ABSTRACT

Title of Thesis:	BIOACTIVITY OF EPHEDRA: INTEGRATING CYTOTOXICITY ASSESSMENT WITH REAL-TIME BIOSENSING
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Ephedrine-type alkaloids (ETA) are major active ingredients of Ephedra, a traditional Chinese medicinal herb used to treat asthma and nasal congestion. Until recently, large amounts of Ephedra were used in dietary supplements for weight loss and athletic performance enhancement. However, indiscriminate consumption of ETA-containing products has resulted in more than 1,000 reported cases of adverse effects. The objective of this study is to evaluate bioactivity of ETA. The toxicities of (-)-ephedrine and (+)-pseudoephedrine were measured using MTT assay on human neuroblastoma (SH-SY5Y) and rat myoblastoma (H9c2 (2-1)), while the stress responses of a panel of biosensing bioluminescent *Escherichia coli* strains were analyzed. SH-SY5Y showed similar sensitivity to (-)-ephedrine and (+)-pseudoephedrine, while H9c2 (2-1) could differentiate the cytotoxicity of (-)-ephedrine and (+)-pseudoephedrine. The biosensing of the *E. coli* strains was highly sensitive to the toxicity of ETA and could yield instantaneous response. The RLU ratios dependent on the construct of strains gave unique fingerprinting pattern of ETA.

BIOACTIVITY OF EPHEDRA: INTEGRATING CYTOTOXICITY ASSESSMENT WITH REAL-TIME BIOSENSING

By

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CHAPTER 1: INTRODUCTION

More than half of the U.S. adult population uses dietary supplement products, and consumers spend approximately $12 billion annually on dietary supplements, according to the 1998 Nutrition Business Journal Annual Industry Overview (Nutrition Business Journal, 1998). In the last decade, large amounts of ephedrine-type alkaloids (ETA) were used in numerous dietary supplements formulated for weight reduction and athletic performance enhancement (Dulloo and Stock, 1993). However, indiscriminate consumption of ETA-containing products has resulted in more than 1,000 reported cases of poisoning and other serious effects since 1993, some of which were fatal (FDA, 2000).

Used in the Far East to treat asthma, nose and lung congestion, and fever with anhidrosis (Lee *et al.*, 2000), Ephedra (ma huang), a traditional Chinese medicine (TCM) derived from *Ephedra sinica* Stapf and other Ephedra species, is the major sources of ETA. To date, the quality of Ephedra has been determined by the contents of total ETA, with higher contents indicating better quality. However, although individual ETA has similar pharmacological activity, they vary significantly in potency (Cetaruk and Aaron, 1994). Both the contents and the profile of ETA in Ephedra vary with plant species and varieties (Cui *et al.*, 1991), plant parts (Liu *et al.*, 1993), seasons of harvest (Kasahara *et al.*, 1986), and geographical origins (Zhang *et al.*, 1989). In addition to ETA, Ephedra also contains other phytoconstituents, which may modify its pharmacological and toxicological activities. Therefore, the toxicity of Ephedra cannot be totally accounted for by its ETA contents alone (FDA, 1997). A

bioassay is needed to determine the total toxicity of Ephedra due to the combined effect of the alkaloids and other constituents.

The overall objective of this study is to evaluate bioactivity of ephedrine-type alkaloids by measuring its cytotoxicity against selected cell lines and analyzing the stress responses of a panel of six genetically engineered biosensing strains capable of producing real-time responses to environmental stresses that cause specific cell damage. To accomplish these objectives, the study will be divided into two parts. The first part involves detecting and profiling the cytotoxicity of ephedrine-type alkaloids against the two cell lines —human neuroblastoma and rat myoblastoma—using MTT cell proliferation assay techniques. The second part involves a panel of six strains of bioluminescent *E. coli* to identify the stress fingerprints induced by ephedrine-type alkaloids.

