. This broth was then diluted in 0.1% peptone water to 10^5 CFU/mL. The population was chosen as optimal concentration that works most consistently with the opacity of the broth during the experiment. The transmission of light was important factor to consider using the equipment. Bioscreen C and plates containing 100 wells in honeycomb pattern

were used. Treatments (including 500 ppm *trans*-cinnamaldehyde positive control, negative control, and triplicate of each medicinal mushroom extract fractions) were conducted over two plates with three replications. Triplicate of blank wells with only nutrient broth, and triplicates of blank well with *L. monocytogenes* were also included.

To set up each plate, first 200 µL of Tryptic Soy broth were added to individual wells in the plates. Then 6 µL of treatments were added to the wells. Lastly, 10 µL of *L. monocytogenes* was added to the wells, except for the three wells that excluded *L. monocytogenes*. Two plates were then loaded to the Bioscreen. Parameter was set to 168 well counts for 48 hours with quantification every 30 minutes. Detection wavelength was set to wideband (420-580 nm). The program was set to shake the plates for 10 seconds prior to detection. After 48 hours, data was imported to excel program to generate growth curves.

Z. bailii was prepared in SDB for 48 hours in 25˚C incubator. Then the culture was diluted to 10^5 CFU/mL in 0.1% peptone water. The method remained same for *Z. bailii* including the control treatment, but differed slight in Bioscreen C parameters. The Bioscreen C parameters were set to 72 hours with data collection every 30 minutes. Detection wavelength was set to wideband (420-580 nm). Also the incubator temperature was set to 25 ˚C. The shaker was also on for 10 seconds prior to detection. Data was transferred to excel program to generate growth curves.

2.8 Statistical Analysis

Statistical tests were done to validate the results from disk diffusion assay and Bioscreen C. For disk diffusion assay, results were collected in millimeters. After calculating the mean and standard deviation of each extracts, data were compared to the negative control using two sample

t-test. The test involved Ho: $\bar{x}_{extracts} = \bar{x}_{negative}$ and Ho: $\bar{x}_{extracts} \neq \bar{x}_{negative}$ where $\bar{x}$ is the sample means. Significance level was chosen at $\alpha = 0.05$.

Looking at the change over the OD of fractionated extracts using Bioscreen C, the graphed change was relative. So the area under the curve was calculated first by following the trapezoidal rule. Then the sample means and standard deviations were calculated from n=9. Calculated values were used to run two sample t tests Ho: $\bar{x}_{extracts} = \bar{x}_{negative}$ and Ho: $\bar{x}_{extracts} \neq \bar{x}_{negative}$ where $\bar{x}$ is the sample means. Error bars were created using two standard deviations, equaling 95.5% confidence interval. Statistical run was compared to the negative control for significance at $\alpha = 0.05$.

3. Results and Discussion

3.1 Disk Diffusion Assay

Qualitative and quantitative analysis of inhibition of microorganisms were tested using the disk diffusion assay. The experiment effectively proved the presence of inhibitory compounds in medicinal mushroom extracts against the selected microorganisms. Preliminary work showed the inability of medicinal mushroom extracts to fully solubilize in water. Thus, all medicinal mushroom extracts were dissolved in ethanol solvent so the same concentration of ethanol was used as a control. Ethanol solvent without extracts also proved to have inhibitory activity against microorganisms. The mean inhibition zone was 13.77 mm $\pm$ 1.99 mm for pathogenic bacteria and 15.14 mm $\pm$ 1.81 mm for spoilage yeasts on TSA and SDA plates respectively. It was necessary to account for the inhibitory activity of ethanol when looking at the results. Further statistical analysis was necessary to exclude the activity provided by ethanol solvent. Two sample t-test was conducted to see the significance of inhibitory activities of

medicinal mushroom extracts beyond the activity seen using only ethanol solution. Different level of inhibition was observed in each microorganism.

Against *E. coli* O157:H7, *A. blazei* Murrill, *I. obliquus,* and *L. edodes* was shown to lack inhibitory activity beyond activity observed from ethanol (Table 1). Against *S.* Typhimurium, statistically significant inhibition was observed in all six medicinal mushroom extracts (Table 2). Against *L. monocytogenes*, *I. obliquus* lacked inhibitory activity (Table 3). In yeasts, medicinal mushroom extracts were most effective against *Z. baili.* Against *S. cerevisiae*, *I. obliquus* did not show significant inhibition (Table 4). Only *L. edodes* extract did not show statistical significant inhibition against *Z. bailii* (Table 5). Against *Z. bailii/bisporus*, *I. obliquus* did not show significant inhibition (Table 6). These results agree with previous research where *G. lucidum* showed inhibition of *Salmonella typhimurium*(MTCC 98), *Escherchia coli*(MTCC 443), and *Bacillus subtilis* (MTCC 411) using hot water or methanol extraction*; L. edodes* have been shown to inhibit the growth of *Escherchia coli* (ATCC 25922), *Micrococcus luteus*(ATCC 9341), *Bacillus cereus*(ATCC 27348), and *Candida albicans* (MY 1055) using organic solvents (petroleum ether and methanol) extraction; *Phellinus spp.* inhibited *Bacillus cereus* (ATCC 6633) using ethyl acetate extraction (Sheena et al., 2003; Suay et al., 2000; Yoon et al., 1994; Rosa et al., 2003). In research done using aqueous extraction, *L. edodes* had antifungal and antibacterial activities in 50% and 85% of species examined, respectively, including *E. coli* O157:H7, and *L. monocytogenes* (Hearst et al., 2009).

The medicinal mushroom extracts were compared to the positive control as well to see if they were comparable in activity. For the pathogenic bacteria *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes,* the extracts were not as effective as the positive control, 50 ppm *trans*-

cinnamaldehyde. For spoilage yeasts, most medicinal mushroom extracts showed relatively strong inhibition compared to the positive control, 500 ppm potassium sorbate. Against *S. cerevisiae*, all extracts inhibited comparable activity to the positive control. Against *Z. bailii*, *A. blazei* Murrill, *G. frondosa,* and *P. linteus* inhibited comparably to the positive control. Against *Z. bailii/bisporus*, *A. blazei* Murrill, *G. lucidum, L. edodes* and *P. linteus* inhibited comparably to the positive control. For spoilage yeasts, the comparison to the positive control shows that extracts worked the best against *S. cerevisiae*. However, the inhibition zone of *S. cerevisiae* by positive control was only 16.25 mm ±3.87 while it was 17.53 ±1.55 and 17.52 mm ±1.72 in *Z bailii* and *Z. bailii/bisporus*, respectively. Therefore, comparing the inhibition zone of extracts to both positive and negative control, only *Z. bailii* was chosen to be tested against fractions of medicinal mushroom extracts using the Bioscreen C. As for pathogenic bacteria, although medicinal mushroom extracts inhibited best against *S.* Typhimurium, there was inconsistency of results between two Gram negative bacteria. Therefore, *L. monocytogenes* was chosen for further research.

One difference in pathogenic bacteria are that *S.* Typhimurium and *E. coli* are Gram negative while *L. monocytogenes* is Gram positive. The difference in their cell wall formation can contribute largely to the outcome of the experiment. Gram negative cell walls has single peptidoglycan layer underneath the lipoprotein bilayer (Beveridge, 1999). This composition of cell wall allows them to withstand extreme temperatures and pH ranges while keeping the cell wall's elasticity. Gram positive cell wall contains multilayered peptidoglycan layer with low lipoprotein and amino acid contents (Salton, 1953). These differences in composition of cell walls makes inhibitory activities vary from microorganism to microorganism. Also these bacteria often build resistance by adapting new mechanisms such as glycopeptide resistance, mutation

through DNA gyrase, or parallel and vertical transfer of already known resistance genes; these adaptation forces organisms to utilize multiple inhibitory compounds (Courvalin, 1994). Unlike bacteria, the different species of yeasts used were similar in their composition and physical characteristics, which probably explains why we found similar inhibitory results for them.

The disk diffusion assay is a quick and simple way to observe inhibition, however it has many limitations. It does not answer questions regarding the mode, effectiveness or duration of inhibition. The disk diffusion assay was used with whole extracts so the concentration of active compound is at much lower level compared to the pure compound used as positive control. Thus further study was necessary to answer some of these questions.

3.2 Optical Density Measurement

OD (turbidity) allows for the determination of growth characteristics of microorganisms. It also allows for determining small differences in growth patterns. Using Bioscreen C, measurements were taken every 30 minutes for 48 hours and 72 hours for *L. monocytogenes* V7 and *Z. bailii* Y03 respectively. However some issues arose from using Bioscreen C verses the disk diffusion assay. Bioscreen C operates at much smaller volume than the disk diffusion assay, thus the pH became an issue. pH allows for certain chemical activities to be masked or present. This was much less noticeable in *L. monocytogenes* V7 however, testing against *Z. bailii* Y03, the positive control previously selected did not inhibit growth. Potassium sorbate is sensitive to the pH change. It loses 50% of its antifungal effect from pH of 3 to 5 (Sauer, 1977). The pKa of sorbic acid is 4.76, and although the salt forms are used more because of their higher water solubility, the acid form is more active as an antimicrobial (activity is greater below pKa, similarly to sodium benzoate and many other antimicrobials). So at low concentration of

potassium sorbate, there isn't much acid dissociated in the solution; therefore, *trans*-cinnamaldehyde was used at 500 ppm instead. This brought an insight about the importance of pH and dissociation of hydrogen ions in the solution. It is known that there are benzoic acids and other compounds that have antimicrobial effect in mushrooms (Lindequist et al., 2005). However they are not effective at neutral pH, which is the pH range of the nutrition broth used in the experiment. So it is understandable that there might be some secondary metabolites, specifically acids, which might not have contributed to the inhibition due to inactivity at neutral pH. An avenue for further research would obviously be to test the extracts at lower pH.

The HPLC chromatograms of fractionated medicinal mushroom extracts are shown in Figures 25 and 26. The research was difficult due to the fact that there were no available methods using high performance liquid chromatography to properly fractionate different secondary metabolites found in medicinal mushrooms. The method used was a gradient to separate based on different polarity of the compounds. There was also an issue with contaminants from the column that had ghosting effect in the chromatography. It was relatively easy to identify these peaks because they were consistently showing up on all extracts including the blank (no injection) run. Obvious ghost peaks can be seen around 17.5 and 59.5 minutes as well as a group of 12 peaks from 23.2 to 33.9 minute mark. These peaks were ignored from fractionation method to minimize their presence in fractions collected.

Results were analyzed looking the line graph of change in OD as well as quantifying the results through area under the curve. Quantification also allowed the comparison of fractionated extracts to the negative (95.5% EtOH) and positive control (500 ppm *trans*-cinnamaldehyde) statistically using two sample t-test. The Bioscreen C method was set up so that the

microorganisms were able to fully utilize the nutrients in the well. So in Figure 1-12, there are plateaus formed by the microorganisms that couldn't further populate. There are some differences in growth characteristics of *L. monocytogenes* compared to *Z. bailii.* In *L. monocytogenes* there was delay in growth which means that compared to negative control, fractionated extracts caused *L. monocytogenes* to take longer to reach the plateau. However, for *Z. bailii,* inhibition forced differences in total population when it reached plateau. It means the total populations with fractionated extracts were less than the total population reached in negative control. The results compared to positive control were significantly different and no fractions came close to the effectiveness of the positive control. It is also interesting that visually, there weren't significant differences from negative control to the fractionated extracts.

Further analysis of results was necessary to make conclusions. Therefore, area under the curve was calculated using the trapezoidal rules. Statistically, some fractions were significantly different from the negative control, proving that there were inhibitory activities from the fractionated medicinal mushroom extracts. Against *L. monocytogenes, A. blazei* Murrill fraction 7 was the only fraction that significantly inhibited the growth; *G. lucidum* fraction 1, 4, 5, and 7 significantly inhibited the growth; *G. frondosa* fraction 4 significantly inhibited the growth; *I. obliquus* fraction 4 and 5 significantly inhibited the growth; *L. edodes* fractions did not yield any inhibitions; and lastly, *P. linteus* did not yield any inhibitions (figure 13-18). Against *Z. bailii, A. blazei* Murrill fraction 7 significantly inhibited growth; *G. lucidum* fraction 1, 2, 3, 4, 5, 6, and 7 significantly inhibited growth; *G. frondosa* fraction 1, 2, 3, 4, 5, 6, and 7 significantly inhibited growth; *I. obliquus* fraction 1, 2, 3, and 6 significantly inhibited growth; *L. edodes* fraction 4 and 6 significantly inhibited growth; and *P. linteus* fractions 1, 2, 3, 4, 5, 6, 7, and 8 significantly inhibited growth (figure 19-24).

The interesting aspect of these results is that some fractions did not have visible peaks in detection at 280 nm in chromatography, but yet it still had inhibitory activities. Fraction 4 from *I. obliquus,* fraction 7 from *A. blazei* Murrill had inhibitory activities in *L. monocytogenes* and yet it did not yield any visible peaks. Fractoin 4, and 6 from *I. obliquus,* fraction 4, 6, and 7 from *G. frondosa,* and fraction 7 from *A. blazei* Murrill had inhibitory activities in *Z.* bailii and yet it did not yield and visible peaks. A lack of peaks represents little or no absorbance at 280 nm, the wavelength used. Most phenolic compounds absorb at 280 nm. So the lack of peaks could represent compounds lacking phenolic moieties or very low concentrations of compounds that do. The issue with choosing proper method was difficult. Because the research was to look at unknown compounds, it was hard to pick out the physicochemical characteristics of the inhibitory compounds. The method was chosen to include phenolic compounds because many phenolic compounds are known to have antimicrobial activities (Alves et al., 2013). However not all antimicrobial compounds are phenolic, which explains why some empty fractions yielded antimicrobial activities. Also because there were some large peaks, small peaks were almost invisible, and/or there could be a compound that is available only at very small concentration.

