Toxicity Assessment of *Clitocybe nuda* as Natural Antimicrobials

by

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Abstract

Clitocybe nuda is an edible mushroom which shows promise to be developed as a novel natural food antimicrobial due to various antimicrobial components found in its fruit body as well as secondary metabolites in recent years. However, no toxicity information is available about this mushroom. The aim of this study is to assess the toxicity of the fruit body and secondary metabolites from *C. nuda* using *in vitro* methods. The cytotoxic effects of its fruit body extract and secondary metabolites were investigated with an optimized cytotoxicity assay. Furthermore, acute oral LD_{50} values were estimated based on results from cytotoxicity assay using an established prediction model. Additionally, genotoxic effects of the fruit body extract and secondary metabolites crude extract were evaluated with two standard *in vitro* genotoxicity assays: a bacteria reverse mutation assay and a cytokinesis-blocked micronucleus cytome assay. Results indicate that both the fruit body extract and secondary metabolites from *C. nuda* can be properly classified as not genotoxic and not causing acute oral toxicity.

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Publications in Support of This Thesis

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List of Abbreviations

2-AAN	2-Aminoanthracene
4-NOP	4-Nitro-*orho*-phenylenediamine
9-AAC	9-Aminoacridine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BN	Binucleated
CBMN-cyt	Cytokinesis-blocked micronucleus cytome
CFR	Code of Federal Regulation
CP	Cyclophosphamide
CPBI	Cytokinesis-block proliferation index
Cyt-B	Cytochalasin B
DMEM	Dulbecco's modification of eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EURL ECVAM	Europe Union Reference Lab for Alternatives to Animal Testing
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMEM	Eagle's minimum essential medium
USDA ERS	United States Department of Agriculture Economic Research Service

USDA FSIS	United States Department of Agriculture Food Safety and Inspection Service
FBS	Fetal bovine serum
FD	Freeze dry
FDA	Food and Drug Administration
G-6-P	Glucose-6-phosphate
GHS	Globally Harmonized System of Classification and Labelling of Chemicals
GRAS	Generally recognized as safe
HD	Heat dry
IC_{50}	Concentrations inhibiting 50% of the growth
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
LD_{50}	Value of the oral dose that produces lethality in 50% of test animals
MIC	Minimum inhibitory concentrations
MNi	Micronuclei
MNvit	*In vitro* micronucleus
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADP	Nicotine adenine dinucleotide phosphate
NBUDs	Nuclear buds
NCS	Newborn calf serum
NIH	National Institutes of Health
NPBs	Nucleoplasmic bridges
NRU	Neutral Red Uptake
OECD	Organisation for Economic Co-operation and Development
PDB	Potato dextrose broth

PI	Pre-incubation
pKa	Acid dissociation constant
SAS	Statistical Analysis System
SLS	Sodium lauryl sulfate
SP	Standard plating

CHAPTER 1: INTRODUCTION

1. Background

Clitocybe nuda (*C. nuda*, also known as *Lepista nuda*) is an edible mushroom that grows throughout Europe, North America, Asia, and Australia (Barros et al., 2008). In recent years, this mushroom has attracted more and more interest, because extracts from its fruit body and secondary metabolites have demonstrated distinctive antimicrobial properties (Alves et al., 2012; Chen and Huang, 2009; Dulger et al., 2002; Mercan et al., 2006; Yamaç and Bilgili, 2006). This mushroom also has been reported to have pharmacical potentials because of its antidiabetic, antihyperlipidemic and antitumor activititives (Beattie et al., 2011; Chen et al., 2014), as well as the possibility for use as a novel adjuvant in cancer therapy and vaccination because of its immunologic effects (Chen et al., 2013). The antimicrobial functions are most intensively explored due to the fact that food industry are looking for novel naturally occurred food antimicrobials to fulfill the consumer's increasing desire for safer and higher quality foods with clean labeling.

Food microbial contamination is one of the most important sources for foodborne illness. Reducing microbial contamination is the major concern for food safety. In our previous research, the fruit body ethanol extract from *C. nuda* fruit body has been proven to show promising antibacterial activities against various foodborne pathogens with good stability (Bo, 2012). Additionally, their potential use in foods like liquid eggs has also been investigated (Gardner, 2015). The culture filtrate of *C. nuda* was also found to have strong inhibitory effect of certain plant pathogenic fungi and bacteria in previous research (Chen and Huang, 2009). Later, the

secondary metabolites from the same *C. nuda* strain culture were also found to have promising inhibitory effects to four prevalent foodborne pathogens – *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aures* and *Escherichia coli* O157:H7 (Hou, 2013). Moreover, the antibacterial efficacies in food models like liquid eggs and ground beef were also investigated in the same study (Hou, 2013). Based on results from current studies, the fruit body extract and secondary metabolites are promising to be used as natural antimicrobial in minimally processed foods with clean labeling.

Toxicity assessment is a multiple-step process requiring a screening strategy based on a number of expensive, time-consuming complementary tests, from microbes to mammals, to draw valid conclusions (Eisenbrand et al., 2002). *In vitro* testing is important to understand the hazardous effects of chemicals, so as to predict those effects on humans (Broadhead and Combes, 2001). For food additives, *in vitro* test data have already been considered for risk assessment purpose in some cases (Eisenbrand et al., 2002). Toxicity information is always lacking for extracts from natural products that are intended to be added into food. Therefore, a special testing system needs to be established, based on weight-of-evidence and stepwise strategies. A review of current *in vitro* techniques and state-of-the-art recommendations indicates that a battery of optimized *in vitro* cytotoxicity and genotoxicity tests can illuminate enough toxicity information with less cost of time, labor, and budget. Therefore, the aim of this study is to assess the toxicity effect of fruit body extract and secondary metabolites from *C. nuda* by using the results of *in vitro* testing.

2. Purpose of Study

The purpose of this study is to assess the toxicity of both fruit body extract and secondary metabolites from *C. nuda* using *in vitro* methods. In order to achieve this goal, several specific

aims are listed: 1) to assess the cytotoxicity and estimated acute oral toxicity of fruit body extract and secondary metabolites crude extract; 2) to assess the genotoxicity of fruit body extract and secondary metabolites crude extract and pure compound; 3) to assess the influence of food processing conditions such as heating to the toxicity of fruit body extract and secondary metabolites; 4) to assess and optimize some current *in vitro* toxicity testing methods and make a suitable system for testing of natural substances which propose to be claimed as generally recognized as safe (GRAS).

3. Significance of Study

For new emerging extracts from nature products, there must be enough scientific data to support the safety of their intended use and those data must be widely available (e.g. peer-review publication). *C. nuda* differs from many other natural antimicrobial extracts of plant origin which are generally recognized as safe (GRAS) and can be used without labeling. This is because *C. nuda* belongs to fungi kingdom, many of which are known to produce hazardous metabolites (including toxins) that may be harmful to humans (Cole et al., 2003). Although *C. nuda* has been consumed by human as food for a very long time, the intake dose level is relatively low. Potential toxicity of the concentrated extract at a higher dose level has never been examined. Therefore, convincing studies about the toxicity information of *C. nuda* are still required to ensure the safety to be approved as a novel naturally occurred food antimicrobial. Also, toxicity data from this study will promote the process of extracts from *C. nuda* to be approved as GRAS substances, which will stimulate more studies of *C. nuda* as new functional food and dietary supplement in the future.

CHAPTER 2: REVIEW OF LITERATURE

1. Natural antimicrobials and regulations

1.1 *Natural antimicrobials and applications in foods*

Food antimicrobial contamination is one of the most important sources for foodborne illness. According to the latest report from United States Department of Agriculture Economic Research Service (USDA ERS) that roughly 9 million people get sick, 50,000 are hospitalized, and 2,300 die of 15 foodborne diseases each year in the US, and about $ 15.6 billion total cost are associated with 15 recognized pathogens each year (ERS, 2014). Reducing microbial contamination is the major concern for food safety. Although a lot of novel technology like processing, packaging and storage have been continually developed, more challenges are faced by today's food industry such as the globalization of food production, processing and distribution, and consumer's demand for minimally processed food products while with high quality and safety. Antimicrobial preservatives have been used in foods for a long history to extend shelf life while today they are more expected to play important roles for inhibiting foodborne pathogens.

Antimicrobials in food can be generally classified as traditional or naturally occurred (Davidson et al., 2005). Traditional antimicrobials are substances that have been approved by the regulatory agencies to be applied in foods. In the US, regulatory-approved food antimicrobials are listed in Title 9 (USDA Food Safety and Inspection Service approved) and Title 21 (FDA approved) of the Code of Federal Regulation (CFR). Extensive reviews about regulatory-

approved antimicrobials can be found in Davidson et al. (2005). All of these regulatory-approved antimicrobials are pure compounds (e.g. acetic acid, nitrite) or molecules (e.g. lysozyme, nisin). Naturally occurred antimicrobials are substances derived from nature products. Since many host defense mechanisms have already existed in animals, plants, fungi and microorganisms, many foods have antimicrobial function in their natural state and their extracts can be added into food directly. Especially in recent years, more and more consumers have the concern of the safety of pure compounds as antimicrobial preservatives in foods, and with the increasing demand for minimally processed foods, more naturally occurred antimicrobials are desired. This is because naturally occurred antimicrobials are from these foods that have been consumed by human for a long history the safety will not be a big obstacle for them. Therefore, the food industry are looking for more novel naturally occurred food antimicrobials to fulfill the consumer's desire for clean labeling. Natural antimicrobials can be derived from animal, plant, and microbial origins and they have been extensively reviewed previously (Davidson et al., 2005).

Natural antimicrobials are applied in food system in various ways: directly adding into foods, coating on the surface of food contact materials, or incorporating into the packaging materials (Lucera et al., 2012). Minimally processed foods, such as fresh-cut fruit and vegetables, are among the important application areas for natural antimicrobials due to the following reasons: first, it is quite challenge for preserving their fresh-like quality after prolonging the shelf-life; second, consumers expect higher quality for minimally processed foods which means the use of synthetic chemical preservatives is not preferred even though they have been approved; third, the relative higher cost of natural antimicrobials is comprised by the consumer willing to pay higher price for higher quality for minimally processed foods. Natural antimicrobials can be applied to protect minimally processed foods in several ways like dipping,

impregnation, coating and spraying. Among those, coating is most frequently used in ready-to-eat fruit and vegetables. For example, some pathogens can be significantly reduced by dipping the fresh-cut cabbage with acetic, lactic and malic acids (Bae et al., 2011). An edible coating based on chitosan and carboxymethyl-cellulose on the surface of minimally processed broccoli can not only inhibit microbial growth and but also keep quality by retarding weight loss, browning, yellowing and reducing stem hardening (Ansorena et al., 2011).

1.2 *Regulations related to natural antimicrobials*

In the U.S., the food additive regulatory system is built based on the Food Additives Amendment of 1958. The Congress has defined food additive as "any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food." (21 USC §321.201(s)) Theoretically, any substance expected to become a component of food should be regulated under "food additives" and premarket approval must be authorized by FDA; however, a substance can be exempt from the regulation under "food additives" if it fell into one of the following 6 categories: generally recognized as safety (GRAS) substances, pesticide chemicals and residues, prior-sanctioned substances, color additives, drugs in animal feed and dietary supplements (21 USC §321.201(s)). Currently, there are more than 10,000 substances allowed to be used in human foods and roughly 45% of them are under the categories of "food additives" and 45% are categorized as "generally recognized as safe" (GRAS) (Neltner et al., 2013). Among all these substances allowed to be used in food, approximately 60% have been decided as safe: federal agencies made approximately 40% safe decisions while manufacturers and the trade association made about 60% generally recognized as safe (GRAS) decisions without FDA review (Neltner et al., 2011).

For those substances derived from natural products and are intended to be used as antimicrobials in food, it can be defined as either "food additives" (e.g. nisin) or "GRAS substances" (e.g. essential oils). If they were intended to be used as new antimicrobial food additives, it always need longer time (usually 10-12 years) and higher cost (usually millions of dollars) to perform detailed safety assessment studies and to get the approval from FDA (Davidson et al., 2005). Currently, there are only 13 groups of compounds that have been approved by FDA to be used as food antimicrobials, and most of them are originally derived from nature products (e.g. nisin, natamycin, lysozyme, lactorferrin and organic acids). However, the process can be much simplified if they were claimed as "GRAS substances", which refer to substances that are generally recognized as adequately shown to be safe under their intended conditions of use. The GRAS status can be claimed based on either routine use in food before 1958 or scientific procedures, and a GRAS status can be reevaluated and removed if any adverse effects indicated (21 CFR Part 182).