CHAPTER 2: LITERATURE REVIEW

2.1 Ephedra

2.1.1 Botanical

Ephedra, also known as ma huang, belongs to the family Ephedraceae that is an evolutionarily primitive plant family (Blumenthal and King, 1995). Ephedra species favor dry, sandy or rocky environments, and are found in the temperate and subtropical regions of China, Mongolia, India, parts of the Mediterranean and Afghanistan as well as regions of North and Central America (Blumenthal and King, 1995). Although the genus Ephedra consists of more than 50 species (Schaneberg *et al.*, 2003), primary species of Ephedra are represented by *Ephedra sinica*, *E. major* Host, *E. gerardiana* Wall, *E. intermedia* Schrenk & Meyer, and *E. equisetina* Bunge (Morton, 1977).

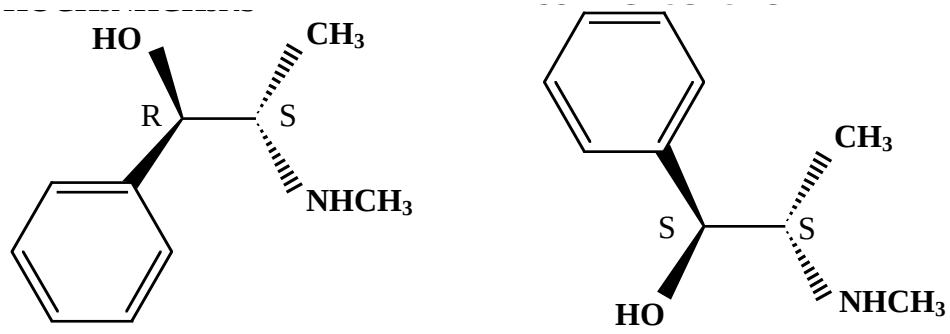
Ephedra species are short, evergreen and almost leafless shrubs that grow about 60 to 90 cm high (23.5 to 35.5 inches high). The stems are green in color, slender, erect or reclining, small ribbed and channeled, about 1.5 mm in diameter and usually terminating in a sharp point. Nodes are 4 to 6 cm apart, and small triangular leaves appear at the stem nodes (Blumenthal and King, 1995). The nodes are characteristically reddish brown. The stems usually branch form the base (Blumenthal and King, 1995). They bear minute, yellow-green flowers and fruits, and emit a strong pine-like odor and have an astringent taste (Blumenthal and King, 1995).

2.1.2. Chemistry

The major active ingredients of Ephedra are alkaloids that constitute 0.5 to 2.5 percent of the total mass, and are referred to as ephedrine-type alkaloids (Blumenthal and King, 1995). The six optically active alkaloids that have been isolated from Ephedra species are (-)-ephedrine, (+)-pseudoephedrine, (-)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (-)-norephedrine, (+)-norpseudoephedrine. Usually, (-)-ephedrine is the major isomer that comprises 30 to 90 percent of total alkaloid fraction accompanied by (+)-pseudoephedrine, with trace amount of other ephedrine-type alkaloids (Blumenthal and King, 1995). The total content of ephedrine-type alkaloids depends on the species of Ephedra, time of year of harvest, weather conditions, and altitude where the plant grows (Blumenthal and King, 1995), and can exceed 2% (Bruneton, 1995). This variation according to environmental conditions explains why some Ephedra containing dietary supplements of the same brand often show alkaloid content markedly differing from label claims and also variation among lots when analyzed chemically (Gurley *et. al.*, 2000).

Preparation of ephedrine-type alkaloids from crude plant material involves an acid/base extraction procedure (Reti, 1953). In addition to the extraction from plants, ephedrine-type alkaloids can also be chemically synthesized, and most of the ephedrine and pseudoephedrine used in western medicine has been manufactured synthetically. However, these synthetic ephedrine-type alkaloids differ from the natural forms in that they are racemic, i.e., optically inactive, because they are made up of two enantiomorphic isomers (Abourashed *et al.*, 2003).