4. Conclusions

The research showed the presence of inhibitory compounds in medicinal mushrooms. The identification of the active compounds requires further fractionation, testing, and analysis with LCMS. It is important to understand the composition of secondary metabolites to understand what compounds could have had the inhibitory activities. It also seems there are multiple compounds that are active as seen by different fractionated extracts within each medicinal mushroom extracts. However, further study is needed to identify the specific compounds that are

responsible for the inhibition. Understanding of the specific compound responsible for the inhibition of microorganism can have immediate impact in food industry requiring new and improved ways to control microorganism contamination and foodborne-related illnesses.

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Tables

Table 1. Inhibition of growth of *E. coli* O157:H7 over 24 hrs in Tryptic Soy agar plate by six medicinal mushrooms extracts.

Treatments	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}cinnamaldehyde}$)
A. blazei Murrill	15.00	$\pm$2.07	$p=9.5x10^{-2}$	$p=2.0x10^{-10}$
G. lucidum	15.64	$\pm$1.66	$p=2.6x10^{-3}$	$p=9.5x10^{-11}$
G. frondosa	15.50	$\pm$1.77	$p=7.3x10^{-3}$	$p=1.3x10^{-10}$
I. obliquus	14.99	$\pm$1.62	$p=5.4x10^{-2}$	$p=1.3x10^{-12}$
L. edodes	15.17	$\pm$2.20	$p=6.3x10^{-2}$	$p=1.6x10^{-9}$
P. linteus	16.16	$\pm$1.36	$p=2.4x10^{-5}$	$p=1.7x10^{-10}$
trans-cinnamaldehyde	20.45	$\pm$2.62	$p=8.9x10^{-18}$	
Ethanol	13.98	$\pm$2.02		$p=8.9x10^{-18}$

Medicinal Mushrooms (5000 ppm; n=18), *trans*-cinnamaldehyde (50 ppm, n=36), Ethanol (n=36)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with *trans*-cinnamaldehyde: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\boldsymbol{trans\text{-}cinnamaldehyde}}$ H_a: $\mu_x \neq \mu_{\boldsymbol{trans\text{-}cinnamaldehyde}}$

Table 2. Inhibition of growth of *S.* Typhimurium over 24 hrs in Tryptic Soy agar plate by six medicinal mushrooms extracts.

Treatments	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}cinnamaldehyde}$)
A. blazei Murrill	14.98	$\pm$2.43	$p=1.7x10^{-2}$	$p=3.9x10^{-5}$
G. lucidum	15.04	$\pm$2.62	$p=2.0x10^{-2}$	$p=1.1x10^{-4}$
G. frondosa	16.30	$\pm$1.76	$p=2.0x10^{-6}$	$p=1.4x10^{-3}$
I. obliquus	15.50	$\pm$2.07	$p=7.9x10^{-4}$	$p=8.7x10^{-5}$
L. edodes	16.12	$\pm$1.33	$p=2.2x10^{-7}$	$p=1.3x10^{-4}$
P. linteus	16.49	$\pm$1.54	$p=9.9x10^{-8}$	$p=2.0x10^{-3}$
trans-cinnamaldehyde	18.36	$\pm$2.66	$p=4.5x10^{-13}$	
Ethanol	13.29	$\pm$2.1		$p=4.5x10^{-13}$

Medicinal Mushrooms (5000 ppm; n=18), *trans*-cinnamaldehyde (50 ppm, n=36), Ethanol (n=36)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with *trans*-cinnamaldehyde: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\boldsymbol{trans\text{-}cinnamaldehyde}}$ H_a: $\mu_x \neq \mu_{\boldsymbol{trans\text{-}cinnamaldehyde}}$

Table 3. Inhibition of growth of *L. monocytogenes* over 24 hrs in Tryptic Soy agar plate by six medicinal mushrooms extracts.

Treatments	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}Cinnamaldehyde}$)
A. blazei Murrill	16.90	$\pm$2.67	$p=3.8x10^{-4}$	$p=5.9x10^{-4}$
G. lucidum	16.35	$\pm$1.88	$p=1.4x10^{-4}$	$p=5.0x10^{-6}$
G. frondosa	16.09	$\pm$2.34	$p=2.9x10^{-3}$	$p=9.0x10^{-6}$
I. obliquus	14.58	$\pm$1.65	$p=2.7x10^{-1}$	$p=2.4x10^{-10}$
L. edodes	16.45	$\pm$1.59	$p=1.3x10^{-5}$	$p=3.0x10^{-6}$
P. linteus	17.43	$\pm$1.71	$p=7.5x10^{-8}$	$p=4.6x10^{-4}$
trans-cinnamaldehyde	20.09	$\pm$3.52	$p=1.8x10^{-12}$	
Ethanol	14.03	$\pm$1.85		$p=1.8x10^{-12}$

Medicinal Mushrooms (5000 ppm; n=18), *trans*-cinnamaldehyde (50 ppm, n=36), Ethanol (n=36)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with *trans*-cinnamaldehyde: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\textbf{\textit{trans}-cinnamaldehyde}}$ H_a: $\mu_x \neq \mu_{\textbf{\textit{trans}-cinnamaldehyde}}$

Table 4. Inhibition of growth of *S. cereivisiae* over 48 hrs in Sabouraud Dextrose agar plate by six medicinal mushrooms extracts.

Treatments	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}Cinnamaldehyde}$)
A. blazei Murrill	16.75	$\pm$1.77	$p=6.3x10^{-3}$	$p=9.1x10^{-1}$
G. lucidum	17.59	$\pm$1.35	$p=1.0x10^{-5}$	$p=2.4x10^{-1}$
G. frondosa	16.52	$\pm$1.38	$p=6.0x10^{-3}$	$p=8.7x10^{-1}$
I. obliquus	16.03	$\pm$2.07	$p=1.4x10^{-1}$	$p=4.9x10^{-1}$
L. edodes	17.34	$\pm$1.28	$p=3.2x10^{-5}$	$p=3.8x10^{-1}$
P. linteus	16.94	$\pm$1.76	$p=2.6x10^{-3}$	$p=7.3x10^{-1}$
Potassium sorbate	16.65	$\pm$3.87	$p=4.9x10^{-2}$	
Ethanol	15.04	$\pm$1.99		$p=4.9x10^{-2}$

Medicinal Mushrooms (5000 ppm; n=15), Potassium Sorbate(500 ppm, n=30), Ethanol (n=30)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with potassium sorbate: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\textbf{potassium sorbate}}$ H_a: $\mu_x \neq \mu_{\textbf{potassium sorbate}}$

Table 5. Inhibition of growth of *Z. bailii* over 48 hrs in Sabouraud Dextrose agar plate by six medicinal mushrooms extracts.

Treatments	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}Cinnamaldehyde}$)
A. blazei Murrill	17.11	$\pm$1.07	$p=8.0x10^{-6}$	$p=2.9x10^{-1}$
G. lucidum	16.54	$\pm$1.29	$p=1.7x10^{-3}$	$p=3.0x10^{-2}$
G. frondosa	17.03	$\pm$1.48	$p=2.4x10^{-4}$	$p=3.0x10^{-1}$
I. obliquus	16.18	$\pm$1.26	$p=1.2x10^{-2}$	$p=3.6x10^{-3}$
L. edodes	16.35	$\pm$2.32	$p=5.9x10^{-2}$	$p=9.0x10^{-2}$
P. linteus	17.46	$\pm$1.55	$p=3.3x10^{-5}$	$p=8.9x10^{-1}$
Potassium sorbate	17.53	$\pm$1.55	$p=1.0x10^{-7}$	
Ethanol	15.01	$\pm$1.66		$p=1.0x10^{-7}$

Medicinal Mushrooms (5000 ppm; n=15), Potassium Sorbate(500 ppm, n=30), Ethanol (n=30)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with potassium sorbate: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\textbf{potassium sorbate}}$ H_a: $\mu_x \neq \mu_{\textbf{potassium sorbate}}$

Table 6. Inhibition of growth of *Z. bailii/bisporus* over 48 hrs in Sabouraud Dextrose agar plate by six medicinal mushrooms extracts.

Z. bailii/bisporus Y108	Inhibition zone (in mm)	1 std. dev.	*T test (α= 0.05,H_a: $\mu_x \neq \mu_{neg}$)	**T test (α= 0.1,H_a: $\mu_x \neq \mu_{trans\text{-}Cinnamaldehyde}$)
A. blazei Murrill	17.00	$\pm$2.03	$p=1.3x10^{-2}$	$p=4.0x10^{-1}$
G. lucidum	17.19	$\pm$1.62	$p=1.6x10^{-3}$	$p=5.1x10^{-1}$
G. frondosa	16.33	$\pm$1.20	$p=3.7x10^{-2}$	$p=1.0x10^{-2}$
I. obliquus	16.31	$\pm$1.93	$p=1.2x10^{-1}$	$p=5.0x10^{-2}$
L. edodes	16.91	$\pm$1.47	$p=3.9x10^{-3}$	$p=2.2x10^{-1}$
P. linteus	17.53	$\pm$1.29	$p=4.0x10^{-5}$	$p=9.8x10^{-1}$
Potassium sorbate	17.52	$\pm$1.72	$p=1.2x10^{-5}$	
Ethanol	15.36	$\pm$1.78		$p=1.2x10^{-5}$

Medicinal Mushrooms (5000 ppm; n=15), Potassium Sorbate(500 ppm, n=30), Ethanol (n=30)
*Two sample t-test with Ethanol: μ = Sample average, α=0.05, H_o: $\mu_x = \mu_{neg}$; H_a: $\mu_x \neq \mu_{neg}$
**Two sample t-test with potassium sorbate: μ = Sample average, α=0.1, H_o: $\mu_x = \mu_{\textbf{potassium sorbate}}$ H_a: $\mu_x \neq \mu_{\textbf{potassium sorbate}}$

Table 7. Fractionated medicinal mushroom extracts by range of time (in minutes) used in HPLC-fraction collector.

	***A. blazei* Murrill**	***G. lucidum***	***G. frondosa***	***I. obliquus***	***L. edodes***	***P. linteus***
Fraction 1	1.8-7.8	1.8-7.8	1.8-7.8	1.8-7.8	1.8-7.8	1.8-7.8
Fraction 2	7.8-13.8	7.8-13.8	7.8-13.8	7.8-13.8	7.8-13.8	7.8-13.8
Fraction 3	13.8-19.8	13.8-19.8	13.8-19.8	13.8-19.8	138-19.8	13.8-19.8
Fraction 4	19.8-25.8	19.8-25.8	19.8-25.8	19.8-25.8	19.8-25.8	19.8-25.8
Fraction 5	25.8-31.8	25.8-31.8	25.8-31.8	25.8-31.8	25.8-31.8	25.8-31.8
Fraction 6	31.8-37.8	31.8-37.8	31.8-37.8	43.8-49.8	31.8-37.8	37.8-43.8
Fraction 7	37.8-43.8	37.8-43.8	37.8-43.8	49.8-55.8		43.8-49.8
Fraction 8						49.8-55.8

Figures

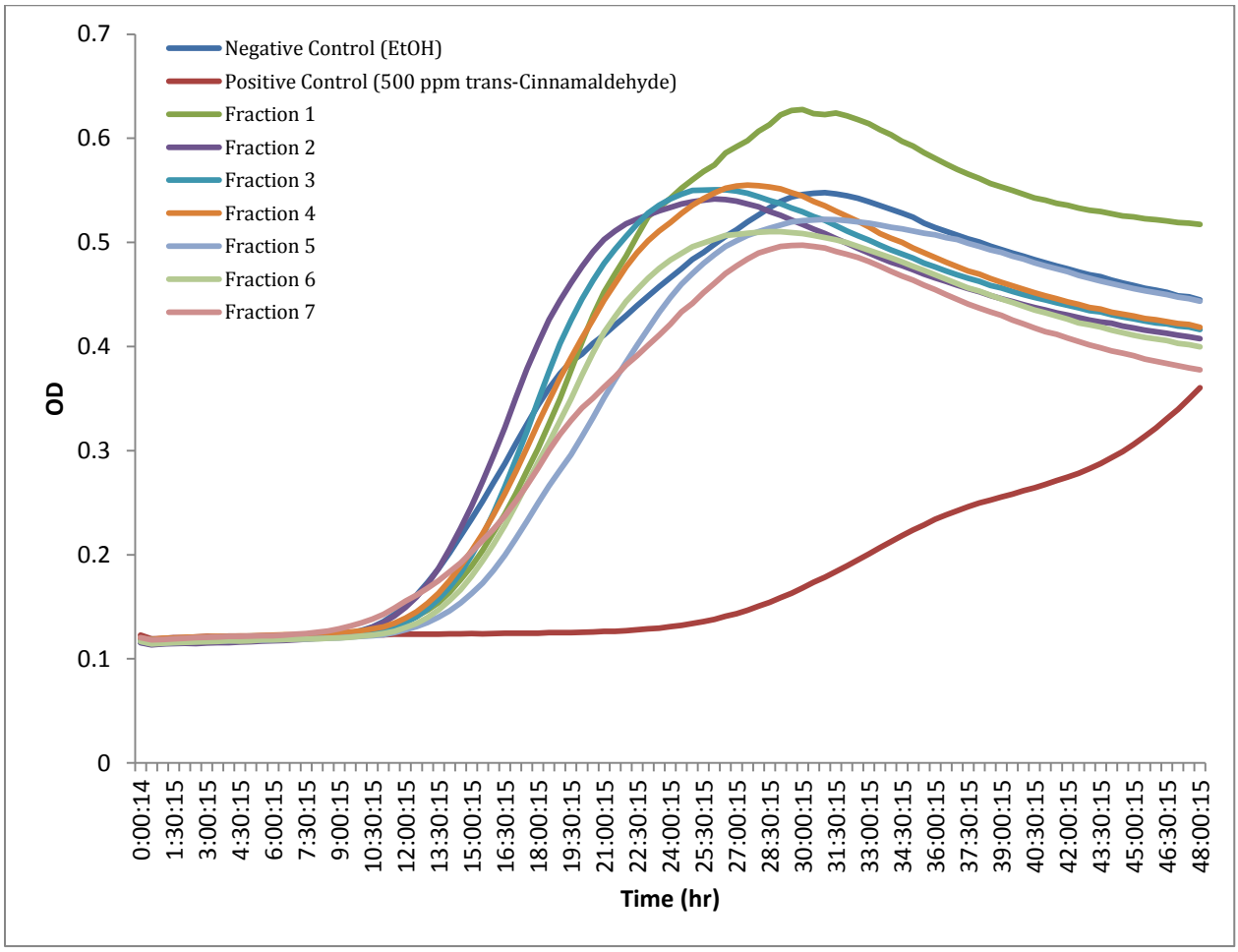

Fig 1. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with seven fractions from *A. blazei* Murrill extracts over 48 hours.