The GRAS status does not need the permission or confirmation of FDA, it is a voluntary action of the persons who intend to use it. However, a company can still choose to inform the FDA and ask FDA to help check their decision under the GRAS Notification Program (USFDA, 1997). FDA has received over 562 GRAS notices from June 1998 to March 2015. For new emerging extracts from nature products, there must be enough scientific data to support the safety of their intended use and those data must be widely available (e.g. peer-review publication). Therefore, toxicity assessment of these extracts are indispensable. However, since the extracts are a mixture of various substances, the regulatory agency does not give universal guideline of how to assess the natural products to be defined as GRAS. They are usually decided on a case-by-case strategy (Abdel-Rahman et al., 2011).

2. *Clitocybe nuda* as novel natural antimicrobials

2.1 *Mushroom extract*

Clitocybe nuda (*C. nuda*), which is also named *Lepista nuda* and commonly known as wood blewit, belongs to Basidiomycota Division in the Fungi Kingdom. It is first described in 1790 and now it is cultured throughout Europe, Australia, Asia and North America (Barros et al., 2008). Fruit body, also known as fruiting body or sporocarp, is the part of fungus where the spores are produced. It is a multicellular structure which constitutes part of the sexual phase during the life cycle of a fungi. If the fruit body of a fungi grows on the ground and is visible by naked eye, it is often referred as mushroom. So this structure is often consumed by human as food, and many bioactive components with nutritional values are found in this part. *C. nuda* is a precious edible mushroom with very strong flavor and special fragrant and it has been recorded as medical mushroom is some oriental countries (Ying, 1987).

In recent years, this mushroom have attracted more and more interest due to its distinctive antioxidant and antimicrobial properties (Alves et al., 2012; Chen and Huang, 2009; Dulger et al., 2002; Mercan et al., 2006; Yamaç and Bilgili, 2006). Additionally, it also has been reported to have several pharmacical potentials like antidiabetic, antihyperlipidemic and antitumor actividitives (Beattie et al., 2011; Chen et al., 2014), and as a novel adjuvant in cancer therapy and vaccination for its immunologic effects (Chen et al., 2013). We are most interested in its antimicrobial activity, so this review will focus on its antimicrobial function. Based on results from current research, the fruit body extract and secondary metabolites from *C. nuda* are very promising to be used as novel natural antimicrobials in minimally processed foods such as vegetables, liquid eggs and ground meats with clean labeling. The antimicrobial active components are found in both of its fruit body extract and mycelial culture filtrates (secondary

metabolites). The chemical compositions of *C. nuda* have been investigated previously, and the predominant bioactive compounds are shown in Table 2-1 (Barros et al., 2008; Mercan et al., 2006). The extract of fruit body from *C. nuda* have been proven to have promising antimicrobial activity and its reported antimicrobial activity are summarized in Table 2-2. In previous research, the fruit body ethanol extract of *C. nuda* has been proven to show promising antibacterial activities against various foodborne pathogens with good stability (Bo, 2012). Additionally, its potential use in foods like liquid eggs has also been investigated (Gardner, 2015).

Table 2-1 Chemical and bioactive compositions of *C. nuda.* Summerized from Barros et al. (2008).

General composition (g/100g*)		**Tocopherol** (ng/100g)		**Total bioactive compounds**	
moisture	93.77	α-tocopherol	7.95	phenols (mg/g)	6.31
total fat	0.11	β-tocopherol	12.13	flavonoids (mg/g)	3.36
crude protein	3.70	γ-tocopherol	14.64	ascorbic acid (mg/g)	0.23
ash	1.15	total	34.72	β-carotene (μg/g)	2.52
carbohydrate	1.55			lycopene (μg/g)	0.98

* All the results are based on fresh wild mushroom.

Table 2-2 Antibacterial activities of fruit body extract from *C. nuda*.

Pathogens	**Antibacterial activity***			**References**
	MIC (mg/mL)	IC_{50} (mg/mL)	IZD (mm)	
Bacillus cereus	0.005	-	> 9	(Barros et al., 2008)
	-	-	-	(Dulger et al., 2002)
Staphylococcus aureus	0.005	-	> 9	(Barros et al., 2008)
	-	-	16.0	(Dulger et al., 2002)
	-	143.6	-	(Bo, 2012)
	-	-	> 15	(Suay et al., 2000)
Escherichia coli	0.5	-	> 9	(Barros et al., 2008)
	-	-	14.6	(Dulger et al., 2002)
	-	105.9		(Bo, 2012)
Salmonella Typhimurium	-	-	6.0	(Dulger et al., 2002)
	-	84.5	-	(Bo, 2012)
Listeria monocytogenes	-	79.2	-	(Bo, 2012)

* MIC: minimal inhibitory concentrations; IZD: internal zone diameter; IC_{50}: concentrations inhibiting 50% of the growth.

2.2 *Secondary metabolites*

Secondary metabolites are generally defined as organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism (Fraenkel, 1959). Fungal secondary metabolites, extracellular secreted by the mycelium, are usually a combination of low-molecular-weight functional metabolites which are used as the chemical signals that help fungi to compete or communicate with other organisms in the environment. The systematical study of fungal secondary metabolites started in 1922; however, it has not drawn widespread attention until the discovery of penicillin as secondary metabolites in fungi (Raistrick, 1950). Since then, thousands of fungal secondary metabolites with bioactive activities have been identified and reviewed elsewhere (Cole and Schweikert, 2003; Turner, 1971; Turner and Aldridge, 1983). A recent survey showed that more than half of these 1,500 secondary metabolites compounds isolated and characterized between 1993 and 20001 were proved to have antibacterial, antifungal or antitumor activity (Pelaez, 2005). However, there are still more secondary metabolites with special bioactive function that awaiting people to discover. Among these, anti-bacterial function is most important considering the fact that more and more antibiotic resistance bacteria emerged in recent years. The classification of fungal secondary metabolites based on the enzyme classes involved in their biosynthesis has been reviewed before (Keller et al., 2005) and is summarized in Table 2-3. Additionally, structures of some represented metabolites in each group were shown in Figure 2-1.

The culture filtrate of *C. nuda* was found to have strong inhibitory effect of certain plant pathogenic fungi and bacteria in a research conducted by Chen and Huang (2009). Later, three compounds (as shown in Figure 2-2) were further isolated and identified from these secondary metabolites responsible for inhibition effects (Chen et al., 2012). The antibacterial activities of

secondary metabolites from the same *C. nuda* strain culture were also investigated using four prevalent foodborne pathogens – *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aures* and *Escherichia coli* O157:H7, and the Minimum Inhibitory Concentrations (MIC) of 50% log reduction were 27, 25, 23 and 40 mg/mL respectively (Hou, 2013). Moreover, the antibacterial efficacies in food models like liquid eggs and ground beef were also investigated in the same study (Hou, 2013).

Table 2-3 Classes of fungal secondary metabolites involved in their biosynthesis. Summarized from previous review by Keller et al. (2005).

Classes	Examples	Enzyme	Substrate
Polyketides	Naphthopyrone, aflatoxin, lovastatin	Type I polyketide synthases	Fatty-acid
Non-ribosomal peptides	Penicillin, cephalosporin	Non-ribosomal peptide synthetases	Amino acids
Terpenes	Aristolochenes, indole-diterpenes	Terpene cyclase	Diphosphates
Indole alkaloids	Ergotamine, fumitremorgens	Dimethylallyl tryptophan synthetase	Tryptophan, dimethylallyl pyrophosphate, amino acids

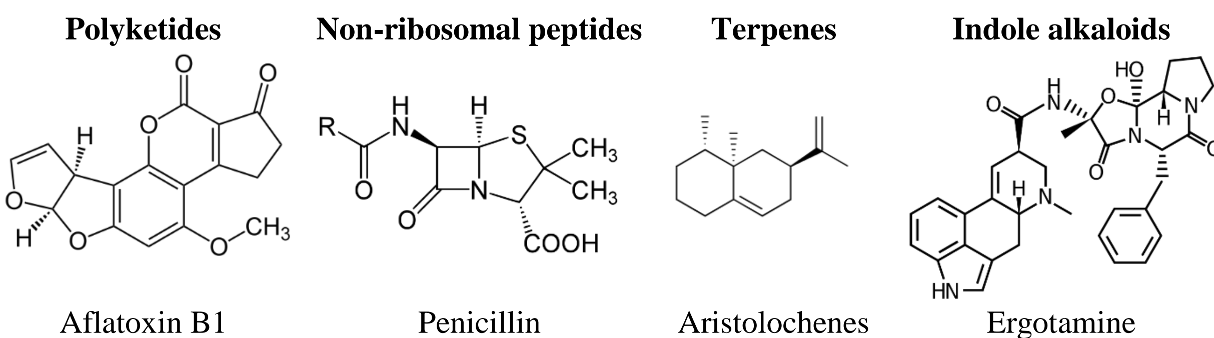
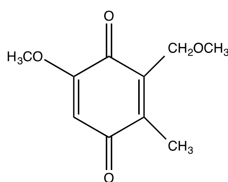
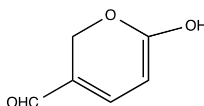
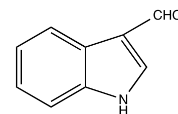


Figure 2-1 Structures of fungal secondary metabolites represent in four classes.







2-methoxy-5- methyl-6-methoxymethyl-p-benzoquinone	6-hydroxy-2H-pyran-3-carbaldehyde	indole-3-carbaldehyde

Figure 2-2 Structures of antibacterial compounds from secondary metabolites of *C. nuda*.

3. Methods of *in vitro* toxicology

3.1 *Toxicology assessment of substances used in foods*

In the U.S., toxicity assessment of substances used in foods are dominated by FDA. FDA has defined "safe" as a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use (21 CFR 170.3(i)). FDA has provided detailed guidance for industry about the toxicological principles for the safety assessment of food ingredients in its Redbook 2000 (USFDA, 2007). Usually, a series of short-term and long-term studies about genetic toxicity, acute oral toxicity, reproductive and developmental toxicity, neurotoxicity, immunotoxicity, and metabolism and pharmacokinetic studies are recommended depend on different situations. However, these toxicity assessments include a number of complementary tests using mammals, which will be highly cost and time consuming to draw concrete conclusions (Eisenbrand et al., 2002). Non-animal methods provide important tools to understand the toxicity of substances that are intended to be added into food and for predicting their potential adverse effects on human (Broadhead and Combes, 2001). For food additives, which are generally considered more strictly regulated than GRAS substances, toxicity data from *in vitro* studies have been considered for risk assessment purpose (Broadhead and Combes, 2001). The primary toxicity concerns for substances that will be added into food

are acute toxicity in the short term and carcinogenicity in the long term. In the past, animal testing has become the “golden standard” for investigating both short-term and long-term toxicity such as acute oral toxicity, genetic toxicity and carcinogenicity. However, in recent years, a lot of efforts have been paid to develop new *in vitro* testing methods that can be used for predicting acute oral toxicity and genotoxicity. The aim of developing these *in vitro* tests is to reduce the use of animals and ultimately to substitute animal tests. One important prerequisite for applying an *in vitro* approach is that the method must have been validated with *in vivo* animal tests using a wide range of chemicals and in different labs to confirm its predictability. Among all these *in vitro* methods that have been developed, 3T3 NRU cytotoxicity test, bacteria reverse mutation test and *in vitro* mammalian cell micronucleus test have been validated by a large amount of studies and all have been recommended by Organisation for Economic Co-operation and Development (OECD) as standard methods for toxicity testing (OECD, 1997, 2010a, 2014)

3.2 *In vitro basal cytotoxicity*

Basal cytotoxicity can provide toxicity information about unknown chemical compounds based on cellular lethal endpoints. However the value of cytotoxicity testing results is limited due to the fact that, compared with whole organisms like rats, cell cultures are in lack of biotransformation process. Moreover, some toxicants may have specific target organs in human body which cannot be well reflected using sole cell culture. In the past two decades, a great deal of interest have been attracted to explore the feasibility to predict acute oral toxicity, subchronic toxicity and chronic toxicity using *in vitro* cytotoxicity testing. A number of studies have been performed to establish the relationship between *in vitro* cytotoxicity and *in vivo* lethality, and various cell lines and endpoints have been tested. Among all these testing systems that have been

validated, 3T3 NRU assay has proven to be one of the most promising methods based on sensitivity, reproducibility and predictability.