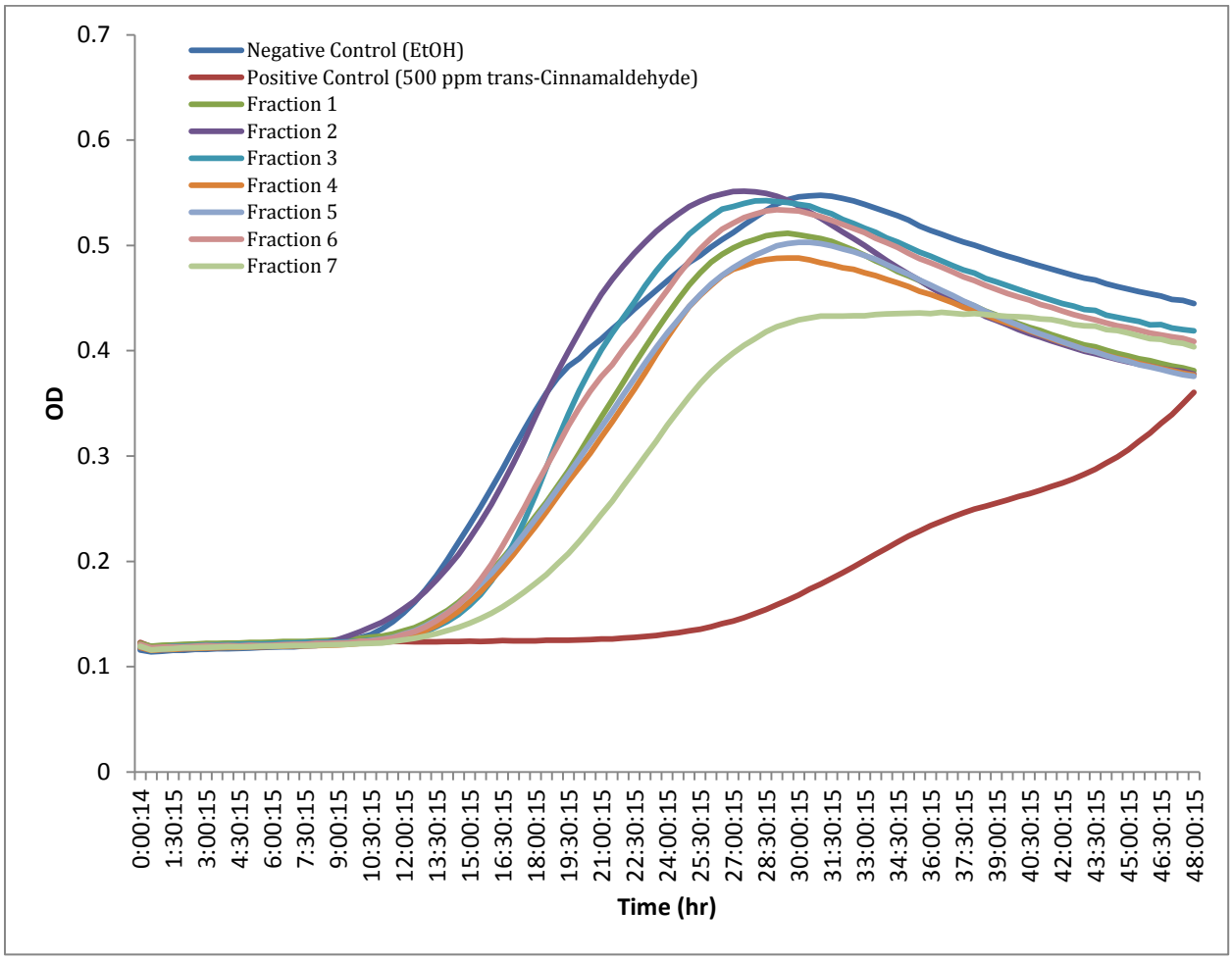

Fig 2. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with seven fractions from *G.lucidum* extracts over 48 hours.

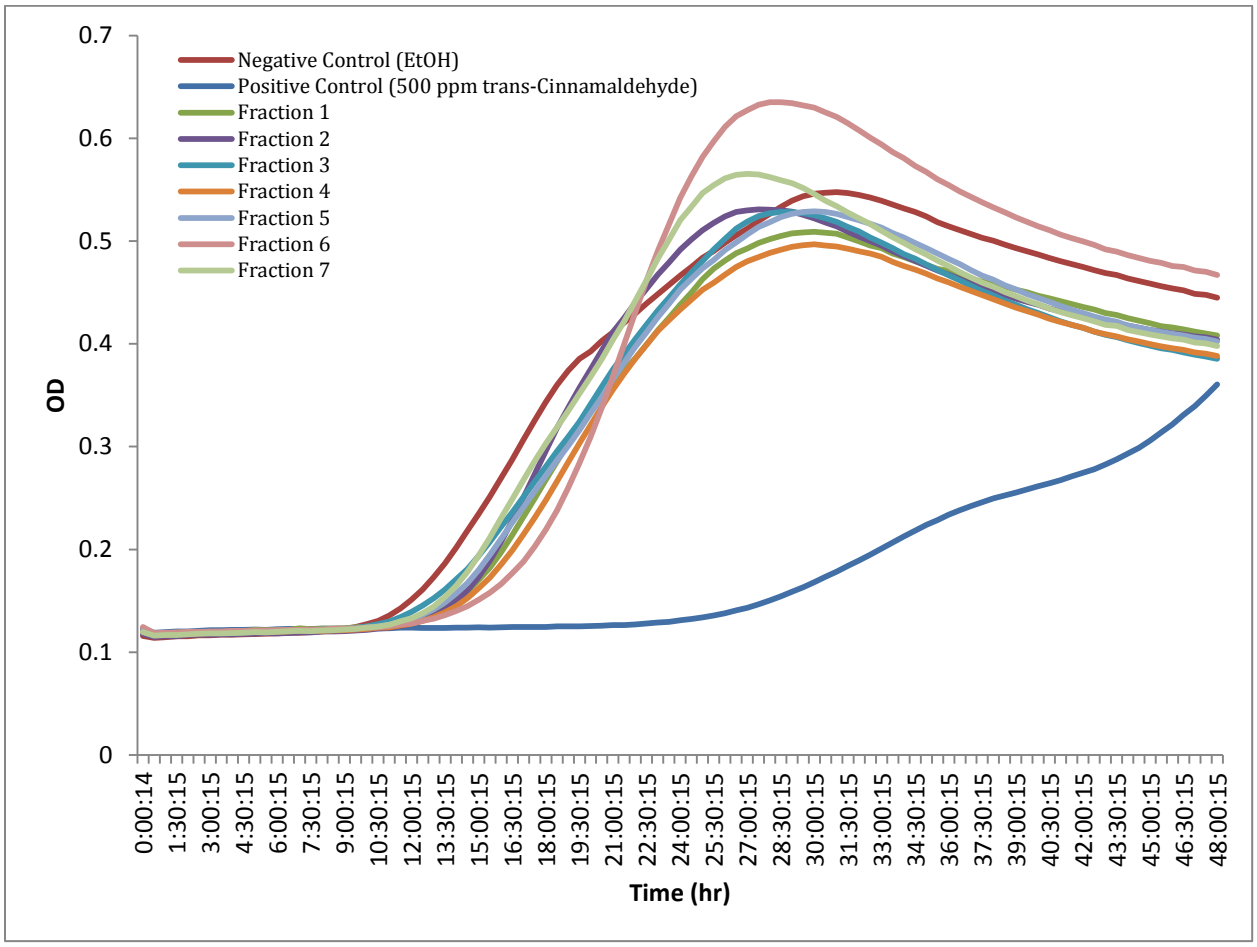

Fig 3. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with seven fractions from *G. frondosa* extracts over 48 hours.

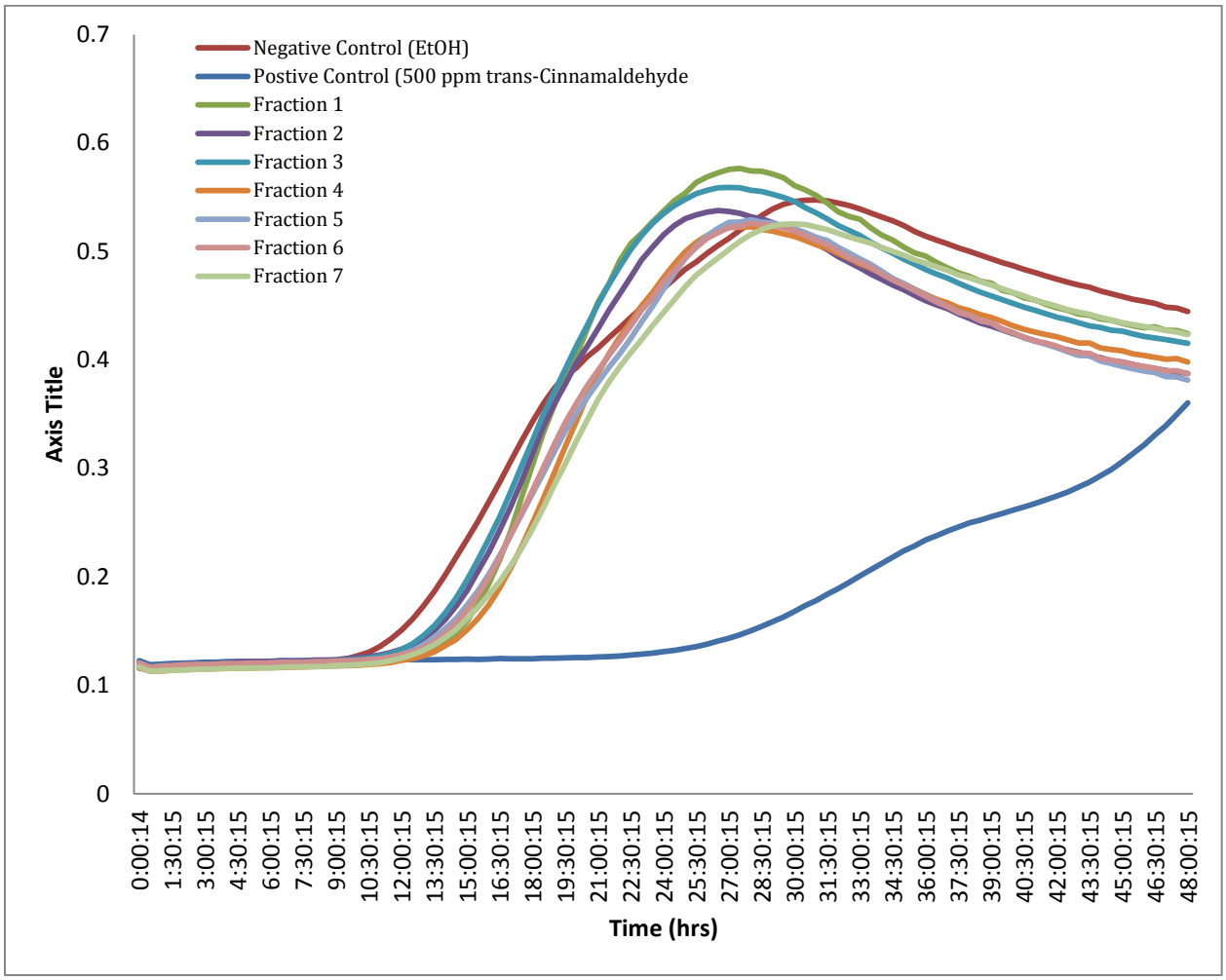

Fig 4. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with seven fractions from *I. obliquus* extracts over 48 hours.