NRU assay is a standard cytotoxicity testing method to measure killing effects of testing substances in animal cells by using neutral red uptake as endpoint. The principle of this method is as illustrated in Figure 2-3. Neutral red is a weakly cationic and highly lipophilic dye which has a pKa at approximately 6.8. It is nonionic in the medium and cytosol (pH=7.2), so it can easily penetrate the phospholipid membranes of cell and lysosome. After entering into the inside of lysosomes where has a lower pH at around 5.0, it will become charged and be retained in the lysosomes through electrostatic bonds with anionic or phosphate groups of the lysosomal matrix (Nemes et al., 1979). The low pH in lysosome need to be retained by ATP so neutral red will only be retained within lysosomes if the cells are alive with integrated membrane and ATP. Finally, the amount of neutral retained within lysosomes will be extracted and measured using spectrometry. Therefore, the number of living cells are proportional to the amount of NR dye that can be desorbed from the culture (Repetto et al., 2008). Balb/c 3T3 (clone A31) is a strain of animal fibroblast cell developed from disaggregated 14- to 17-day-old BALB/c mouse (*Mus musculus*) embryos in 1968. The 3T3 NRU assay has been officially recommended by both of NIH (2006) and OECD (2010) as an *in vitro* cytotoxicity test method for estimating starting doses for acute oral systemic toxicity after a number of validation studies and discussions. Furthermore, in April 2013, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) suggested that 3T3 NRU cytotoxicity assay can be used to predict the acute oral toxicity classification of unknown chemicals (ECVAM, 2013). However, at the same time, it is also addressed that this prediction method should be used with caution by adequately being aware of its limitations. One limitation is that NR is a weak cationic dye which

concentrates in lysosomes, which will generate bias in some situation where the cytotoxic target is not lysosomes. Therefore, another commonly used endpoint, MTT reduction based on NAD(P)H-dependent cellular oxidoreductase enzymes in live cells (as shown in Figure 2-3), was used as a complementary indicator. Another limitation is that BALB/c 3T3 cell is an animal cell and without enzymatic metabolizing system, which cannot give enough information on human and the situation with metabolizing activation. Therefore, a human derived hepatoma cell line (hepG2) with phase I and II enzyme activities was applied to illuminate more information on human and the situation with metabolization.

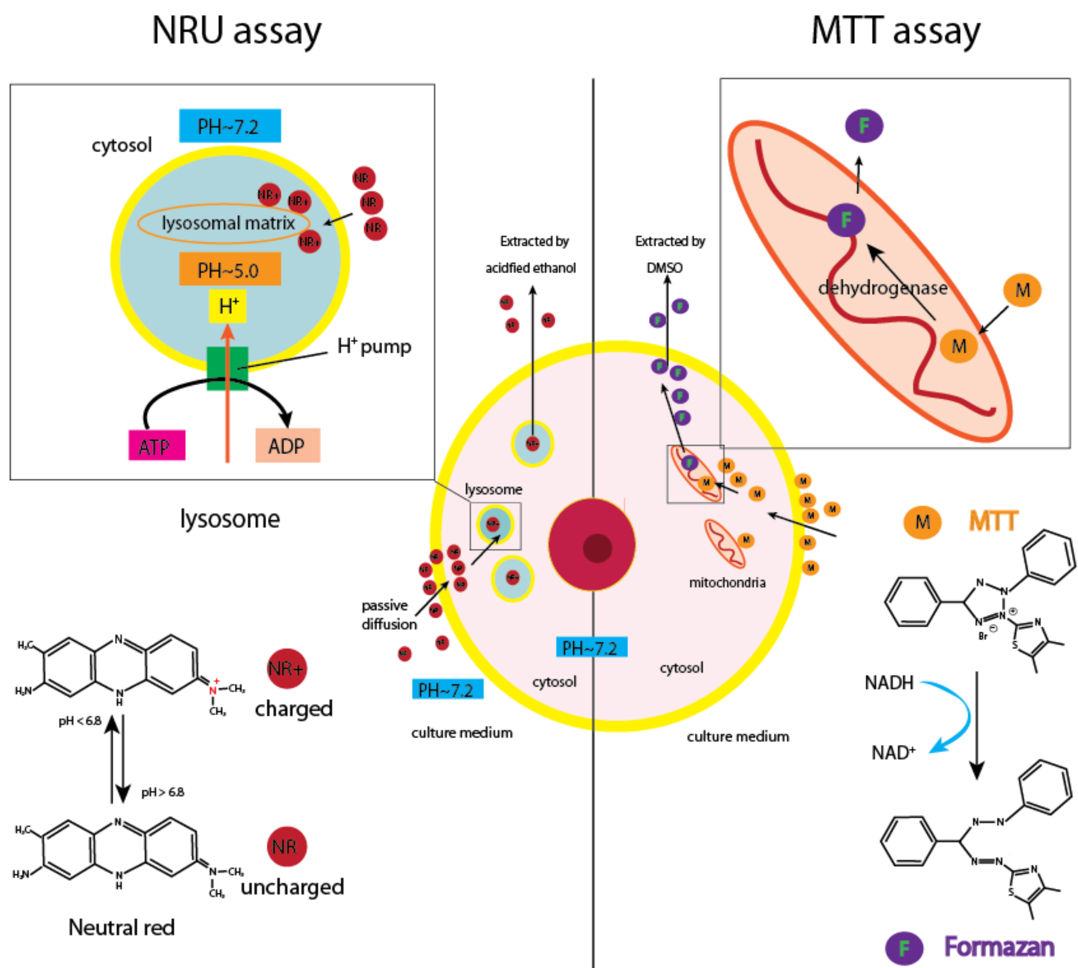


Figure 2-3 Mechanism of NRU and MTT assay.

3.3 *In vitro genotoxicity*

Genotoxicity is used to describe the damage of genetic information caused by chemical agents. Genotoxicity may happen in four levels: gene level (single base modified, few bases inserted or deleted), DNA level (large deletion or rearrangement), chromosome level (break or rearrangement), and whole chromosomes (gain or lose). Genotoxicity may lead to cancer and a variety of genetic diseases if the mutation happened on regulatory genes related to oncogenes, tumor suppressor genes or damage repair genes (Erickson, 2010). Some other diseases like accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases are also related to accumulation of DNA damage caused by genotoxicity (De Flora and Izzotti, 2007; Frank, 2010; Hoeijmakers, 2009; Slatter and Gennery, 2010). Moreover, these damages caused by genotoxicity can be heritable to the offspring and subsequent generations (EFSA, 2011). For those substances that are proposed to be added into food, the testing of genotoxicity has been routine for many years (EFSA, 2011). The European Food Safety Authority (EFSA) scientific committee recommends to use a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test as the first step to investigate genotoxic potential of substances that will be used in foods. The scientific committee further conclude that "these two tests are reliable for detection of most potential genotoxic substances and if all *in vitro* endpoints are clearly negative in adequately conducted tests, then it can be concluded with reasonable certainty that the substance has no genotoxic potential" (OECD, 1997). Therefore, a combination of *Salmonella*/microsome mutagenicity assay (Ames test) and cytokinesis-block micronucleus cytome (CBMN-cyt) assay are used for genotoxicity testing in this study.

The bacterial reverse mutation test is a short-term test to detect point mutations using mutated bacteria strains. The principle of bacteria reverse mutation test is that a series of test

strains are mutated to lose the function of synthesizing an essential amino acids, and then these bacteria are treated with testing substances and cultured in the absence of that amino acids, only those back reverted strains which recover the ability to synthesis the amino acids themselves can grow and be counted (Mortelmans and Zeiger, 2000). Usually a series of bacteria are mutated to cover different mechanisms of point mutation like substitution, deleting or addition of DNA base pairs. *Salmonella* Typhimurium is one of the most frequently used bacteria in the Ames test. A combination of five *S.* Typhimurium strains (TA98, TA100, TA1535, TA1537 and TA1538) covering all these point mutation events as described were used in this study and their locations on the gene structure of *S.* Typhimurium *histidine* operon are shown in Figure 2-4. TA1535, TA1537 and TA1538 strains are mutated directly from wild strains while TA98 and TA100 are obtained from TA1535 and TA1538 respectively by introducing a plasmid (pKM101) which results in higher reversion rate. Some mutagenic chemicals, such as aromatic amines, are only biologically active after being metabolized by cytochrome-based P450 metabolic oxidation system in human's organs like liver, lung and kidneys. However, bacteria do not have this metabolic capacity. Therefore, a rodent metabolic activation system (S9 mix) consisting of supernatant fraction of a rat liver homogenate, NADP and oxidation cofactors was also added into the testing system to detect potent indirect mutagens (Maron and Ames, 1983).

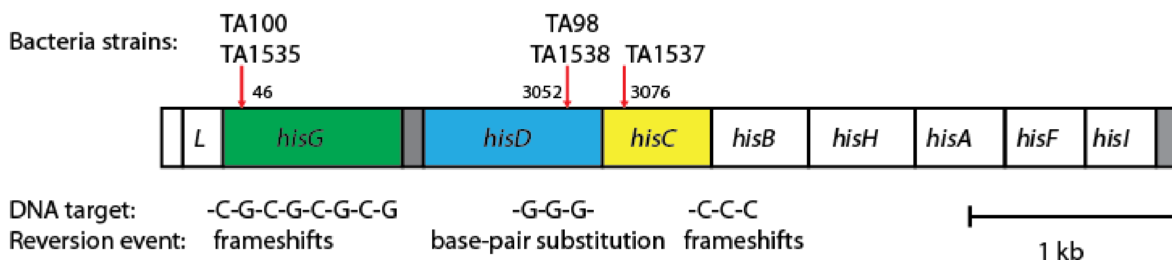


Figure 2-4 Structure of *S.* Typhimurium *histidine* operon. Locations of mutated genes of five mutated *S.* Typhimurium strains (TA98, TA100, TA1535, TA1537, and TA1538) are shown.

The *in vitro* micronucleus (MNvit) assay is a standard genotoxicity test for screening genotoxic compounds. Micronuclei (MNi) are small cytoplasmic bodies that fail to reach the poles of anaphase cells during mitosis (Fenech, 2000). MNi were first described by Scott and Evans (1967) to assess chromosomal damage in the root tips of braod beans (*Vicia faba*) and now it has been developed as the one of the most successful and reliable biomarkers for detecting genotoxic substances. Several improvements to the micronucleus assay have been made by Dr. Fenech's group in the past two decades (Fenech, 2007; Fenech et al., 2003; Thomas et al., 2003). One improvement is the introduction of cytochalasin-B (Cyto-B) to eliminate the bias caused by altered cell division kinetics: Cyto-B can inhibit the assembling of microfilament ring which is necessary for the completion of cytokinesis, so the scoring of micronuclei can be restricted in once-divided cells with binucleated (BN) appearance (Fenech, 2007). Additionally, other genotoxic biomarkers like nucleoplasmic bridges (NPBs) induced by DNA misrepair, and nuclear buds (NBUDs) induced by gene amplification are also introduced to be included in the system to enable it as a comprehensive method for detecting genotoxic events (Thomas et al., 2003). Various fates of cultured cytokinesis-blocked cells after treated with genotoxic agents are illustrated in Figure 2-5 and the mechanisms of MNi, NPBs, and NBUDs formation are also shown. Usually a variety of primary cell cultures or cultures of cell lines of human (e.g. peripheral blood lymphocytes) and rodent (e.g. CHO, V9 and L5178Y) are used for *in vitro* micronucleus assay (OECD, 2014). However, these cells lack metabolic activation system and an exogenous metabolic activation mixtures (e.g. S9 mix) as described earlier is always introduced. Another appealing way for solving this problem is through using cells that have metabolic activation enzymes themselves such as human heptoma cell line hepG2, which will make the procedure simpler (Nikoloff et al., 2014).

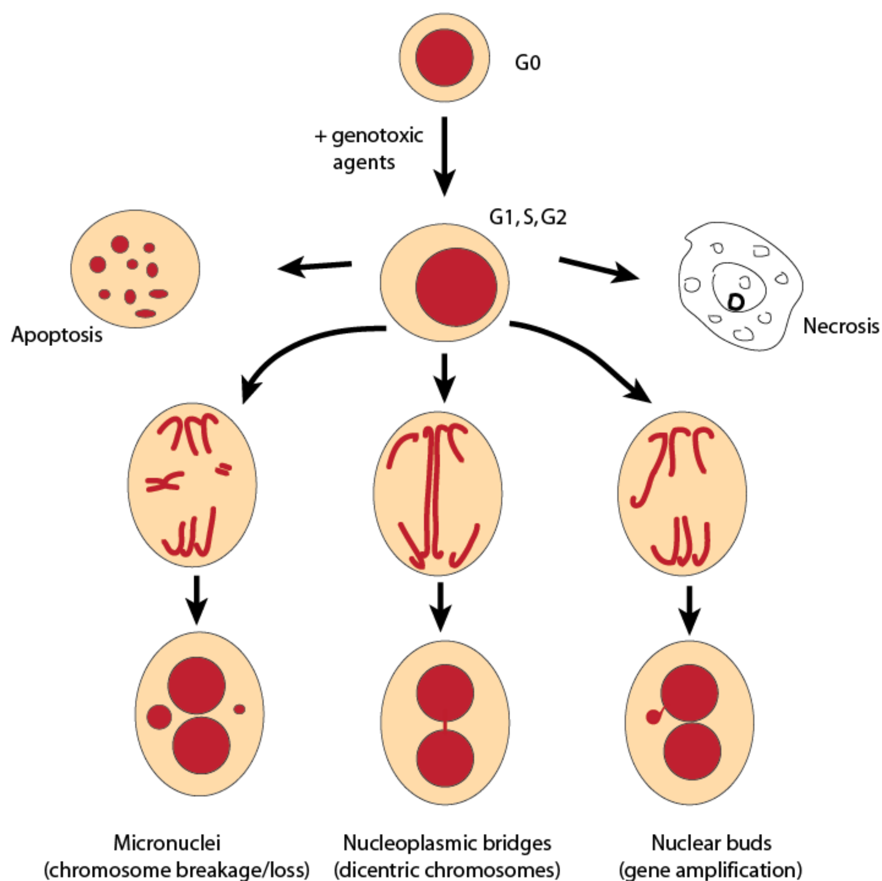


Figure 2-5 Mechanism of *in vitro* micronucleus (MNvit) assay.

3.4 *Human liver metabolizing system*

Compared with *in vivo* tests using animals, one major limitation of *in vitro* toxicity methods is that they are lacking of biotransformation system. One direction of improving *in vitro* methods is to incorporate human metabolization system into the testing system. Therefore, human metabolizing system is also reviewed here. In human, the metabolism of chemicals are mainly located in liver which has a variety of enzymes. Chemical metabolism in the liver can be divided into two phases (Williams, 1959) and the mechanisms of chemical metabolization in liver are shown in Figure 2-6. In phase I, the substrate is added a functional group (e.g. –OH, -SH, or $–NH_2$) through oxidative, reductive and hydrolytic pathways. In phase II, the modified

functional group introduced in phase I will be increased with polarity by further modifying into *O*- and *N*-glucuronides, sulfate esters, various α-carboxyamides, and glutathionyl adducts (Parkinson, 1996). Most chemicals will be transformed into more water soluble format after this two-step process and are easier to be excreted. Most toxic chemical will become non-toxic after detoxification of liver, however some indirect mutagens like bazo[a]pyrene and cyclophosphamide are only activated after activation. Although hepG2 cells are reported to have lower levels of phase I enzymes compared with human primary hepatocytes (Wilkening et al., 2003), hepG2 cells are more preferred due to the fact that it is easier to be cultured as a culture of cell line. Therefore, in this study, hepG2 cells are chosen for genotoxicity test and complementary cell line for cytotoxicity test to investigate the potential toxic effect of testing samples after metabolizing.

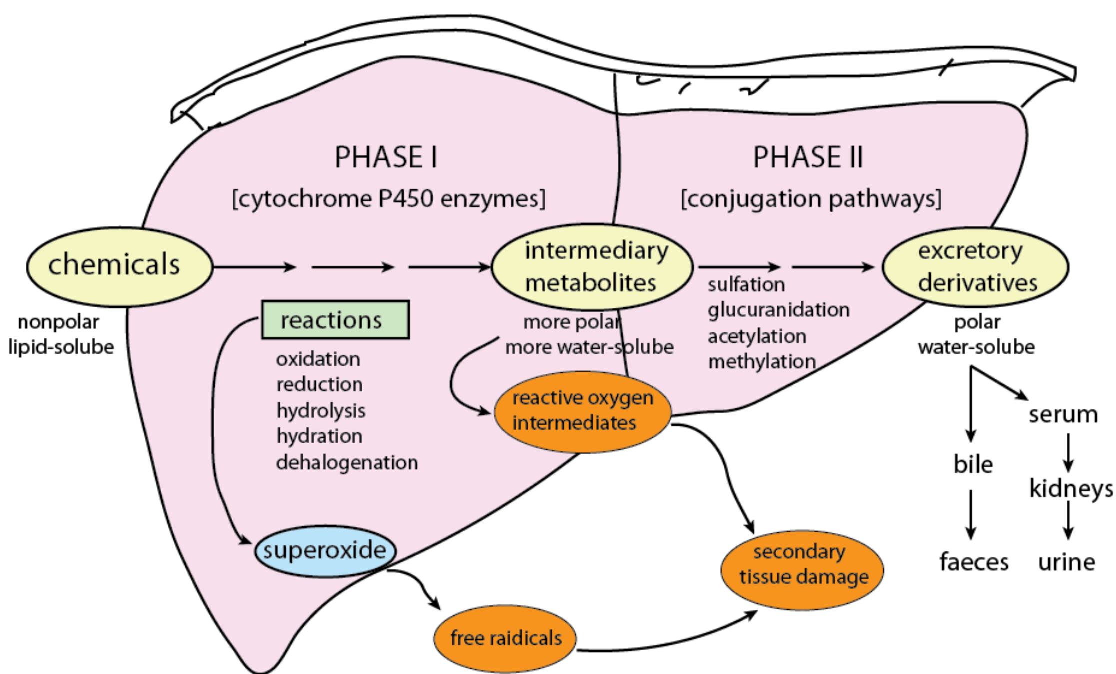


Figure 2-6 Mechanism of chemical metabolization in human liver.

CHAPTER 3: MATERIALS AND METHODS

1. Chemicals and reagents

Sodium azide, 9-aminoacridine, 2-aminoanthracene, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cyclophosphamide were purchased from Alfa Aesar (Ward Hill, Massachusetts). D-biotin was purchased from Chem-Implex (Wood Dale, Illinois). Nicotine adenine dinucleotide phosphate (NADP) was supplied by Amresco (Solon, Ohio). 4-Nitro-o-phenylenediamine (4-NOPD), neutral red (NR) and poly-L-lysine solution were supplied by Sigma-Aldrich (St. Louis, Missouri). L-histidine was purchased from Acros Organics (New Jersey). Glucose-6-phosphate (G-6-P) sodium salt and rat liver S9 (Aroclor-1254-induced) were purchased from MOLTOX (Boone, North Carolina). Cytochalasin B (Cyt-B) was purchased from EMD Chemicals (San Diego, California). Newborn calf serum (NCS) was purchased from HyClone Laboratories (South Logan, Utah). Non-heat inactivated fetal bovine serum (FBS) was purchased from Seradigm (West Providence, Utah). Eagle's minimum essential medium (EMEM) without L-glutamine was purchased from Quality Biological (Gaithersburg, Maryland). Dulbecco's modification of eagle's medium (DMEM) without L-glutamine (4.5 mg/L glucose) was purchased from Lonza (Walkerville, Maryland). Accutase® cell detachment solution (containing 0.5 mM EDTA) was supplied by Innovative Cell Technologies (San Diego, California). Protocol® HEMA3® STAT PACK for differential staining was purchased from Fisher Scientific Company (Kalamazoo, Michigan).

2. Bacterial strains and cell cultures

All five *S.* Typhimurium tester strains (TA98, TA100, TA1535, TA1537 and TA1538) were obtained from MOLTOX (Boone, NC). All tester strains were confirmed for their phenotype characteristics and analyzed for their genetic integrity and spontaneous mutation rates as shown in Table 3-1 and Table 3-2 before use (Mortelmans and Zeiger, 2000). Mouse embryonic fibroblast cells BALB/c 3T3 clone A31 (ATCC® CCL163™) and human heptoma HepG2 (ATCC® HB-8065™) cell line were purchased from the American Type Culture Collection (ATCC; Manassas, VA). 3T3 cells were maintained in DMEM (high glucose at 4.5 g/L) supplemented with 10% of NCS and 4 mM of L-Glutamine. HepG2 cells were cultured in EMEM supplemented with 10% of FBS, 100 IU/mL Penicillin and 100 μg/mL Streptomycin. Both cells were maintained 75 cm^2 cell culture flasks in a humidified incubator at 37 °C under an atmosphere containing 5% CO_2. 3T3 cells were routinely passaged every 2-3 days while hepG2 cells were subcultured at 3-4 days intervals.

Table 3-1 Genotype characteristics of tester strains.

Strains	Allele	Reversion event	DNA target	References
TA98 TA1538	*HisD3052*	Frameshifts	-C-G-C-G-C-G-C-G-	(Isono and Yourno, 1974)
TA100 TA1535	*HisG46*	Base-pair substitution	-G-G-G-	(Barnes et al., 1982)
TA1537	*HisC3076*	Frameshift	+1 frameshift (near -C-C-C-)	(Ames et al., 1973)

Table 3-2 Genetic integrity and spontaneous mutation rates of tester strains.

Strains	*bio chlD uvrB gal*	LPS defect	Plasmid	Spontaneous revertants*	
				Without S9	With S9
TA98	Deletion	*rfa*	pKM101	20-50	20-50
TA1538	Deletion	*rfa*	No plasmid	5-20	5-20
TA100	Deletion	*rfa*	pKM101	75-200	75-200
TA1535	Deletion	*rfa*	No plasmid	5-20	5-20
TA 1537	Deletion	*rfa*	No plasmid	5-20	5-20

*Range considered valid in the author's laboratories.

3. Preparation of fruit body extract from *C. nuda*

Dry mushroom (*C. nuda*) was grounded using a coffee grinder, and 25 g of the powder were extracted in one liter of 95% ethanol for 24 h at 25 °C with 400 rpm stirring and then filtered through Whatman™ No. 4 filter paper. The residue was extracted two more times using the same procedure. All extracts were combined, and ethanol was evaporated completely from the combined extract by evaporating at 40 °C. The dried residue was re-suspended in 5 mL deionized water and placed in a dialysis tube with a molecular weight cut-off of 500 Dal and then was dialyzed at 25 °C with 100 mL of distilled water with 3 changes of water in a 24-h period. The water solutions outside the dialysis tube were combined (400 mL) and filtered through 10 μm cellulose filter paper and 0.45 μm sterile filter membrane. The water in the filtrate was removed in two methods, freeze-dry (FD) and heat-dry (HD). For the FD extract sample, the filtrate was freeze-dried under a vacuum. In the HD method, the filtrate was heated on a hot plate (82 °C) to remove the majority of the water and then dried at 95 °C to completely remove the water. All dried samples were stored in desiccator at room temperature. Both FD and HD samples were dissolved in sterile deionized water at 200 mg/mL, and the pH was adjusted to 7.0 using NaOH before use. The antimicrobial activity of the FD samples were tested by following the Hou procedure (2013) and the IC_{50} of FD sample to *Salmonella* Typhimurium was about 30-60 mg/mL.

4. Preparation of secondary metabolites extract from *C. nuda*

A high yield *C. nuda* strain (LA82) obtained from Dr. Jin-Wen Huang in the Taiwan Agricultural Research Institute (Taiwan). Culture from slant tube was grown on potato dextrose agar (PDA) plates at 25 °C for 21 days. Then, culture block (10×10×3 mm) was placed in 200 mL of potato dextrose broth (PDB) and incubated at 24 °C on a shaker with 120 rpm for 21 days.