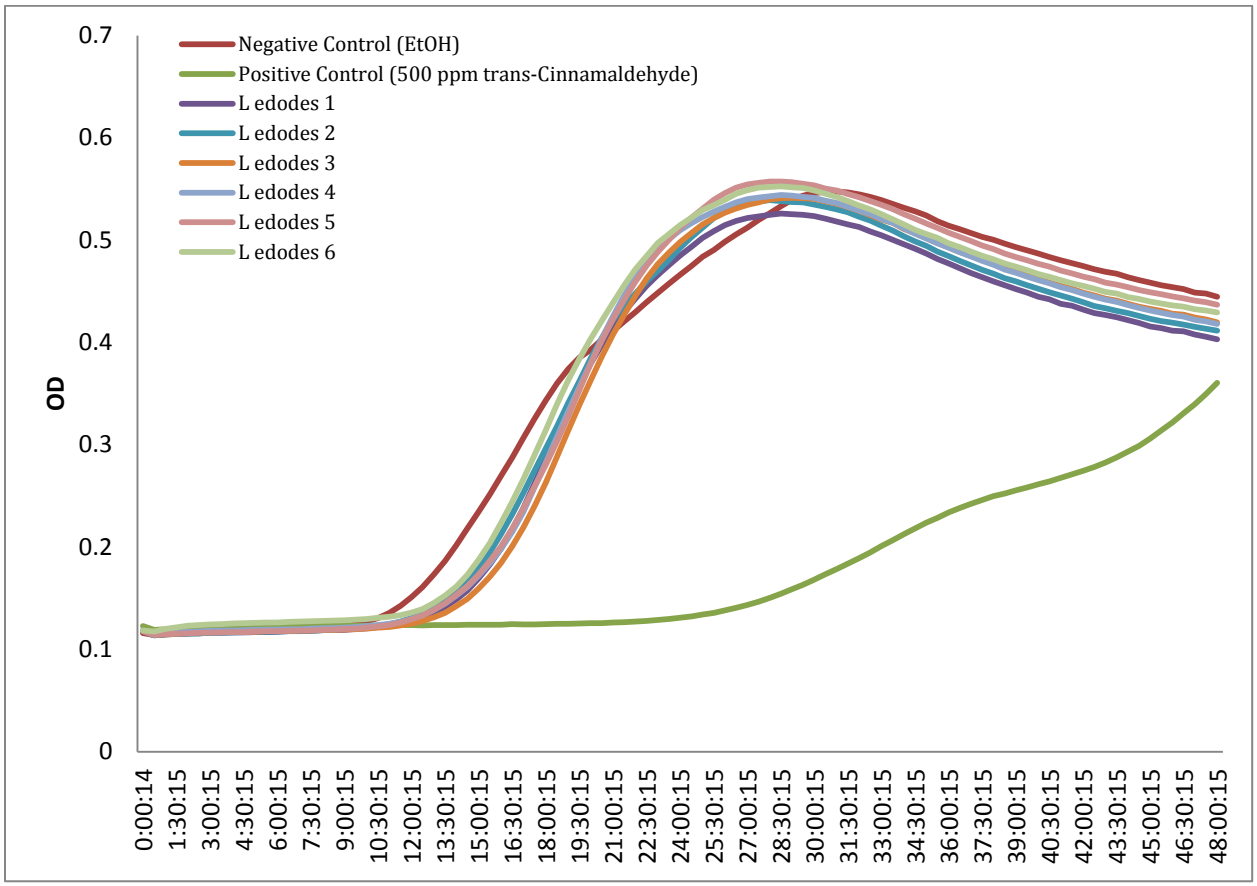

Fig 5. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with six fractions from *L. edodes* extracts over 48 hours.

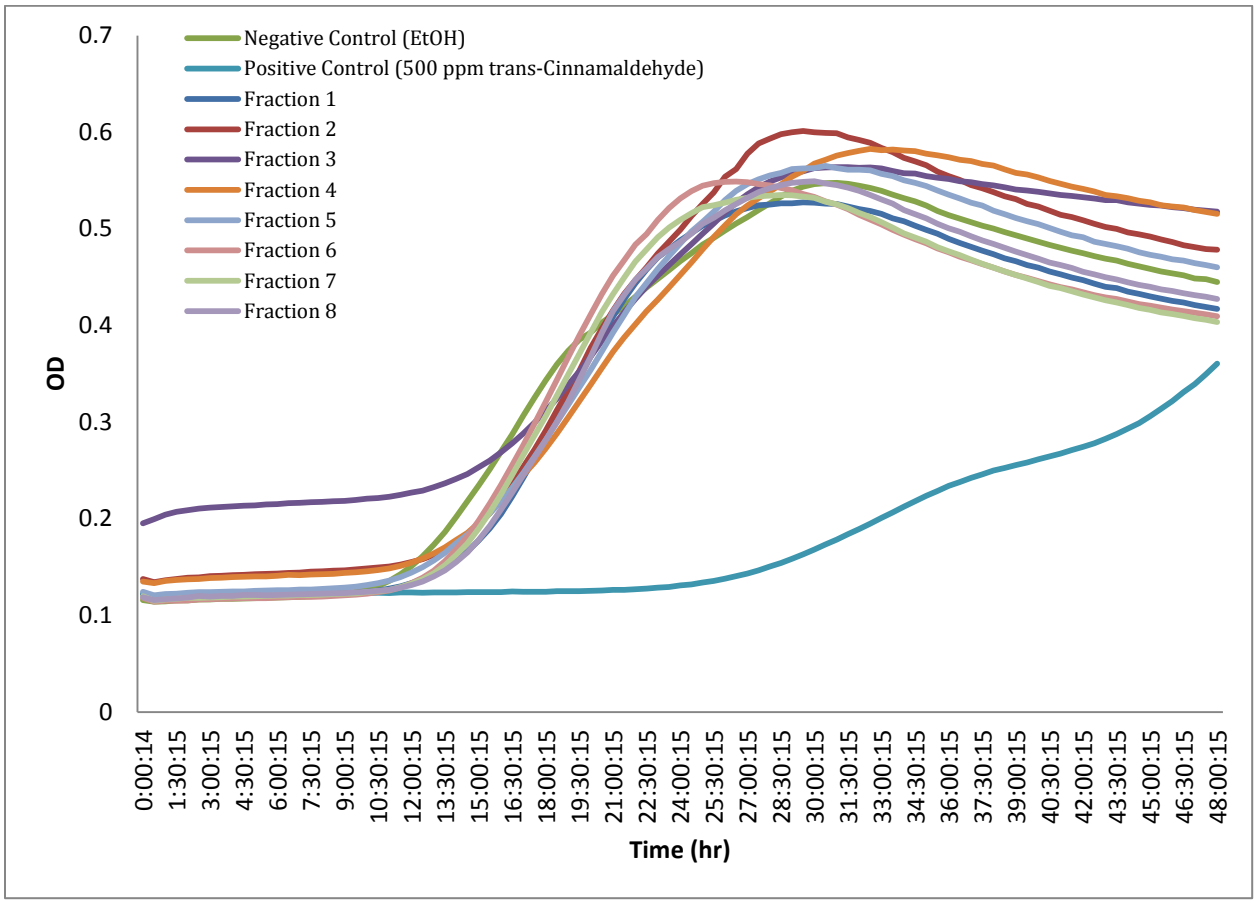

Fig 6. Optical density measurements generated by Bioscreen C of *L. monocytogenes* grown in Tryptic Soy broth with eight fractions from *P. linteus* extracts over 48 hours.

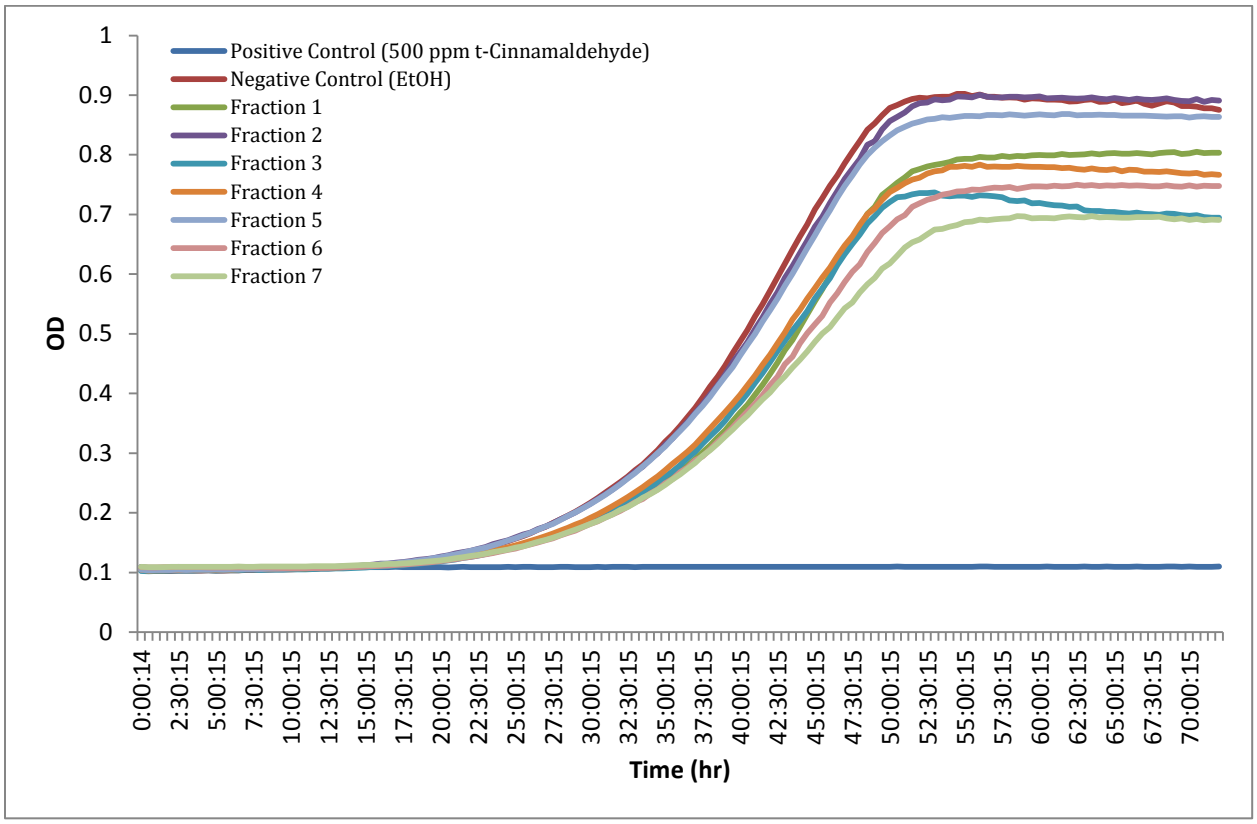

Fig 7. Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with seven fractions from *A. blazei* Murrill extracts over 72 hours.

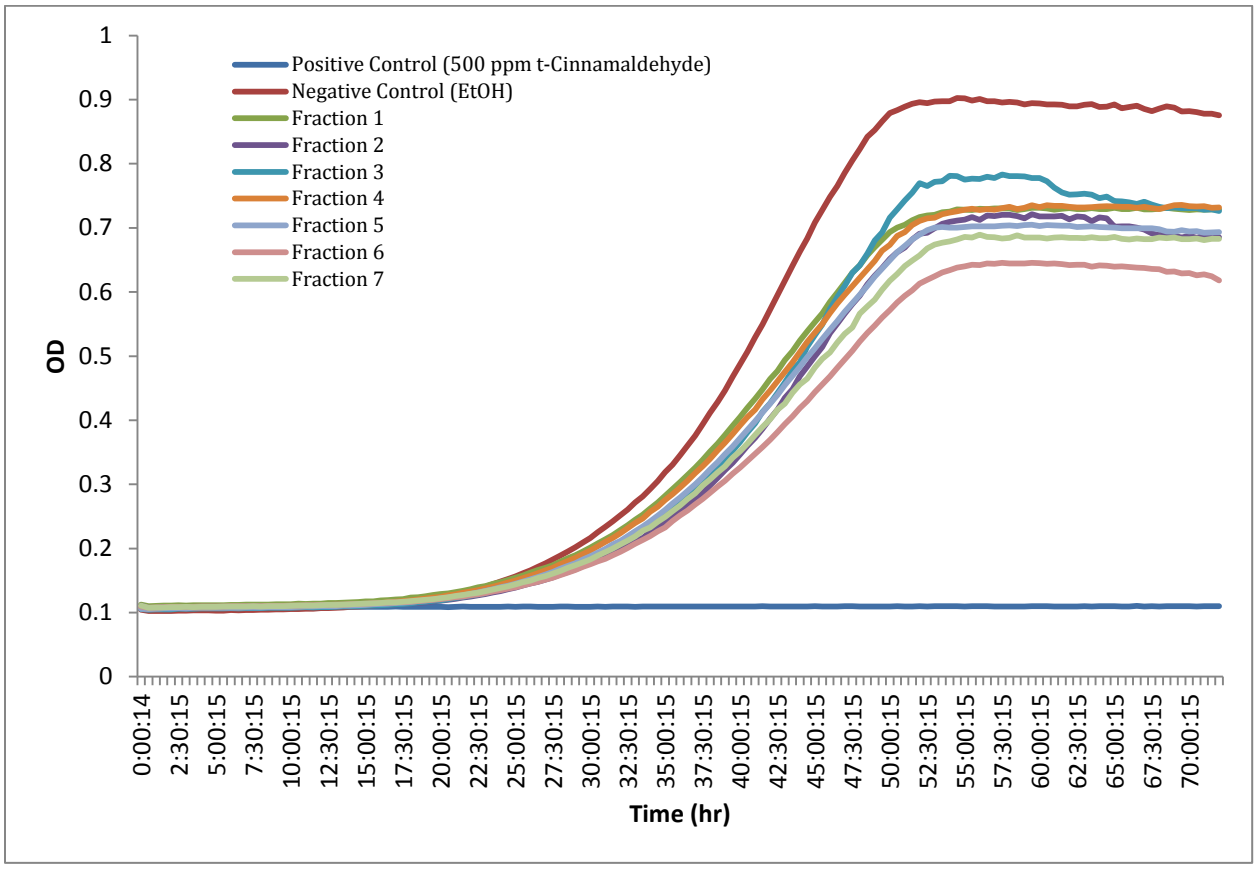

Fig 8. Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with seven fractions from *G. lucidum* extracts over 72 hours.

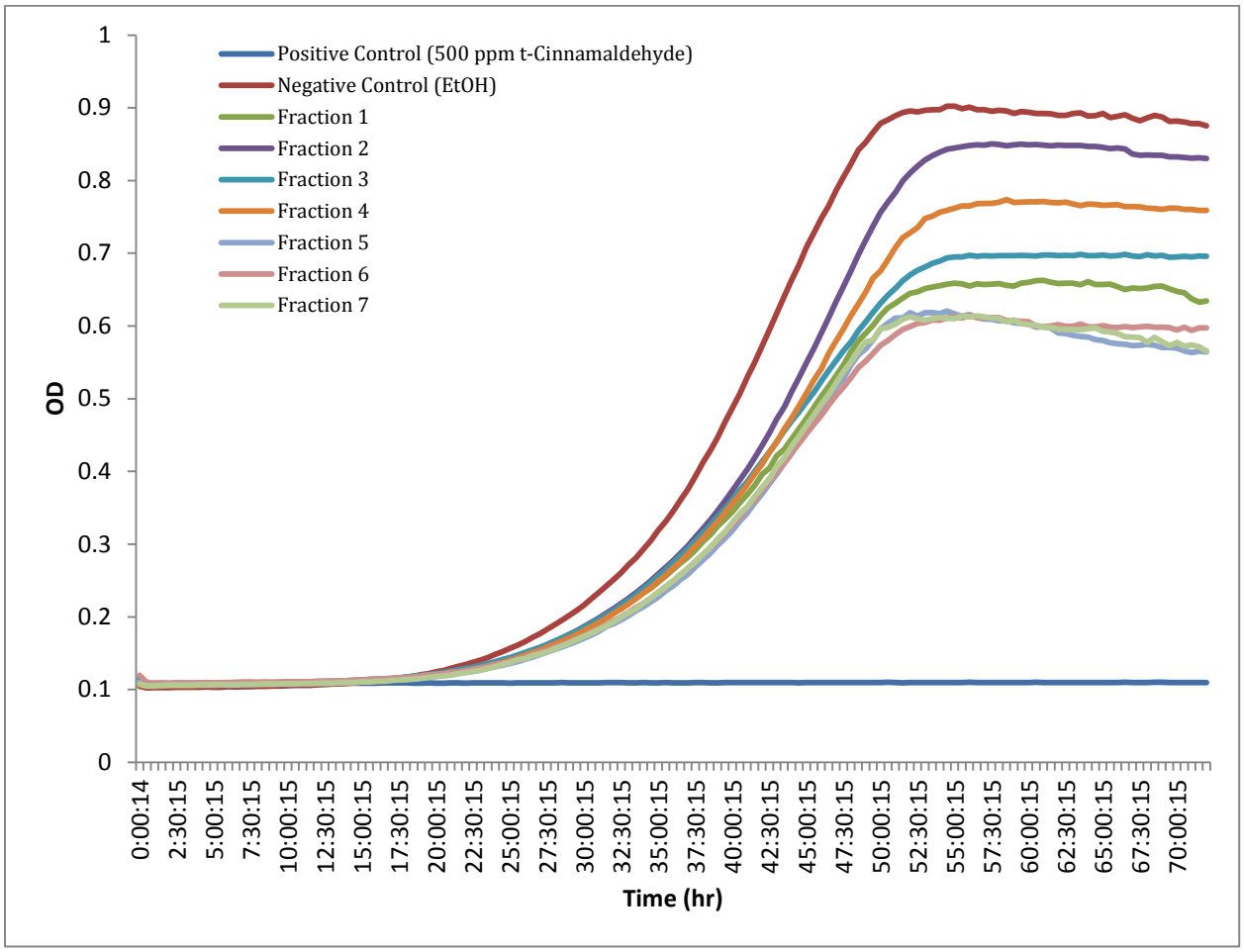

Fig 9. Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with seven fractions from *G. frondosa* extracts over 72 hours.

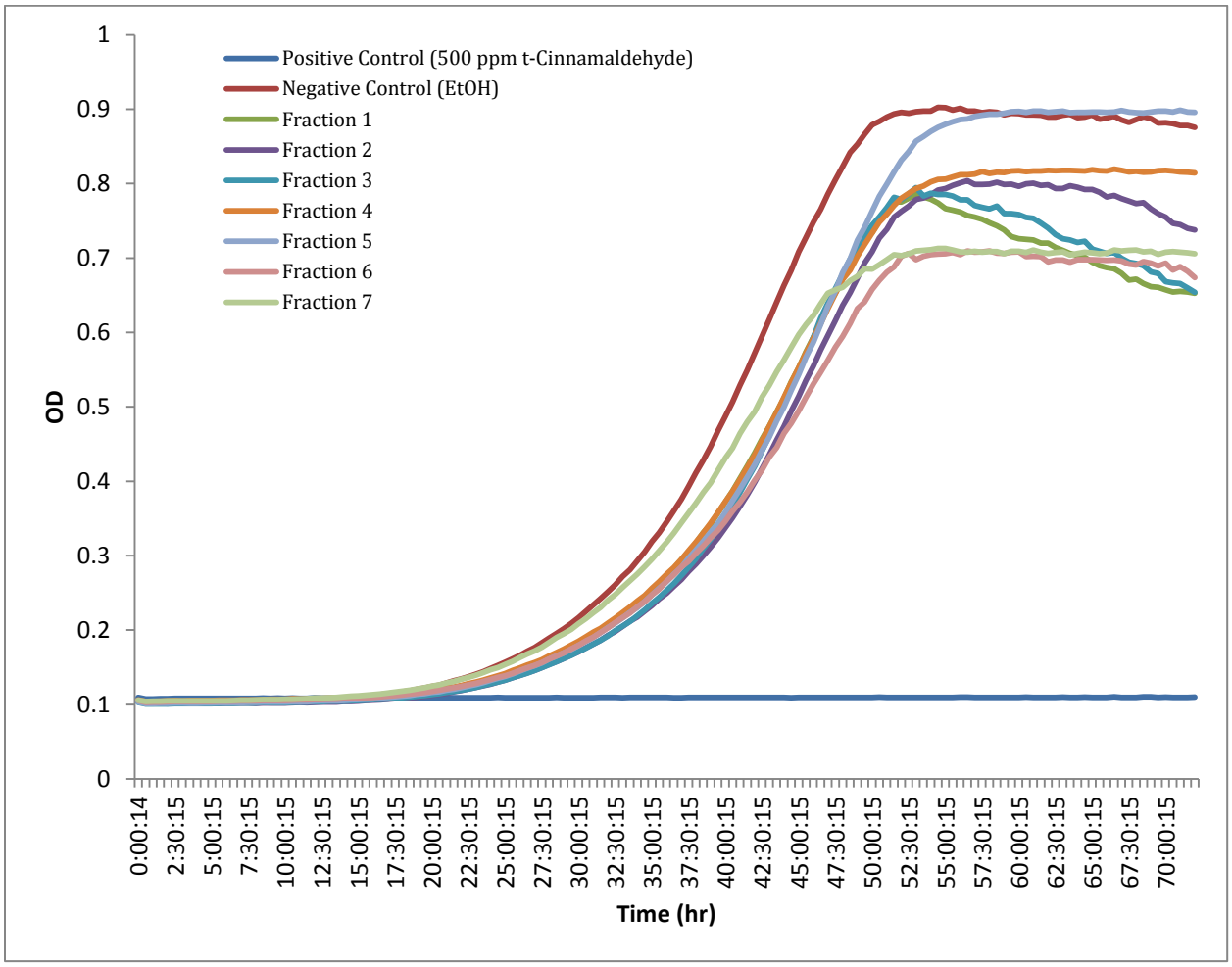

Fig 10. Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with seven fractions from *I. obliquus* extracts over 72 hours.

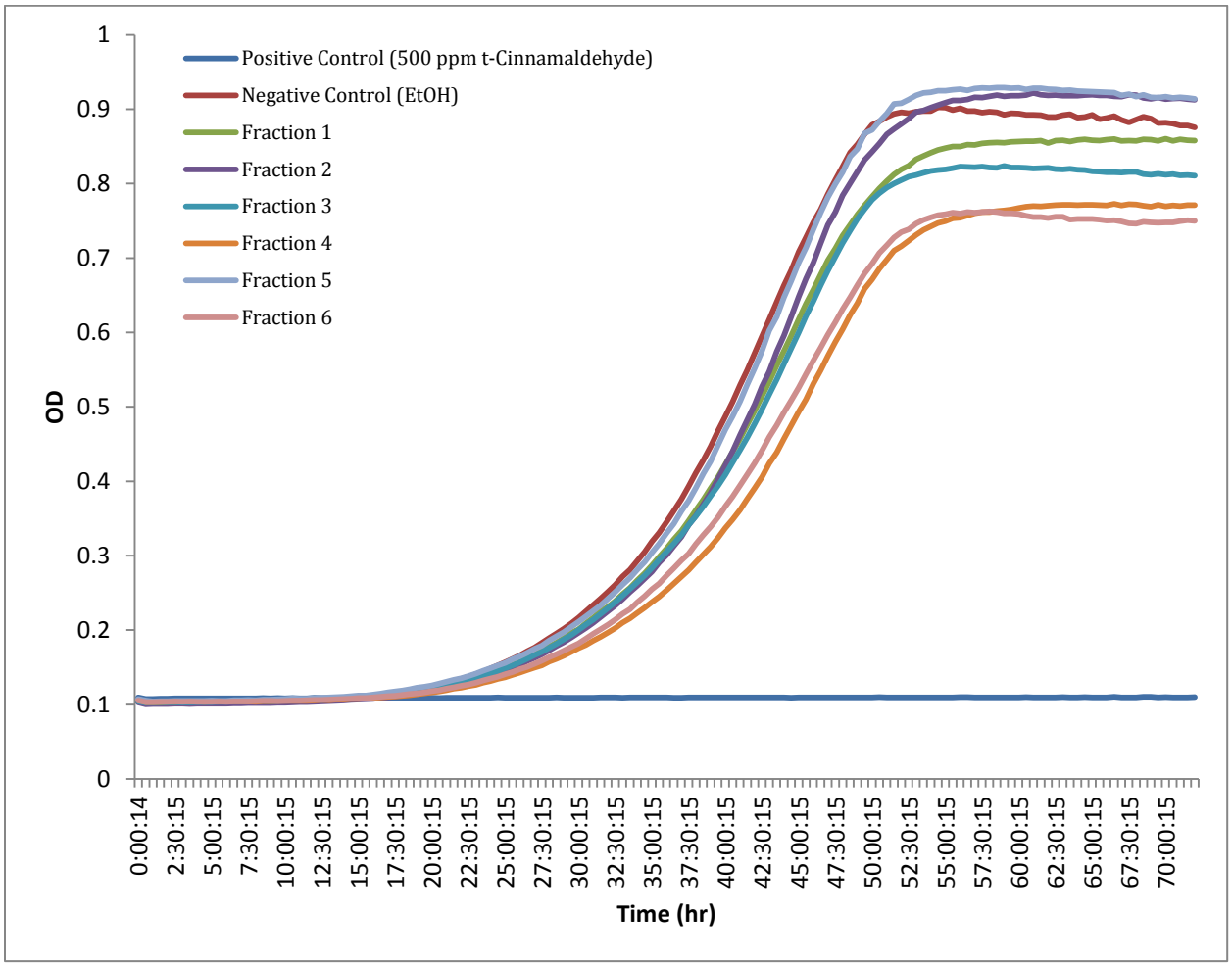

Fig 11 Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with six fractions from *L. edodes* extracts over 72 hours.