The culture fluid was filtrated through Whatman™ no. 1 filter paper and 0.45 μm sterile filter membrane. After that the culture fluid was dried with two different methods: freeze dry or heat dry. For heat dry extract, the volume of the filtrate was reduced from 600 mL to 100 mL by heating on a hot plate at about 82 °C and dried completely in an oven at 95 °C. For freeze dry extract, the fluid was dried under vacuum. Concentrations of working samples were prepared by dissolving the freeze dried or heat dried extract in deionized water. The antimicrobial activity of FD and HD samples were tested by following the procedure of Hou (Hou, 2013) and the MIC_{50} (minimum inhibition concentration at 50%) of HD sample to *Salmonella* Typhimurium was approximately 27 mg/mL.

5. NRU and MTT assay

NRU assay was performed following the protocols recommended by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) for basal cytotoxicity evaluation test using BALB/c 3T3 cells (NIH, 2006) and with modifications for hepG2 cells. Briefly, 100 μl of 3T3 cells (2.7×10^4 cells/mL) or hepG2 cells (1.0×10^5 cells/mL) were seeded in 96-well plates and incubated for 24 h at 37 °C under 5% CO_2 atmosphere. After the culture medium was removed, the cells were treated with 50 μL of culture medium and 50 μL of fruit body extract or secondary metabolites extract at designated dose solutions which were freshly prepared by dissolving in medium without serum at 8 different concentrations between 0.045-100 mg/mL and incubated for 48 h. Afterward, the medium with the mushroom extract was removed, cells were rinsed with D-PBS, and then 200 μL of NR medium (5% serum, 25 μg NR dye/mL) were added and the cells were incubated in dark for another 3 h. After that, the NR medium was removed, 100 μL of ETOH/Acetic Acid (49:1) desorb solution were added to all wells after rinsing with D-PBS and shaken rapidly for 20 min to extract NR. Then, samples

were set stand still for 5 min, absorptions at $O.D._{540}$ of samples were measured by a microtiter plate reader (Multiskan Ascent, Thermo Fisher Scientific). Sodium lauryl sulfate (SLS) was used as positive control through the whole process. A MTT assay was performed following the same procedures as NRU assay except that MTT at 5 mg/mL (dissolved in DPBS) was used instead of NR, and 100 μL of DMSO were used to substitute ETOH/Acetic Acid. For each concentration, cytotoxicity was expressed as the mean percentage of cell viability from three replicates. Dose response curves were constructed and fitted using non-linear regression Hill function (GraphPad PRISM® 6 Demo, GraphPad Software, La Jolla, CA) with $R^2 > 95\%$. IC_{50} values (50% cell viability inhibition concentrations) were calculated using the fitted Hill equation and were presented as mean±SD from three independent experiments. Estimated acute oral LD_{50} was calculated based on IC_{50} (μg/mL) from 3T3 NRU assay using an established regression model: $\log LD_{50}$ (mg/kg) = $0.372 \times \log IC_{50}$ (μg/mL) + 2.024 (ICCVAM, 2006).

6. Bacterial reverse mutation assay

This assay was designed following instructions of the OECD TG471 (OECD, 1997) and pre-incubation procedure was chosen (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). Briefly, tester cultures were incubated in nutrient broth (EMD) overnight at 37 °C to a population of $1\text{-}2\times10^9$ colony forming units (CFU)/mL. Test samples consisted of 100 μL overnight bacterial suspension (~10^8 CFU of bacteria), 50 μL of fruit body extract (freeze dry), secondary metabolites extract (freeze dry and heat dry), or indole-3-carbaldehyde (final concentration at 50, 500, 1,000, 2,500, 5,000 μg/plate), and 500 μL phosphate buffer (for the tests without external metabolizing system) or S9 mix (for the tests with an external metabolizing enzyme system) were pre-incubated for 20 min at 37 °C. Sodium azide (NaN_3, 5 μg/plate), 9-aminoacridine (9-AAC, 50 μg/plate) and 4-nitro-o-phenylendiamine (4-NOP, 2.5 μg/plate) were

used as positive controls for those without S9 mix tests while 2-aminoanthracene (2-AAN, 10 μg/plate) was applied as positive control for the test with S9 mix. Samples containing only water or DMSO solvent were used as negative controls for all tests. After preincubation, each sample was added into 2 mL of top agar containing limited quantities of histidine and biotin (0.5% agar with 0.05 M biotin/histidine) at 45 °C, and poured onto a pre-poured minimal agar plate after vortex. All plates were incubated at 37 °C for two days and bacterial colonies were enumerated and recorded. Triplicate plating was used at each dose level. The tests without S9 mix were repeated three times and the tests with S9 mix were repeated twice. Data were presented as the mean ±SD of revertants for each treatment.

7. Cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay

The CBMN-cyt assay was performed following the procedures of OECD (OECD, 2010b) and previous protocol (Nikoloff et al., 2014) with some modifications. HepG2 cells were subcultured in a new flask for 24 h, and detached using Accutase® cell detachment solution to prevent clumping. Then, 3 mL of cell suspension (2×10^4 cells/mL) were seeded onto precleaned 22 × 22 mm poly-L-lysine coated coverslip in 35 mm diameter petri dish and incubated for 24 h. Afterward, the medium was removed, and cells were treated with fruit body extract (freeze dry) or secondary metabolites extract (freeze dry) at final concentrations of 0.75-12 mg/mL. Cells were then incubated for 24 h and washed twice with D-PBS before treated with Cyto-B (6 μg/mL), then incubated for additional 16 h to acquire binucleated cells. Coverslips were then treated with 0.075 M KCl at 4 °C for 5 min, fixed with methanol for 15 min. Then, slides were stained with Protocol® HEMA3® STAT PACK (equivelant to Giemsa-Wright staining) with 6 s for solution II and 3 s for solution III and air-dried for 30 min. Finally, the coverslips were placed down onto precleaned microscope slides using mounting medium. Cyclophosphamide

(CP, 0.2 mg/mL) was used as positive control. Each experiment was repeated two times with duplicated cultures in each repeat. A CBPI (cytokinesis-block proliferation index) was calculated for each experimental point according to the method described by OECD in 500 viable cells at 1,000 × magnification (OECD, 2010b), and the numbers of micronucleus (MNs), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored in 1,000 binucleated cells following the criteria reported previously (Fenech, 2007; Fenech et al., 2003).

8. Statistical analysis

Data were analyzed using the GLIMMIX procedure as implemented in SAS 9.3 software (SAS Institute Inc., Cary, NC). Tukey adjustment was applied to the least square means (LSMEANS) statement of SAS software to analyze the differences among treatments.

CHAPTER 4: RESULTS

1. Toxicity of fruit body extract from *C. nuda*

1.1 *Cytotoxicity and estimated acute oral toxicity*

The dose-response curves of BALB/c 3T3 and HepG2 cells using NRU and MTT assay after treated with FD fruit body extract from *C. nuda* are illustrated in Figure 4-1, and the HD fruit body extract curves are illustrated in Figure 4-2. Their interpolated IC_{50} values and estimated acute oral LD_{50} values are shown in Table 4-1. Moreover, the influences of drying methods (FD and HD), cytotoxicity endpoints (NRU and MTT) and cell lines (3T3 and HepG2) on the results of cytotoxicity are presented in Figure 4-3. From these results, both of the FD and HD fruit body extracts can cause toxicity in a dose-dependent manner and there is no difference ($P > 0.05$) between the two drying methods in terms of cytotoxicity. For BALB/c 3T3 cells, the IC_{50} obtained from NRU assay is significantly lower ($P < 0.05$) than that in MTT assay; however, this phenomenon was not observed in HepG2 cells. The estimated oral acute LD_{50} were 2,617±139 mg/kg and 2,610±98 mg/kg b.w. of rat respectively for FD and HD fruit body extracts. Both of them are higher than the cut-off value of 2,000 mg/kg b.w., so they are suitable to be classified as non-toxic.

1.2 *Genotoxicity in CBMN-cyt assay*

Cytostatic/cytotoxic and DNA damage effects of *C. nuda* fruit body FD extract in binucleated cytokinesis-blocked hepG2 cells are presented in Table 4-2. The values of positive and negative controls are consistent with previous research (Chequer et al., 2012; Nikoloff et al., 2014). The percentages of binucleated cells, as indicated by CBPI value, decreased in a dose-

dependent manner as the concentrations of extract increased from 0.75 to 12 mg/mL. A significant difference ($P < 0.01$) was observed for MNs in the positive control cells when compared with negative control cells; however, there is no significant difference ($P > 0.05$) between the negative control and each concentration of the fruit body extract as for the number of MNs. Also, no increase in the numbers of NPBs and NBUDs was observed in the fruit body extract treated cells when compared with the negative control. Additionally, no dose-response phenomenon was observed in cells treated with fruit body extract as for MNs, NPBs, and NBUDs. Therefore, the fruit body extract did not induce chromosome breaks and gain/loss in cultured human cells under the conditions in this research.

1.3 *Mutagenicity in bacteria reversion test*

The mutagenic effects of the fruit body extract (FD) from *C. nuda* on five *S.* Typhimurium strains in the Ames assay are shown in Table 4-3. The results show that revertant numbers of all positive control chemicals (with or without S9) were significantly increased compared with negative or solvent controls and they are consistent with historical data in our lab and other researchers (Kouvelis et al., 2011). However, no differences were observed ($P > 0.05$) between the numbers of revertants in the fruit body extract treated samples and negative controls (H_2O) in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system. Additionally, no dose-response relationship of revertant number was observed within 50 to 5,000 μg/plate of the fruit body extract. In addition, the potential influence of methods chosen (preincubation method and standard plating method) and S9 doses (high and low) were also investigated in TA1538 and no influences of these factors were observed. All these results indicate that under the test conditions in this research, the fruit body extract of *C. nuda* is not mutagenic in the tested strains.

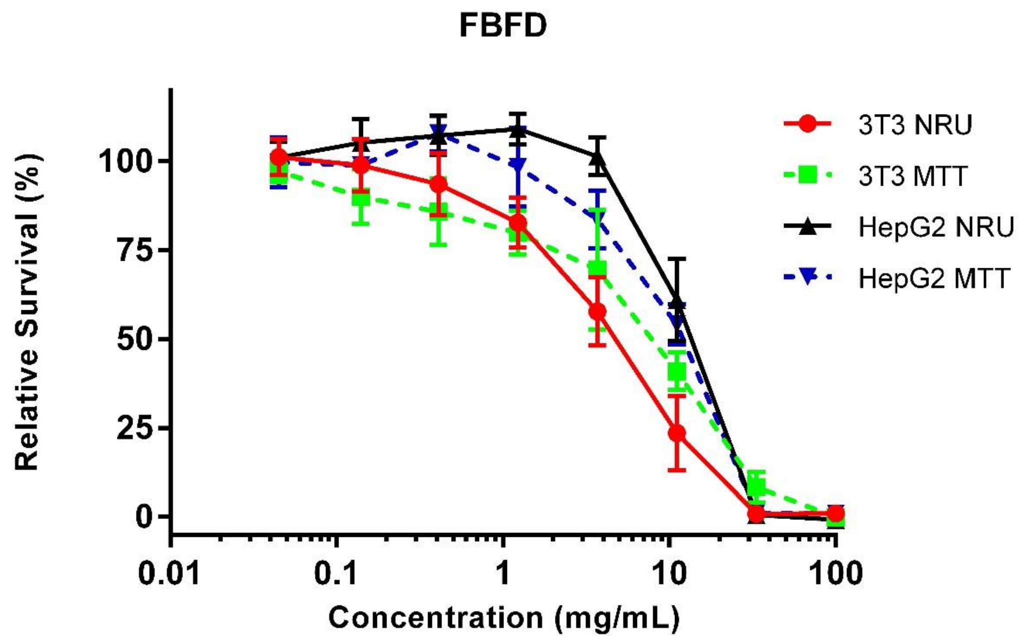


Figure 4-1 Cytotoxic effects of fruit body extract (freeze-dry) from *C. nuda* in BALB/a 3T3 and HepG2 cells using NRU and MTT assay.