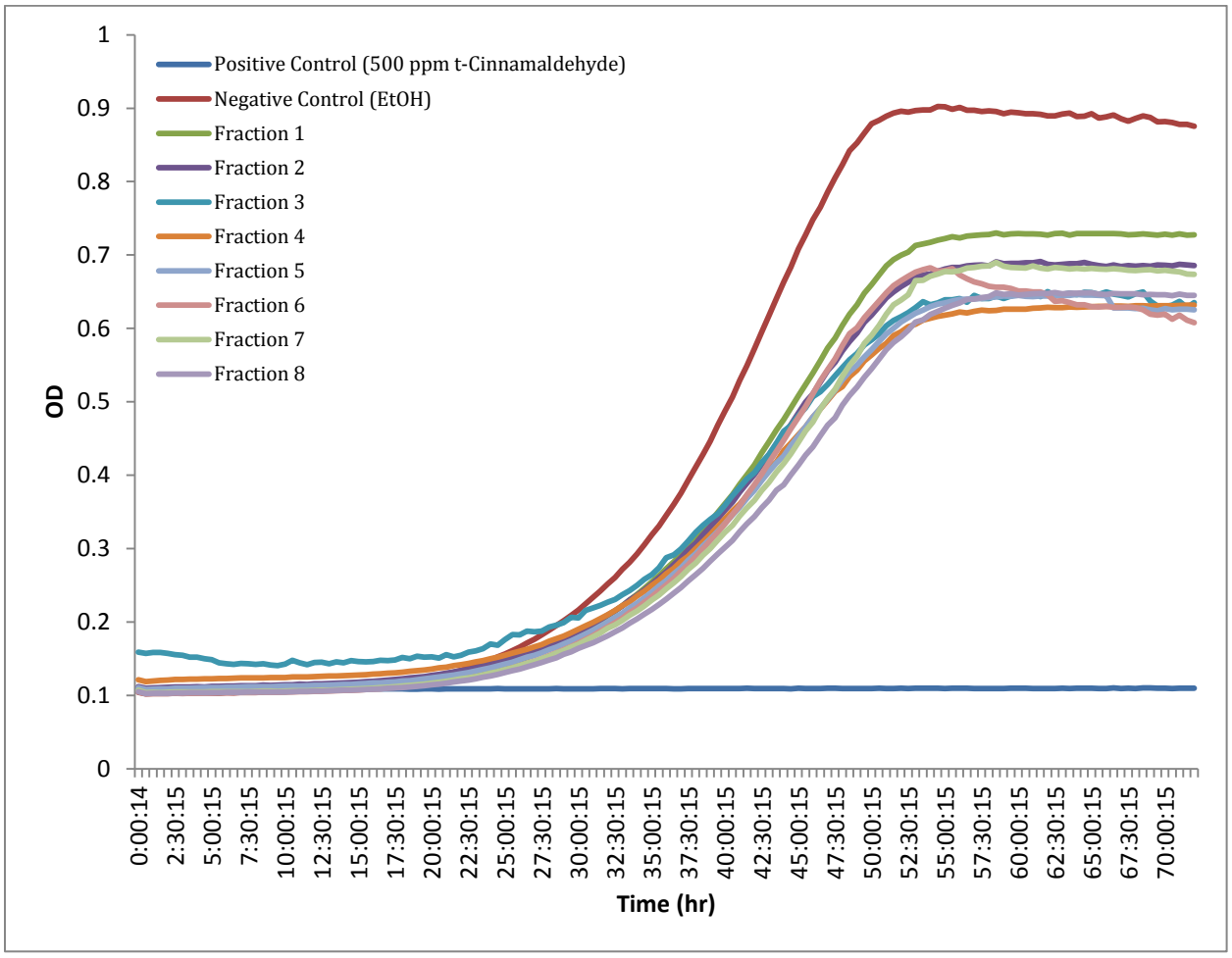

Fig 12. Optical density measurements generated by Bioscreen C of *Z. bailii* grown in Sabouraud Dextrose broth with eight fractions from *P. linteus* extracts over 72 hours.

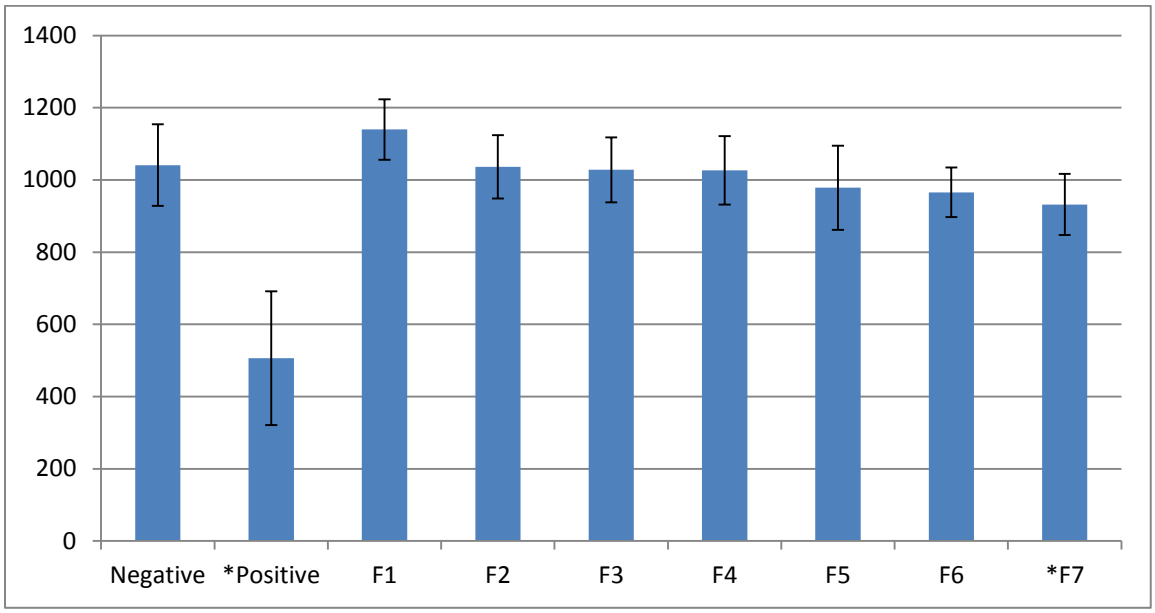

Figure 13. Area under the growth curve comparison for *L. monocytogenes* in seven fractions from *A. blazei* Murrill extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

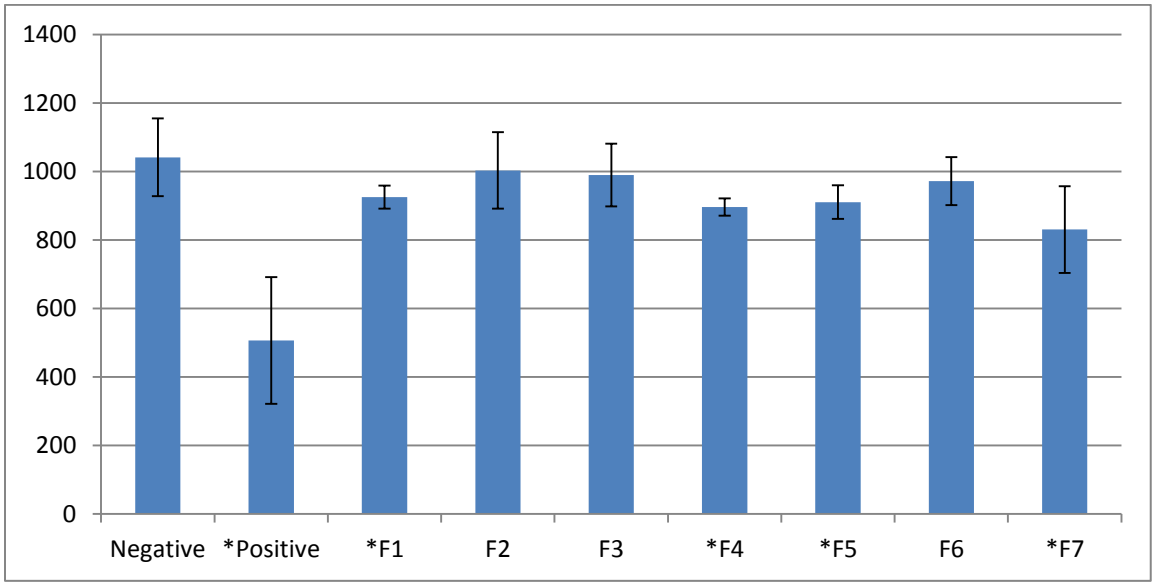

Figure 14. Area under the growth curve comparison for *L. monocytogenes* in seven fractions from *G. lucidum* extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

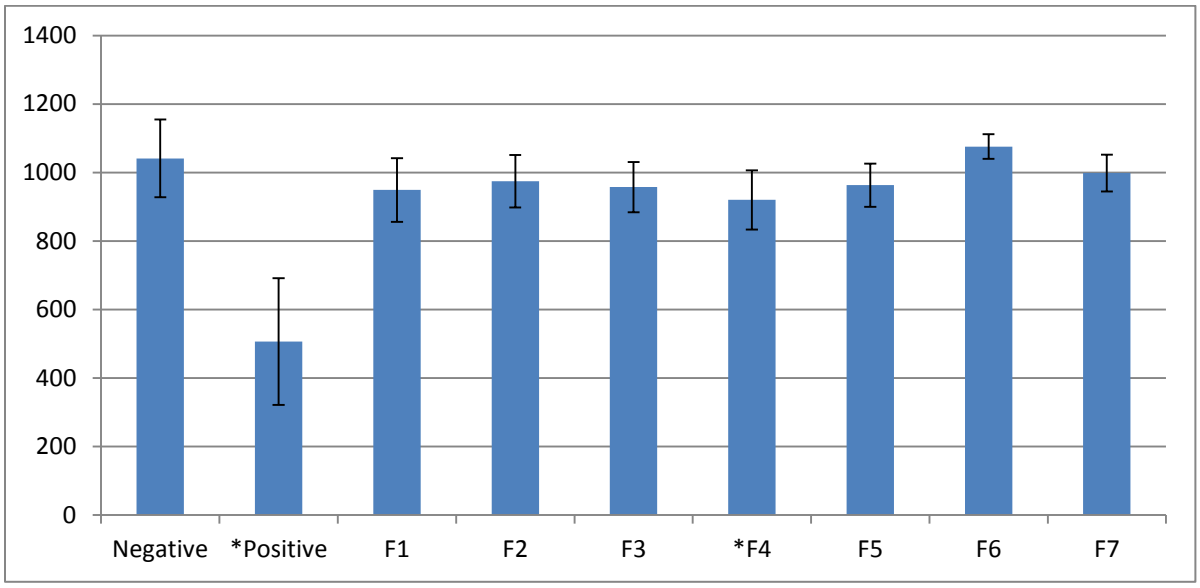

Figure 15. Area under the growth curve comparison for *L. monocytogenes* in seven fractions from *G. frondosa* extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

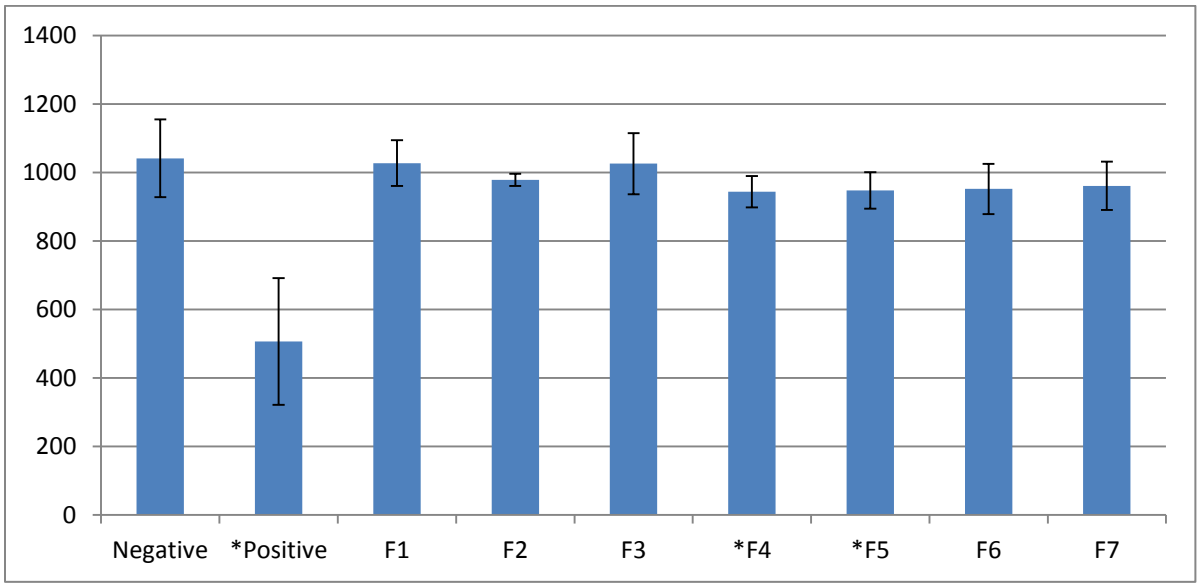

Figure 16. Area under the growth curve comparison for *L. monocytogenes* in seven fractions from *I. obliquus* extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