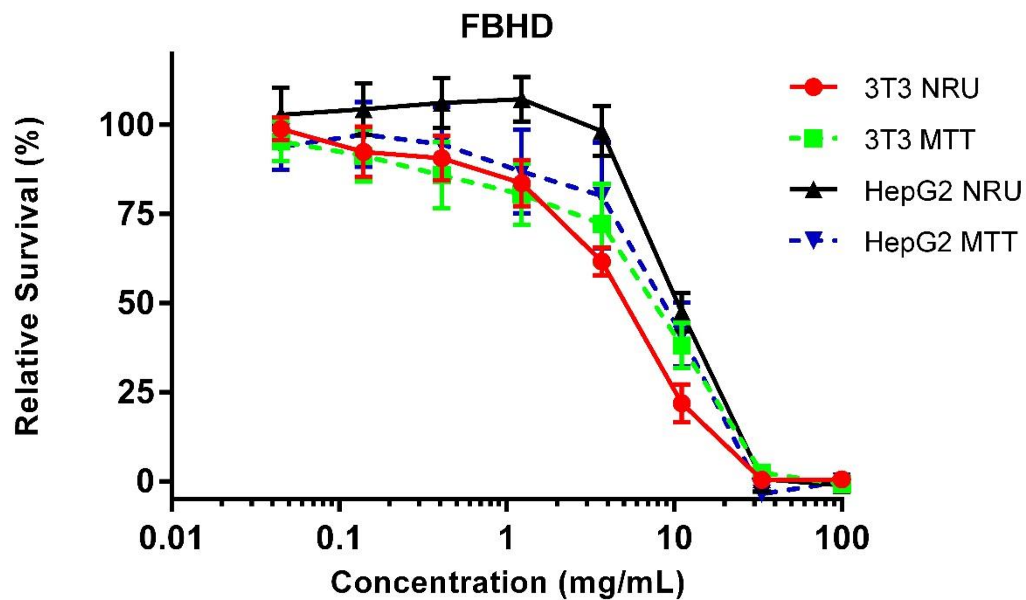


Figure 4-2 Cytotoxic effects of fruit body extract (heat-dry) from *C. nuda* in BALB/a 3T3 and HepG2 cells using NRU and MTT assay.

Table 4-1 Cytotoxicity and estimated acute oral toxicity of fruit body extract from *C. nuda.*

Fruit body extract	IC_{50} (mg/mL), 48 h exposure				Estimated acute oral LD_{50}* (mg/kg b.w.)
	BALB/c 3T3 cells		HepG2 cells		
	MTT	NRU	MTT	NRU	
Freeze-dry	11.2 ± 2.17^b	5.60 ± 0.80^a	11.4 ± 1.33^b	12.0 ± 1.81^b	2617 ± 139
Heat-dry	9.34 ± 1.59^b	5.56 ± 0.56^a	9.91 ± 2.11^b	10.4 ± 1.12^b	2610 ± 98

* Estimated acute oral LD_{50} was calculated based on IC_{50} (μg/mL) value from 3T3 NRU assay using an established RC rat-only weight regression: $\log LD_{50}$ (mg/kg) = $0.372 \times \log IC_{50}$ (μg/mL) + 2.024 (ICCVAM, 2006).
[ab] Numerical values in both of row and column are significantly different ($P < 0.05$) if superscript letters were different.

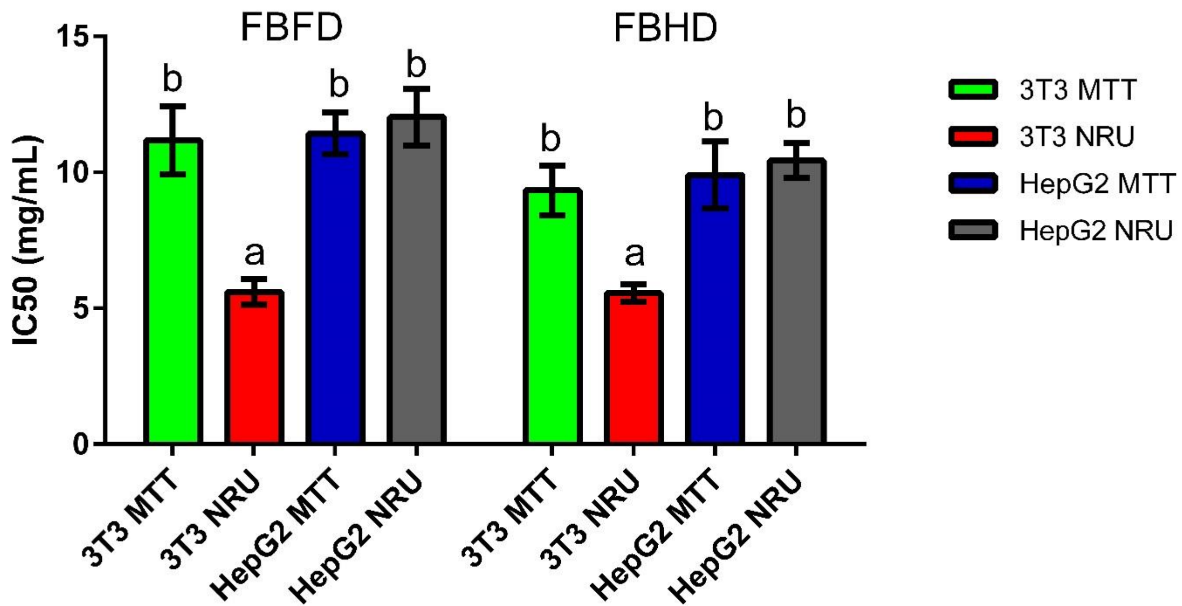


Figure 4-3 Influences of drying methods (FD and HD), cytotoxicity endpoints (NRU and MTT) and cell lines (3T3 and HepG2) on the evaluations of cytotoxicity of fruit body extract from *C. nuda*. [ab] Significant different ($P < 0.05$) between different bars if letters were different.

Table 4-2 Genotoxicity of fruit body extract from *C. nuda*.

Sample [a]	Con. (mg/mL)	Cytostatic/cytotoxicity		DNA damage in 1000 BN cells		
		Viability (%)	CBPI	MNs	NPBs	NBUDs
NC	-	97.4 ±1.8[b]	1.42 ±0.09	19.0 ±6.8	8.0 ±7.2	15.8 ±7.2
PC	0.2	97.4 ±1.6	1.42 ±0.07	49.3 ±7.3**	6.0 ±2.7	26.9 ±9.7
FB	0.75	98.3 ±1.4	1.36 ±0.05	24.5 ±6.7	5.7 ±3.9	12.5 ±6.8
extract	1.5	98.6 ±0.2	1.27 ±0.04*	15.8 ±7.1	1.3 ±1.1	9.4 ±7.4
	3	97.8 ±1.5	1.28 ±0.02*	23.4 ±4.9	5.5 ±2.7	18.3 ±4.8
	6	98.5 ±1.4	1.27 ±0.05*	18.4 ±2.5	6.9 ±2.8	18.1 ±4.4
	12	97.3 ±1.3	1.24 ±0.03**	19.7 ±5.1	5.5 ±3.3	18.9 ±2.3

a. Cyclophosphamide (CP, 0.2 mg/mL) was used as positive control (PC). Water was used as negative control (NC). CBPI, cytokinesis-block proliferation index; BN, binucleated cell; MNs: micronucleus; NPBs, nucleoplasmic bridges (NPBs); NBUDs, nuclear buds.

b. All tests were repeated twice with duplicated for each repeat, values are presented as the mean±SD. Numerical values in the column are significantly different from the negative control group: * ($P < 0.05$) and ** ($P < 0.01$).

Table 4-3 Mutagenicity of fruit body extract from *C. nuda*.

Treatment (μg/plate)		*S. Typhimurium strains*											
		TA 98		TA 100		TA 1535		TA 1537		TA1538			
		-S9[a]	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		+S9	
		PI	4%	PI	4%	PI	4%	PI	4%	PI	SP	4%	10%
Positive controls[b]													
2-AAN	10	-	1237±279[c]	-	1867±103	-	1074±14	-	540±36	-	-	411±101	1834 ±453
9-AAC	50	-	-	-	-	-	-	2408±1112	-	-	-	-	-
NaN_3	5	-	-	1479±123	-	1163±159	-	-	-	-	-	-	-
4-NOP	10	424±67	-	-	-	-	-	-	-	595±85	377±55	-	-
Solvent/negative controls													
DMSO	-	15±3	21±3	87±11	90±18	13±2	14±3	10±6	10±3	6±3	11 ±2	14±5	13 ±3
H_2O	-	15±3	22±6	78±8	82±11	12±3	11±3	13±9	9±3	8±3	9 ±3	15±3	13 ±3
Samples													
Fruit body extract (freeze-dry)	5000	17±3	19±5	82±13	78±3	14±4	13±8	14±8	12±3	7±4	12 ±3	24±7	17 ±5
	2500	17±4	16±4	95±26	77±9	11±3	10±3	10±5	12±3	8±2	11 ±1	16±6	12 ±1
	1000	19±6	17±3	96±8	87±13	12±5	13±4	11±8	12±4	7±2	13 ±6	12±2	9 ±2
	500	14±3	17±3	96±31	74±8	11±3	12±7	10±7	12±4	6±3	8 ±1	14±4	10 ±4
	50	15±3	14±1	91±24	77±15	9±2	13±2	12±7	10±5	9±3	9 ±3	10±4	7 ±2

a. Tests were carried out in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system (rat liver S9 mix). For preincubation test without S9 (PI, -S9), all tests were repeated three times with triplicated plating for each test; for preincubation test with standard concentration of S9 (PI, +S9, 4%), all tests were repeated two times with triplicated plating for each test. PI: pre-incubation method; SP: standard plating method.

b. Positive control chemicals: 2-AAN, 2-Aminoanthracene; 9-AAC, 9-Aminoacridine; NaN_3, Sodium azide; 4-NOP, 4-Nitro-*orho*-phenylenediamine.

c. Data are presented as number of revertants (mean ±SD).

2. Toxicity of secondary metabolites from *C. nuda*

2.1 *Cytotoxicity and estimated acute oral toxicity*

The dose-response curves of BALB/c 3T3 and HepG2 cells using NRU and MTT assay after treated with the freeze-dried secondary metabolites crude extract from *C. nuda* (SMFD) are illustrated in Figure 4-4, and the heat-dried secondary metabolites crude extract (SMHD) curves are illustrated in Figure 4-5. Their interpolated IC_{50} values and estimated acute oral LD_{50} values are shown in Table 4-4. Moreover, the influences of drying methods (FD and HD), cytotoxicity endpoints (NRU and MTT) and cell lines (3T3 and HepG2) on the results of cytotoxicity are reflected in Figure 4-6. The results show that, both of the FD and HD secondary metabolites crude extracts can cause toxicity in a dose-dependent manner. However the IC_{50} of HD sample are significantly lower ($P < 0.01$) than FD sample in both of the two tested cell lines, which means the HD sample is more cytotoxic than FD sample. As for cell types, BALB/c 3T3 cells have lower ($p < 0.05$) IC_{50} value than hepG2 cells in both of the FD and HD samples. The two cytotoxic endpoints (NRU and MTT) are in agreement with each other if the sample and cells are the same with one exception of the HD sample in HepG2 cells. The estimated oral acute LD_{50} is 3,397 ±74 mg/kg and 1,772 ±45 mg/kg b.w. respectively for the FD and HD fruit body extracts. The former one is higher than the cut-off value of 2,000 mg/kg b.w. while the latter one is lower than that. Therefore, the freeze dried secondary metabolites crude extract is suitable to be categorized as non-toxic while more *in vivo* studies needed to be performed on the heat dried one to confirm the toxicity testing result.

2.2 *Genotoxicity in CBMN-cyt assay*

Cytostatic/cytotoxic and DNA damage effects of secondary metabolites crude extract from *C. nuda* in binucleated cytokinesis-blocked hepG2 cells are reflected in Table 4-5. The values of positive and negative controls are comparable to previous research (Chequer et al., 2012; Nikoloff et al., 2014). The percentage of binucleated cells, as indicated by CBPI value, did not change ($P > 0.05$) as the concentrations of extract increased from 0.75 to 6 mg/mL. A significant difference ($P < 0.01$) was observed for MNs in positive control cells when compared to negative control; however, there is no significant difference ($P < 0.05$) between the negative control and each of the extract sample treated cells. Additionally, no dose-response phenomenon was observed for MNs, NPBs, and NBUDs in cells treated with the secondary metabolites crude extract. Therefore, the secondary metabolites crude extract did not induce chromosome breaks and gain/loss in cultured human cells under the conditions in this research.

2.3 *Mutagenicity in bacteria reversion test*

The mutagenic effects of secondary metabolites crude extract from *C. nuda* (FD and HD) on five *S.* Typhimurium strains in the Ames assay are shown in Table 4-6. The results show that revertant numbers of all positive control chemicals (with or without S9) were significantly increased compared with negative or solvent controls and consistent with historical data in our lab and by other researchers (Kouvelis et al., 2011). However, no differences were observed ($P > 0.05$) between the numbers of revertants in fruit body extract treated samples and negative controls (H_2O), in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system. Additionally, no dose-response relationship of revertant numbers was observed within 50 to 5,000 μg/plate of fruit body extract. In addition, the potential influences of methods chosen (preincubation method and standard plating method) and S9 doses (high and low) were also investigated in TA1538 and no influences from these factors were observed. All these results

indicate that under the test conditions in this research, secondary metabolites crude extract of *C. nuda* is not mutagenic in the tested strains.

Furthermore, one of the pure secondary metabolite compounds, indole-3-carboldehyde, has also been tested in the Ames assay and the results are shown in Table 4-7. Also, there is no indication of mutagenicity observed. It should be mentioned that, indole-3-carboldehyde has very low solubility in water, therefore, it forms visible precipitate when the concentration reaches 1,000 μg/plate.

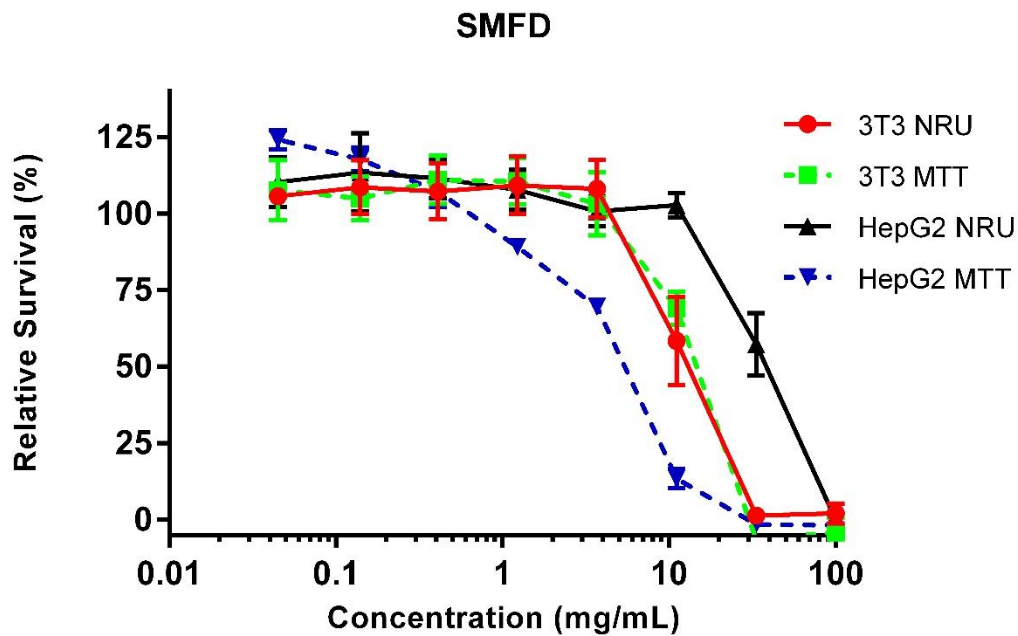


Figure 4-4 Cytotoxic effects of secondary metabolites crude extract (freeze-dry) from *C. nuda* in BALB/a 3T3 and HepG2 cells using NRU and MTT assay.

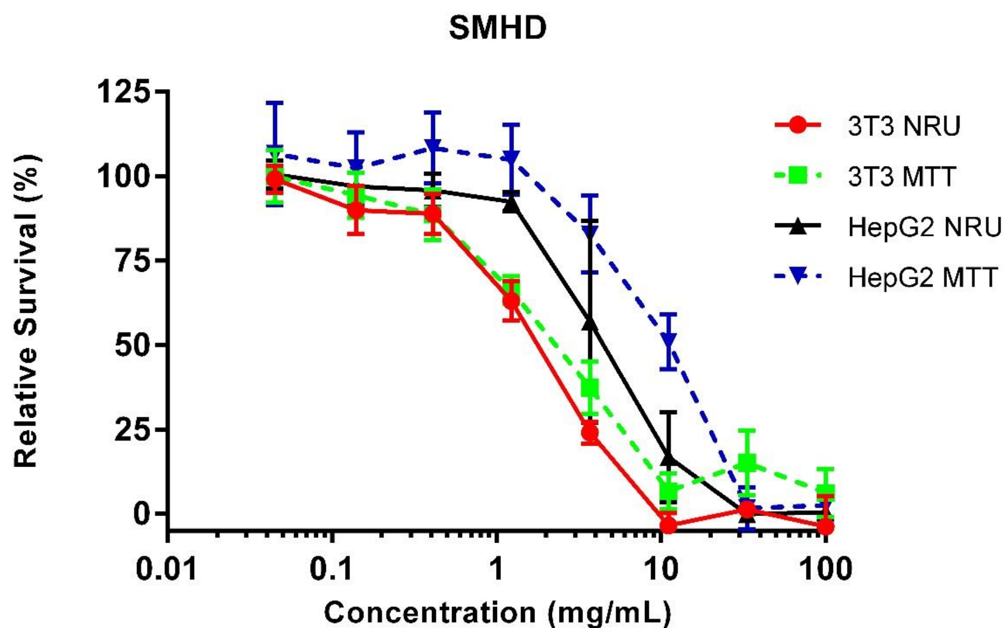


Figure 4-5 Cytotoxic effects of secondary metabolites crude extract (heat-dry) from *C. nuda* in BALB/a 3T3 and HepG2 cells using NRU and MTT assay.

Table 4-4 Cytotoxicity and estimated acute oral toxicity of secondary metabolites crude extract from *C. nuda.*

Fruit body extract	IC_{50} (mg/mL), 48 h exposure				Estimated acute oral LD_{50}* (mg/kg b.w.)
	BALB/c 3T3 cells		HepG2 cells		
	MTT	NRU	MTT	NRU	
Freeze-dry	12.9 ±2.17[a]	11.3 ±0.81[a]	49.6 ±1.33[c]	39.2 ±1.81[c]	3397 ±74
Heat-dry	1.98 ±0.27[b]	1.96 ±0.13[b]	9.80 ±1.10[a]	5.47 ±2.60[d]	1772 ±45

* Estimated acute oral LD_{50} was calculated based on IC_{50} (μg/mL) from 3T3 NRU assay using an established RC rat-only weight regression: $\log LD_{50}$ (mg/kg) = 0.372 × $\log IC_{50}$ (μg/mL) + 2.024 (ICCVAM, 2006).
[abcd] Numerical values in row and column are significantly different (P < 0.05) if superscript letters were different.

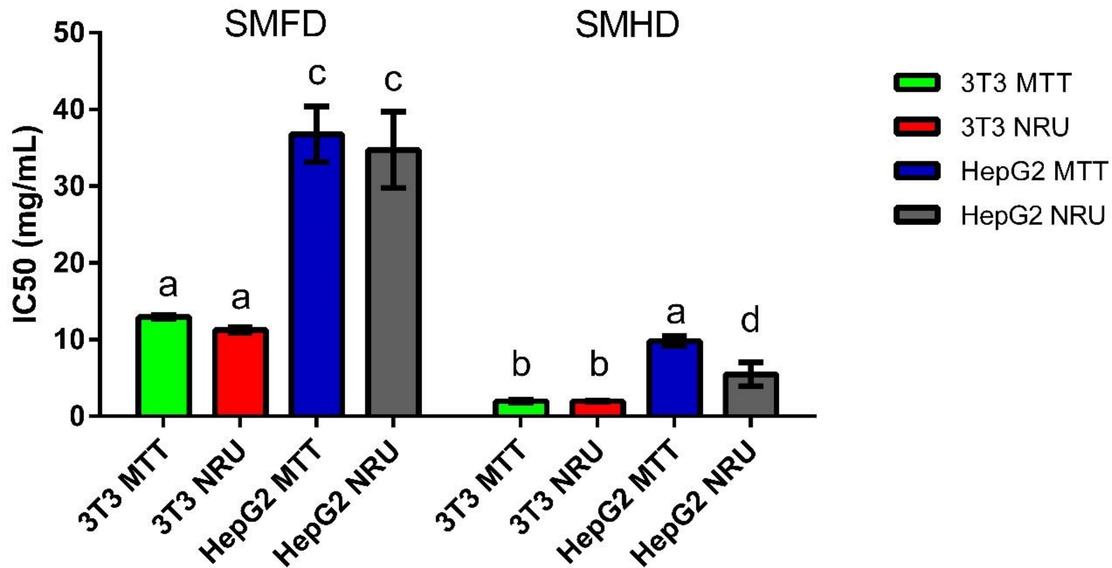


Figure 4-6 Influences of drying methods (FD and HD), cytotoxicity endpoints (NRU and MTT) and cell lines (3T3 and HepG2) on the evaluations of cytotoxicity of secondary metabolites extract from *C. nuda*. [abcd] Significant different (P < 0.05) between different bars if letters were different.

Table 4-5 Genotoxicity of secondary metabolites crude extract from *C. nuda*.

Sample [a]	Con. (mg/mL)	Cytostatic/cytotoxicity		DNA damage in 1000 BN cells		
		Viability (%)	CBPI	MNs	NPBs	NBUDs
NC	-	98.5 ±1.1[b]	1.32 ±0.03	13.0 ±2.0	1.3 ±1.4	6.6 ±3.9
PC	0.2	99.0 ±0.9	1.30 ±0.06	35.9 ±7.1**	7.0 ±3.5	8.0 ±2.6
FB	0.75	99.4 ±0.4	1.36 ±0.05	16.4 ±4.5	1.8 ±2.5	3.5 ±1.9
extract	1.5	98.8 ±0.9	1.33 ±0.06	15.9 ±3.5	3.0 ±2.9	5.3 ±4.7
	3	98.9 ±1.2	1.32 ±0.04	12.5 ±3.5	3.0 ±3.5	4.5 ±3.8
	6	98.2 ±0.9	1.27 ±0.04	15.6 ±4.4	2.8 ±2.9	5.9 ±4.9
	12	95.2 ±0.1*	1.13 ±0.03**	16.2 ±3.5	4.4 ±4.9	4.6 ±4.1

a. Cyclophosphamide (CP, 0.2 mg/mL) was used as positive control (PC). Water was used as negative control (NC). CBPI, cytokinesis-block proliferation index; BN, binucleated cell; MNs: micronucleus; NPBs, nucleoplasmic bridges (NPBs); NBUDs, nuclear buds.

b. All tests were repeated twice with duplicated cultures for each repeat, values are presented as the mean±SD. Numerical values in the column are significantly different from the negative control group: * ($P < 0.05$) and ** ($P < 0.01$).