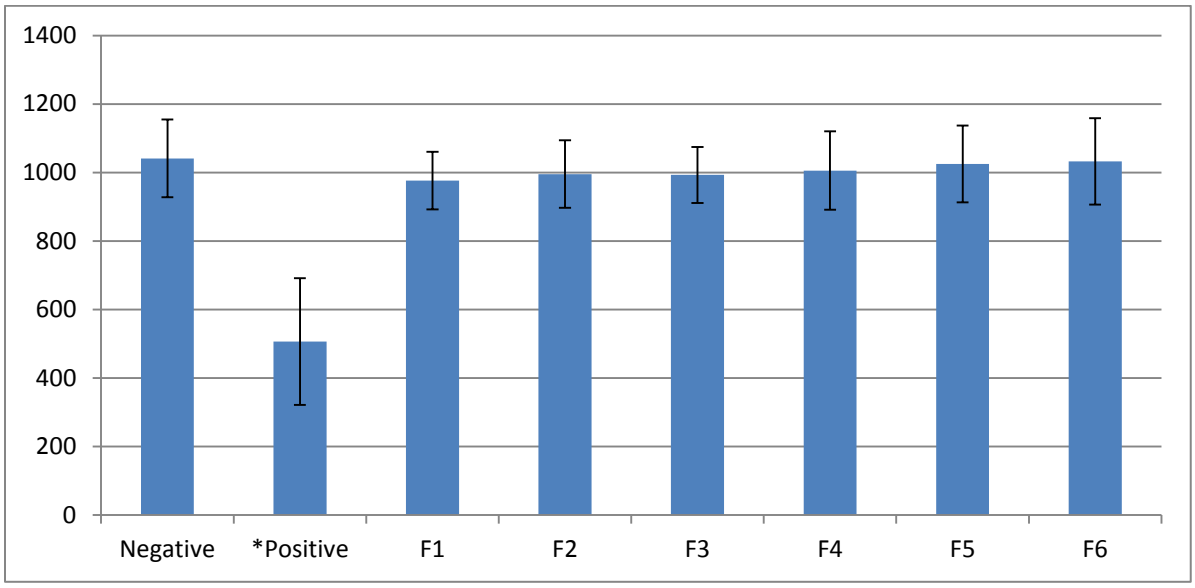

Figure 17. Area under the growth curve comparison for *L. monocytogenes* in six fractions from *L. edodes* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

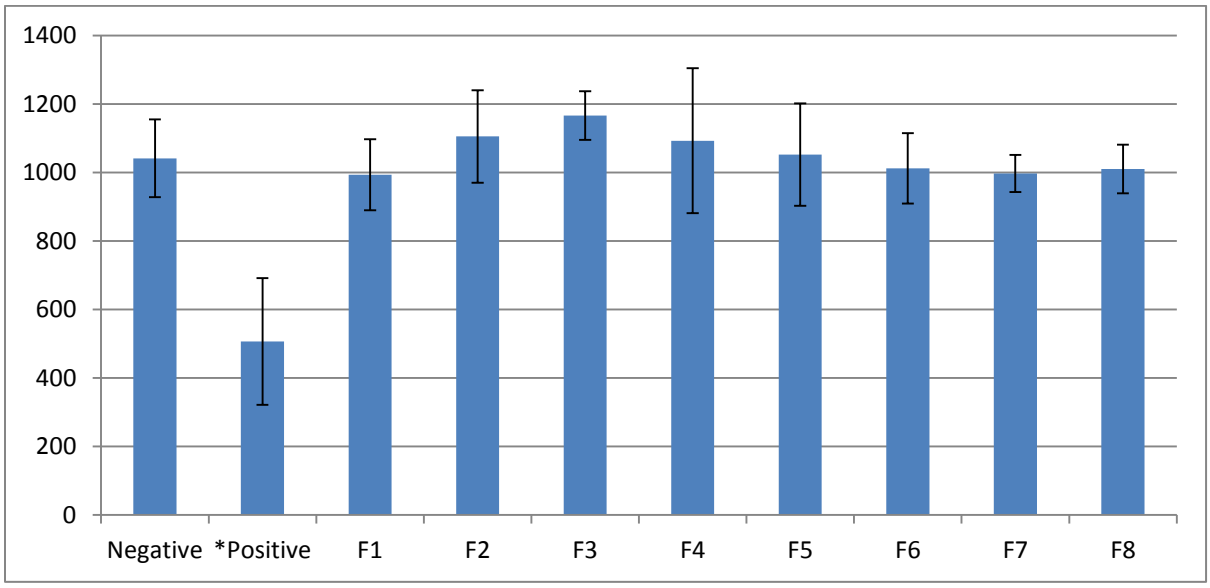

Figure 18. Area under the growth curve comparison for *L. monocytogenes* in eight fractions from *P. linteus* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

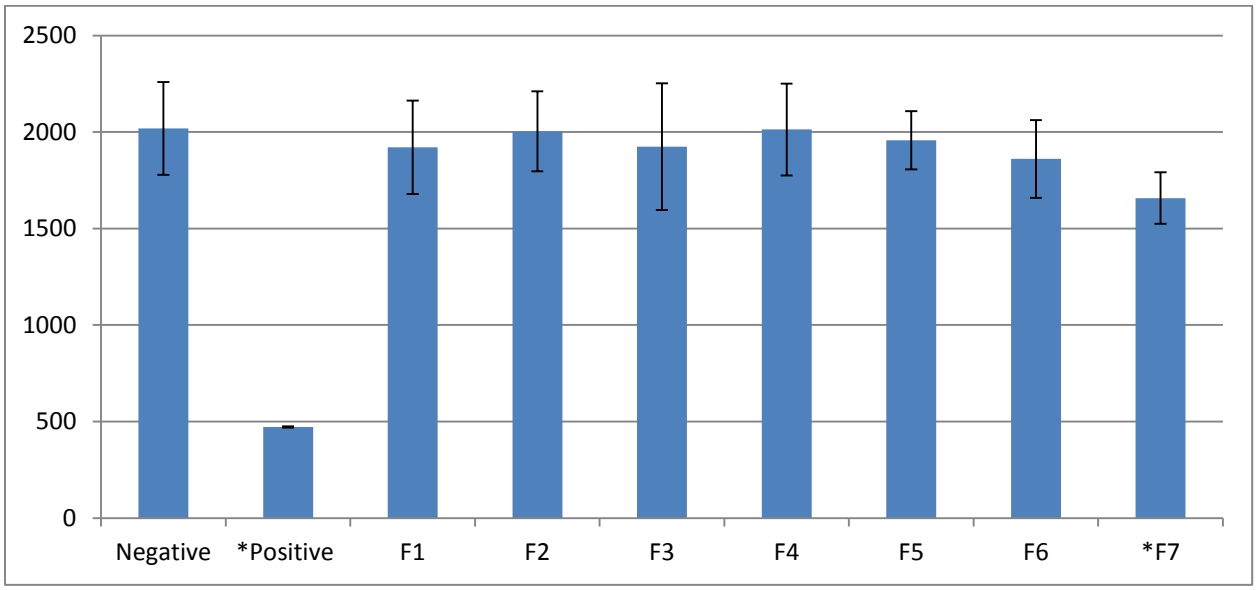

Figure 19. Area under the growth curve comparison for *Z. bailii* in seven fractions from *A. blazei* Murrill extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

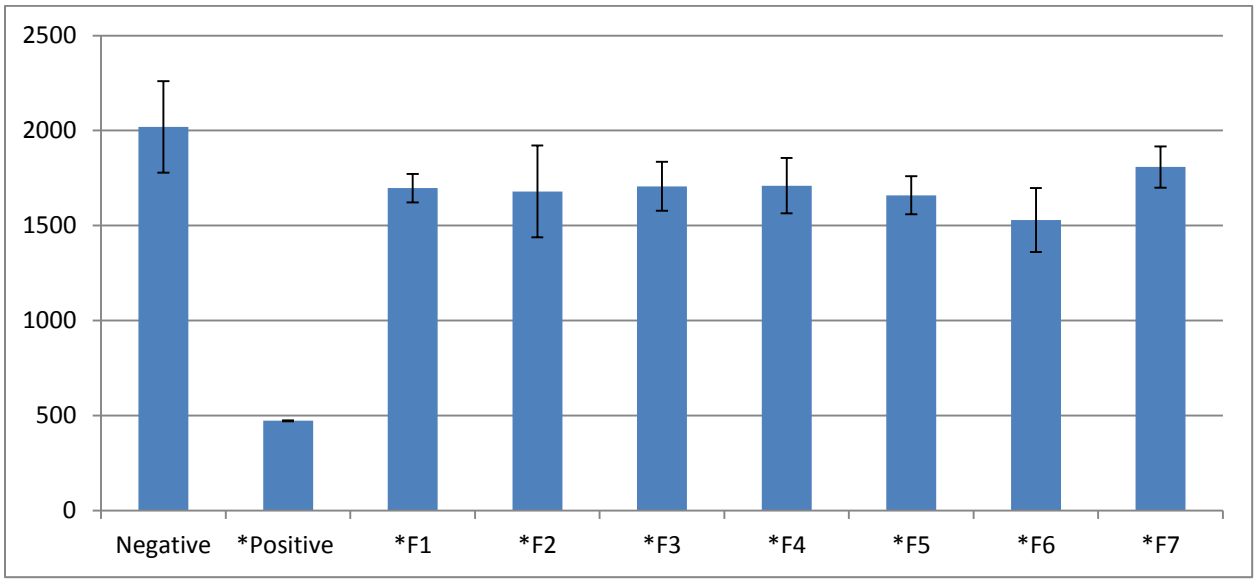

Figure 20. Area under the growth curve comparison for *Z. bailii* in seven fractions from *G. lucidum* extracts. *significant at α=0.05 to negative control; $^{++}$insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

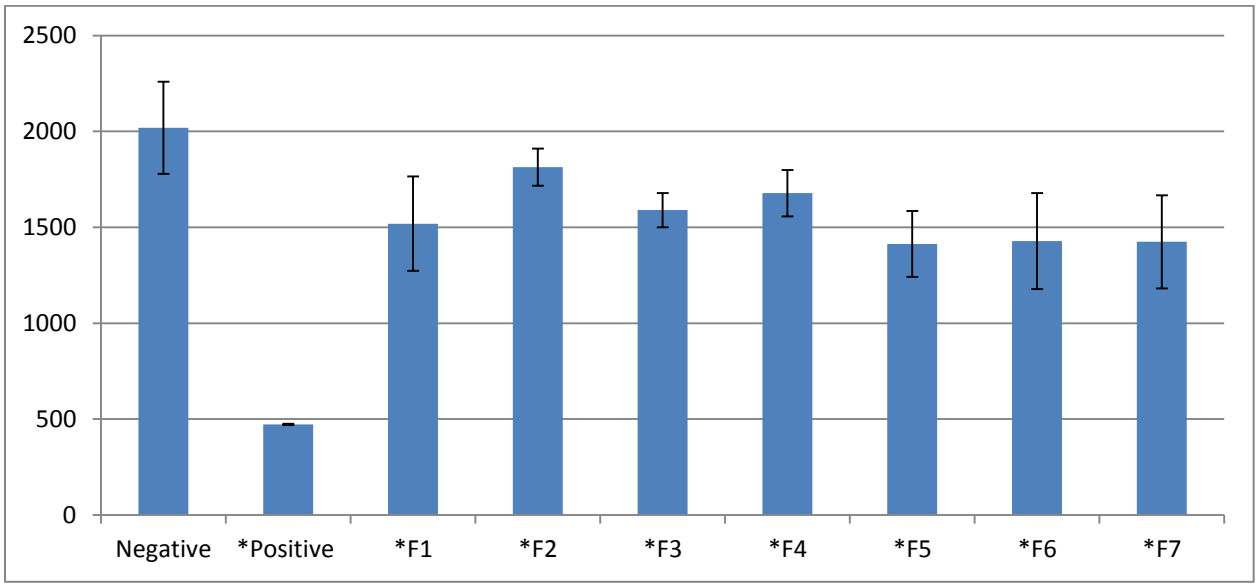

Figure 21. Area under the growth curve comparison for *Z. bailii* in seven fractions from *G. frondosa* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

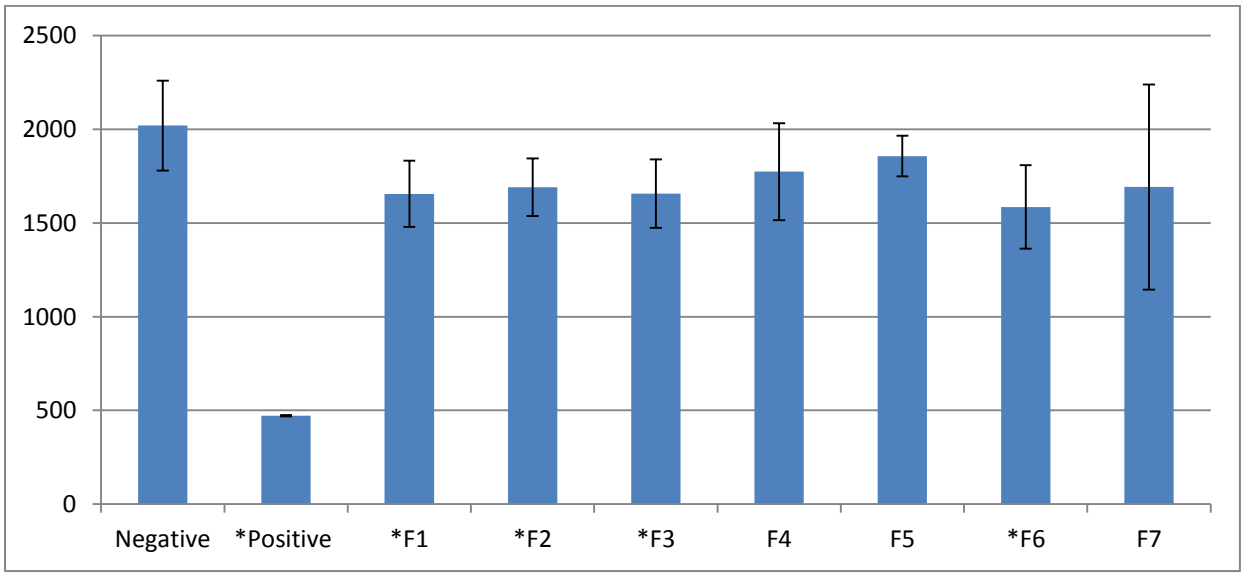

Figure 22. Area under the growth curve comparison for *Z. bailii* in seven fractions from *I. obliquus* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

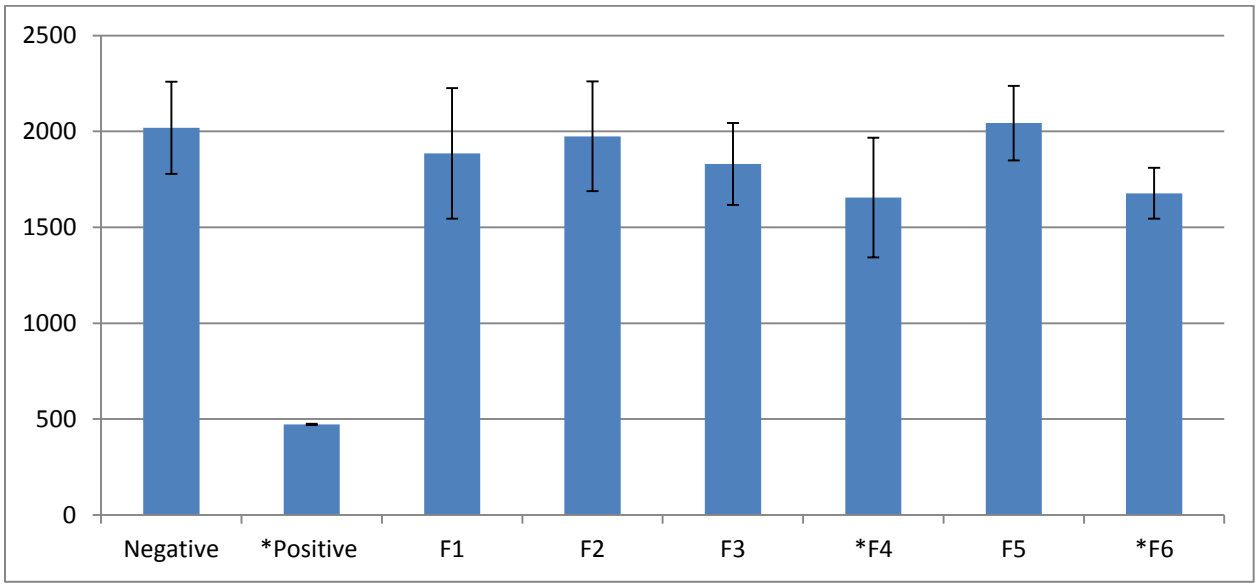

Figure 23. Area under the growth curve comparison for *Z. bailii* in six fractions from *L. edodes* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

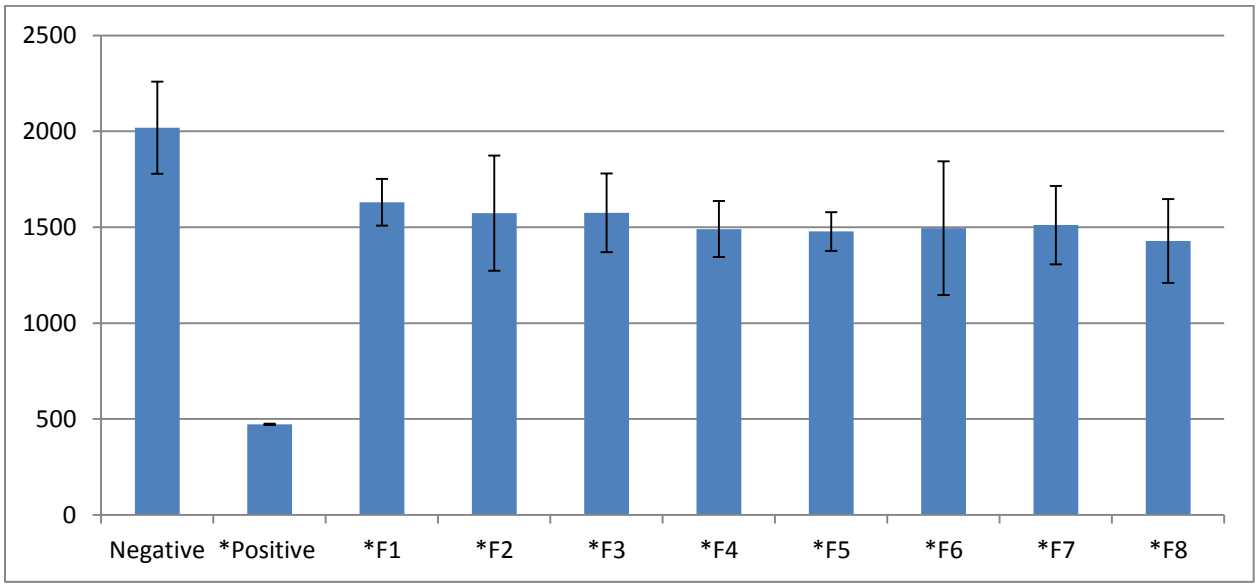

Figure 24. Area under the growth curve comparison for *Z. bailii* in eight fractions from *P. linteus* extracts. *significant at α=0.05 to negative control; ++insignificant at α=0.10 to positive control; negative control = 95.5% ethanol; positive control = 500 ppm *trans*-cinnamaldehyde. Error bar signifies ±1 standard deviation from sample mean (n=9).

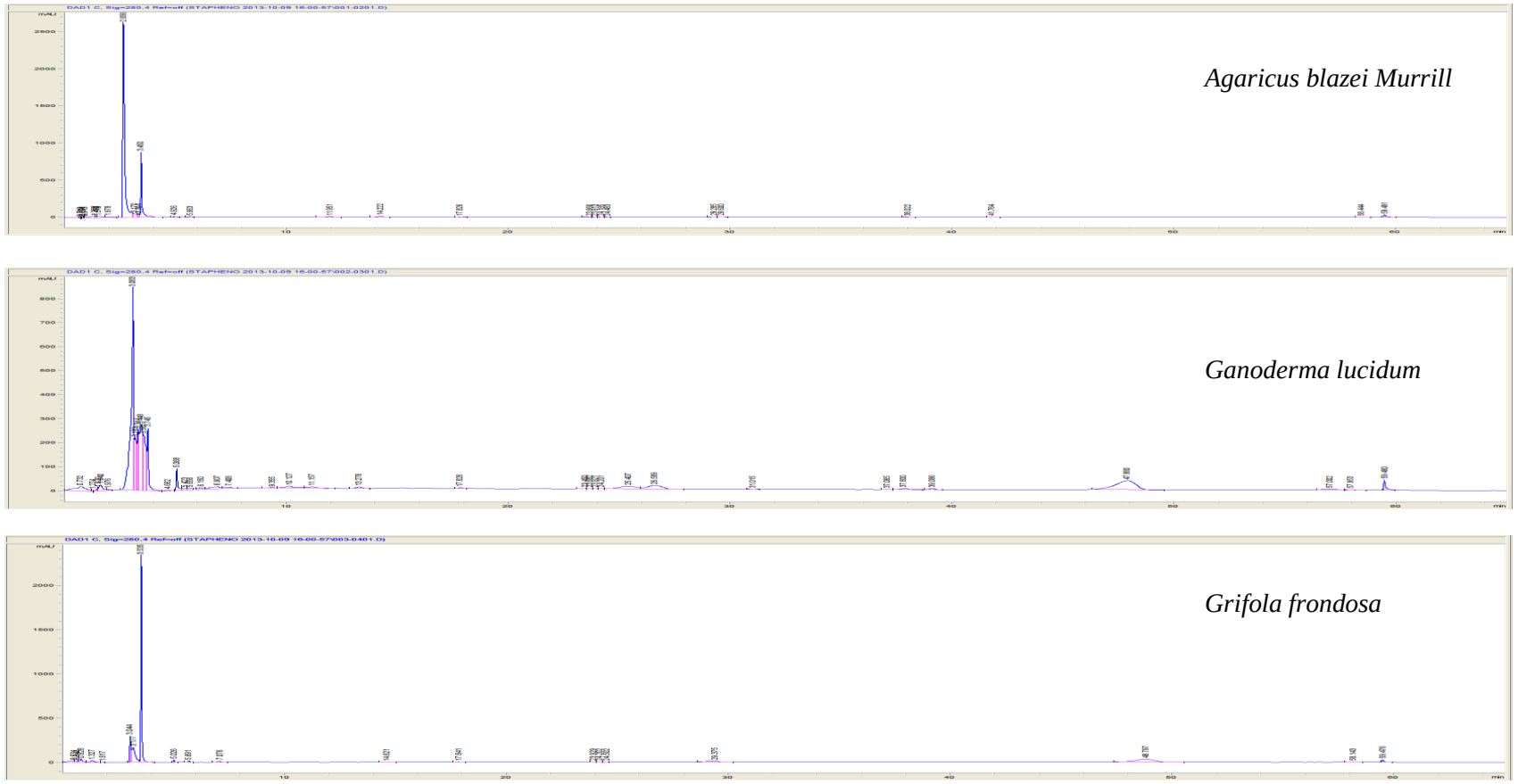

Figure 25. Chromatography of ethanol extracts of *A. blazei* Murrill, *G. lucidum*, and *G. frondosa* over 65 minutes at 280 nm in HPLC. These extracts were further fractionated. Y-axis represents the concentration (mAU); scale of y-axis is distinct of each chromatograph. Except for the ghosting peaks, the peaks represent the compounds visible in 280 nm wavelength.

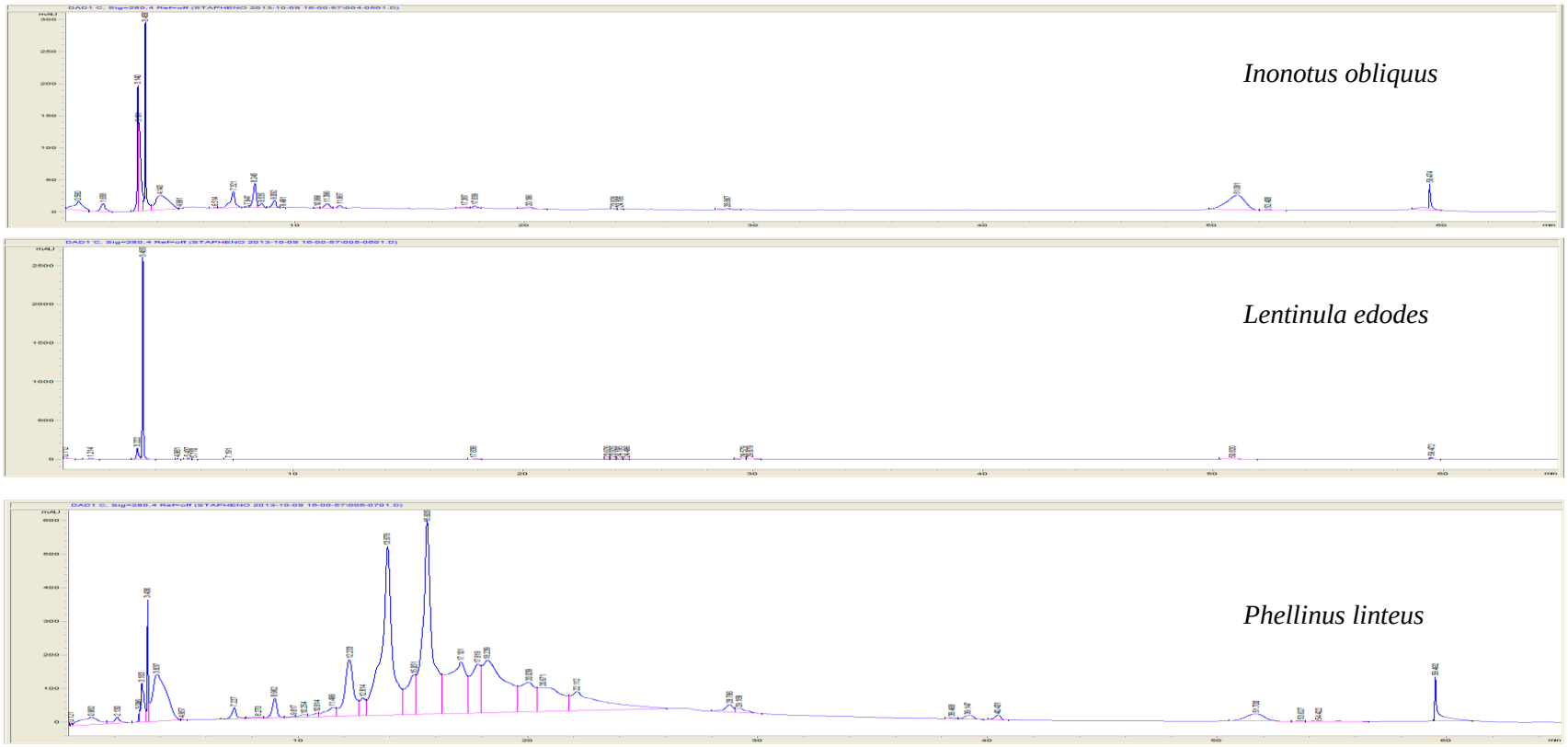

Figure 26. Chromatography of ethanol extracts of *I. obliquus*, *L. edodes*, and *P. linteus* over 65 minutes at 280 nm in HPLC. These extracts were further fractionated. Y-axis represents the concentration (mAU); scale of y-axis is distinct of each chromatograph. Except for the ghosting peaks, the peaks represent the compounds visible in 280 nm wavelength.