Table 4-6 Mutagenicity of secondary metabolites crude extract from *C. nuda.*

Treatment (μg/plate)		*S.* Typhimurium strains											
		TA 98		TA 100		TA 1535		TA 1537		TA1538			
		-S9[a]	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		+S9	
		PI	4%	PI	4%	PI	4%	PI	4%	PI	SP	4%	10%
Positive controls[b]													
2-AAN	10	-	3146±1767[c]	-	2240±593	-	598±434	-	339±223	-	-	411±101	1834 ±453
9-AAC	50	-	-	-	-	-	-	2408±1112	-	-	-	-	-
NaN_3	5	-	-	1479±123	-	1163±159	-	-	-	-	-	-	-
4-NOP	10	424±67	-	-	-	-	-	-	-	595±85	377±55	-	-
Negative/solvent controls													
DMSO	-	15±3	21±3	87±11	90±18	13±2	14±3	10±6	14±3	6±3	11 ±2	14±5	13 ±3
H_2O	-	15±3	22±6	78±8	82±11	12±3	11±3	13±9	11±3	8±3	9 ±3	15±3	13 ±3
Samples													
Crude extracts (Freeze dry)	5000	17±3	18±3	111±29	79±8	13±5	14±5	11±7	14±3	8±4	8 ±4	14±4	8 ±2
	2500	18±5	16±3	88±16	80±13	12±6	10±6	10±5	8±4	6±2	10 ±4	11±3	11 ±1
	1000	13±3	16±5	95±22	77±6	12±4	9±4	13±8	10±3	7±2	13 ±2	11±5	9 ±3
	500	15±4	15±3	91±26	76±6	12±4	12±4	8±6	12±4	7±2	9 ±2	12±4	9 ±2
	50	13±4	16±4	90±12	82±11	11±3	11±5	10±9	14±4	8±4	10 ±5	13±1	11 ±2
Crude extracts (Heat dry)	5000	15±4	18±2	88±9	76±8	13±6	13±4	12±6	13±6	6±3	7 ±2	12±2	12 ±1
	2500	16±5	22±4	89±11	74±8	12±4	9±4	10±5	11±3	6±2	8 ±3	14±4	10 ±3
	1000	15±3	19±4	91±20	73±8	9±4	10±4	10±4	10±3	6±3	8 ±3	13±4	11 ±0
	500	15±3	18±3	91±10	74±10	12±4	10±5	8±5	13±2	8±5	11 ±2	9±3	10 ±2
	50	15±3	18±5	94±23	70±8	11±3	12±3	12±8	13±6	7±4	12 ±6	12±3	7 ±1

a. Tests were carried out in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system (rat liver S9 mix). PI: pre-incubation method; SP: standard plating method. Data are presented as number of revertants (mean ±SD).

b. Positive control chemicals: 2-AAN, 2-Aminoanthracene; 9-AAC, 9-Aminoacridine; NaN_3, Sodium azide; 4-NOP, 4-Nitro-*orho*-phenylenediamine.

c. For preincubation test without S9 (PI, -S9), all tests were repeated three times with triplicated plating for each test; for preincubation test with standard concentration of S9 (PI, +S9, 4%), all tests were repeated two times with triplicated plating for each test.

Table 4-7 Mutagenicity of indole-3-carbaldehyde from *C. nuda*.

Treatment (μg/plate)		*S.* Typhimurium strains											
		TA 98		TA 100		TA 1535		TA 1537		TA1538			
		-S9[a]	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		+S9	
		PI	4%	PI	4%	PI	4%	PI	4%	PI	SP	4%	10%
Positive controls[b]													
2-AAN	10	-	3146±1767[c]	-	2240±593	-	598±434	-	540±36	-	-	411±101	1834 ±453
9-AAC	50	-	-	-	-	-	-	2408±1112	-	-	-	-	-
NaN_3	5	-	-	1479±123	-	1163±159	-	-	-	-	-	-	-
4-NOP	10	424±67	-	-	-	-	-	-	-	595±85	377±55	-	-
Negative/solvent controls[d]													
DMSO	-	15±3	21±3	87±11	90±18	13±2	14±3	10±6	14±3	6±3	11 ±2	14±5	13 ±3
H_2O	-	15±3	22±6	78±8	82±11	12±3	11±3	13±9	11±3	8±3	9 ±3	15±3	13 ±3
Samples													
Indole-3-carbaldehyde	5000	13±3	16±2	70±10	58±13	14±4	12±5	7±3	14±4	7±4	n.t.	16±2	n.t.
	2500	12±3	16±2	75±11	64±9	11±3	15±2	10±5	15±4	6±2	n.t.	15±5	6 ±2
	1000	14±4	19±2	70±10	68±9	12±5	17±4	11±6	16±4	7±3	n.t.	13±3	7 ±5
	500	16±5	15±3	83±14	82±4	11±3	13±2	12±8	13±4	9±4	10 ±2	16±5	6 ±8
	50	13±3	17±2	83±13	86±16	9±2	12±5	11±6	14±4	8±3	8 ±3	15±2	8 ±2

a. Tests were carried out in the presence (+S9) or absence (-S9) of an external metabolizing enzyme system (rat liver S9 mix). For preincubation test without S9 (PI, -S9), all tests were repeated three times with triplicated plating for each test; for preincubation test with standard concentration of S9 (PI, +S9, 4%), all tests were repeated two times with triplicated plating for each test. PI: pre-incubation method; SP: standard plating method.

b. Positive control chemicals: 2-AAN, 2-Aminoanthracene; 9-AAC, 9-Aminoacridine; NaN_3, Sodium azide; 4-NOP, 4-Nitro-*orho*-phenylenediamine.

c. Data are presented as number of revertants (mean ±SD). n.t. (not tested).

d. Indole-3-carboldehyde is dissolved in dimethyl sulfoxide (DMSO).

CHAPTER 5: DISCUSSION

1. Rationality of using *in vitro* methods for toxicity assessment

Although *in vitro* approaches are still in development and cannot substitute the role of animal and human studies for valid risk assessment at this stage, they can uncover more mechanism-derived information which is considered pivotal for adequate risk assessment (Eisenbrand et al., 2002). It is the trend that consumers are curious to know more detailed safety information about all substances that are added into foods, therefore more toxicity studies will be needed. Different from toxicity assessment of drugs, the cost of toxicity testing is an important consideration for those substances that are intended to be added into foods, which is one of the important reasons that they were not subjected to toxicity testing before. Therefore, new testing systems with low cost, high throughput screening capacity and good predictability are needed for assessing the safety of these natural extracts that are intended to be added in food products, and *in vitro* methods can fulfill this trend. Although many of *in vitro* endpoints and models have been continually developed for general toxicity assessment in recent years, each of them has both advantages and disadvantages. A suitable testing system for natural food antimicrobials can be achieved through using current validated *in vitro* testing system and making some adaptions to compensate the shortcoming of each testing system. The primary toxicity concerns for *C. nuda* as natural antimicrobial which will be added into food are acute toxicity in the short term and carcinogenicity in the long term. Therefore, in this research, we introduced a combination of *in vitro* cytotoxicity and genotoxicity testing methods for toxicity assessment.

Cytoxicity has been considered as a good indicator to predict acute systemic toxicity because a good correlation between them has been validated in a wide range of chemicals in many studies (Clemedson et al., 2000). Among all these *in vitro* toxicity testing methods that have been proposed to previous validating studies, the 3T3 NRU assay has been shown to be one of the most promising methods based on predictability, complexity and cost. According to the recommendation from the Europe Union Reference Lab for Alternatives to Animal Testing (EURL ECVAM), "the 3T3 NRU test method shows a high sensitivity (ca 95%) when employed in conjunction with a prediction model to distinguish potentially toxic versus non-toxic substances" (ECVAM, 2013). Thus, the 3T3 NRU assay was chosen in this study to evaluate the basal cytotoxicity of mushroom fruit body extract and to estimate its acute oral toxicity effect. It has been concluded that "substances found to be negative in this test (3T3 NRU assay in combination with prediction model, author's note) would most likely not require classification for acute oral toxicity based on a cut-off value of $>$ 2,000 mg/kg b.w. and no additional animal test is needed " (ECVAM, 2013). This cut-off value (2,000 mg/kg b.w.) is based on the threshold of UN GHS (Globally Harmonized System of Classification and Labelling of Chemicals) acute toxicity hazard Category 4 and Category 5 (UN, 2011).

Genotoxicity testing has been a routine assay in many years for those substances to be used in foods, and some *in vitro* genotoxicity testing methods have already been established and validated (EFSA 2011). European Food Safety Authority (EFSA) scientific committee recommends to use a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test as the first step for genotoxicity testing. The scientific committee concluded that "these two tests are reliable for detection of most potential genotoxic substances and if all *in vitro* endpoints are clearly negative in adequately conducted tests, then it can be concluded with

reasonable certainty that the substance has no genotoxic potential" (EFSA 2011). Since in this research, both an Ames test and a micronucleus test showed clearly negative results, it can be concluded with reasonable certainty that the fruit body extract and secondary metabolites crude extract obtained from *C. nuda* through the freeze dry method have no genotoxic potential. In this study, besides MNs, the number of NPBs and NBUDs were also examined considering there are many compounds in a mixture. This can exclude potential genotoxic mechanism like gain or rearrangement of chromosome other than chromosome breakage (Fenech, 2007; Thomas et al., 2003).

2. Development of current *in vitro* methods

In this research, we also made several adaptions of current *in vitro* methods. First, the 3T3 NRU assay was complemented with MTT assay and HepG2 cells. Second, hepG2 cells was introduced for the MNvit assay and the procedure was simplified. There are some limitations of the 3T3 NRU assay which will affect its predictability. One limitation is that NR is a weak cationic dye which concentrates in lysosomes, which will generate bias in some situation where the cytotoxic target is not the lysosomes; therefore, another commonly used endpoint, MTT reduction based on NAD(P)H-dependent cellular oxidoreductase enzymes in live cells, was used as a complementary indicator. Another limitation is that BALB/c 3T3 cell is an animal cell and without an enzymatic metabolizing system, which cannot give enough information on human and the situation with metabolizing activation. Therefore, a human derived hepatoma cell line (hepG2) with phase I and II enzyme activities was applied to illuminate more information on human and the situation with metabolization. The influences of these two factors were well reflected from the results. However, the 3T3 NRU assay still gives the lowest IC_{50} values among all endpoints applied in this study, which is in good accordance with previous conclusions that

3T3 NRU assay was one of the most sensitive endpoints for predicting cytotoxicity (NIH, 2006). Therefore, the 3T3 NRU assay can be used as a primary endpoint for basal cytotoxicity and be used to predict the estimated acute oral toxicity. Additionally, based on the results from this research, no enhanced cytotoxic effect was observed from the fruit body extract after metabolized with a human hepatic cell metabolizing enzyme system.

This battery of tests is also suitable to analyze other extracts from botanical and animal origins. Even though positive results may be in any one of these tests and *in vivo* test are still needed, this battery of tests is still valuable. Because the estimated oral toxicity data can be used to calculate the starting dose for acute oral toxicity test using animals, which can reduce the number of animals to be used. Therefore, this battery of tests is a good start for testing the toxicology of extracts from nature that are intended to be used as food additives or ingredients. However, more validation tests should be performed between *in vitro* and *in vivo* toxicity studies to obtain more information for building up a database in the future to form a database suitable for extracts from different type of natural resources.

3. Toxicity of *C. nuda* as natural antimicrobials

According to previous research, both of fruit body extract and secondary metabolites are promising to be developed as novel antimicrobials. Therefore, in this study, both of them have been subjected to toxicity assessment. The primary toxicity concerns for substances that will be added into food are acute toxicity in the short term and carcinogenicity in the long term. As a result, a battery of cytotoxicity and genotoxicity tests are chosen. In addition, since natural antimicrobials are edible, and are unnecessary to be washed before cooking, therefore heat treatment on the toxicity of them is also investigated. Moreover, since antimicrobial will be intake by human, the influence of metabolizing are also investigated.

CHAPTER 6: CONCLUSIONS

1. 3T3 NRU assay showed better sensitivity and stability for testing cytotoxicity compared with hepG2 cell line and MTT endpoint.

2. For fruit body extract from *C. nuda*, heat or freeze drying method has no influence on the cytotoxicity of the extract. And the predicted acute oral toxicities (in rat) for freeze dry sample and heat dry sample are 2,167 $\pm$139 mg/kg b.w. and 2,610 $\pm$98 mg/kg b.w. respectively. Both of them are expected to be classified as non-acute-oral-toxic. The fruit body extract *C. nuda* prepared through freeze dry has no genotoxicity.

3. For secondary metabolites from *C. nuda*, heating may probably induce cytotoxicity and even acute-oral-toxicity of the crude extract. The predicted acute oral toxicity (in rat) for freeze dry is 3,397 $\pm$74 mg/kg b.w., which is expected to be classified as non-acute-oral toxic. However, the toxicity of heat dried extract is still not clear based on *in vitro* studies and more *in vivo* toxicity tests are need to be performed in order to draw valid conclusion. The freeze dried secondary metabolites crude extract is not genotoxic and heating will not induce mutagenicity. In addition, one of the most active antimicrobial secondary metabolites, indole-3-carboldehyde, is not mutagenic.

4. Human liver metabolizing system is not expected to induce either acute toxicity or genotoxicity of both fruit body extract and secondary metabolites crude extract from *C. nuda*.

5. The fruit body and secondary metabolites extracts from *C. nuda* may be suitable to be classified as GRAS substances and are expected to be used safely as natural antimicrobials with clean labeling.

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