In addition to the ephedrine-type alkaloids, other alkaloids and amino compounds have been isolated from different species of Ephedra. The macrocyclic spermine alkaloids, Ephedradines A-D, kynurenic acid derivatives, cyclopolyglycine, methanoproline amino acids, flavones, flavanols, tannins, carboxylic acids, volatile terpenes (Schaneberg *et al.*, 2003).



(-)-ephedrine **(+)-pseudoephedrine**

Figure 2-1. Chemical structures of (-)-ephedrine and (+)-pseudoephedrine (adapted from Abourashed *et al.*, 2003)

2.1.3. Pharmacology

Ephedrine is a sympathomimetic substance that stimulates both α- and β-adrenergic receptors (Abourashed *et al.*, 2003). Stimulation of α_1-adrenergic receptors produces contraction of vascular smooth muscle, increased contractile force of the heart and arrhythmias, glycogenolysis, gluconeogenesis, hyperpolarization, and

relaxation of intestinal smooth muscle. Stimulation of α_2-adrenergic receptors decreases insulin secretion, platelet aggregation and the release of norepinephrine from the nerve terminals, and causes contraction of vascular smooth muscle. Stimulation of β_1-adrenergic receptors increases force and rate of contraction of the heart, increased velocity of conduction through the atrioventricular node, and increased rennin secretion. When used in therapeutic doses, stimulation of β_2-adrenergic receptors causes relaxation of the smooth muscle of the blood vessels and bronchi (Mack, 1997). Stimulation of β-adrenergic receptors including β_3-subtype involve in lypolysis and non-shivering thermogenesis (Abourashed *et al*., 2003). In addition to its direct effects, ephedrine also displays indirect sympathetic activation releasing norepinephrine from sympathetic neurons (Abourashed *et al*., 2003). Ephedrine also has central nervous system (CNS) stimulant effects similar to those of amphetamines, but less pronounced (McEvoy, 2000).

2.2. Use of Ephedra

2.2.1. Traditional Use

The Ephedra species have been dispensed in traditional Chinese medicines (TCM) for at least 5,000 years (Morton, 1977). In traditional Chinese medicines, dried stems of Ephedra species are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hay fever. They were also used for a treatment of arthritis, fever, hives, lack of perspiration, headache, aching joints and bones, wheezing, and low blood pressure (Leung and Foster, 1996). The tissue used in TCM is the dried green stem of one of three Ephedra species (*Ephedra*

sinica, *E. equisetina* and *E. intermedia*), which are usually boiled in water and administered as a hot tea. In contrast to the diaphoretic uses properties of ma-huang (stem part), the root and rhizome of Ephedra species, called *mahuanggen*, have antiperspirant property and are employed to treat spontaneous and night sweating (Leung, 1990).

In Western medicine, ephedrine is used for the treatment of nasal congestion due to hay fever, allergic rhinitis, asthma, and common cold (WHO, 1999). Also, ephedrine salts are prescribed in the form of nasal sprays to relieve congestion and swelling. When injected subcutaneously, ephedrine prevents hypotension during anesthesia. Orally, it has been used in treating certain forms of epilepsy, nocturnal enuresis, myasthenia gravis, and urticaria accompanying angioneurotic edema. Pseudoephedrine, taken orally, is an effective nasal decongestant (Morton, 1977).

2.2.2. As Dietary Supplements

Approximately a decade ago, a new usage of Ephedra different from tradition directions had been widespread in the United States. Focusing on the thermogenic and lypolytic effects of Ephedra, dietary supplements containing Ephedra extracts have been commercially promoted and used as a mean of weight reduction and energy enhancement (Josefson, 1995). These dietary supplements are often combined with other botanical ingredients such as St John's wort and stimulants including guarana (caffeine source), carnitine, creatine (CANTOX, 2000).

However, since 1994, there has been increasing number of reports of adverse reactions associated with the use of Ephedra containing products to the U.S. Food and

Drug Administration (FDA). Reported reactions varied from the milder adverse effects such as nervousness, dizziness, tremor, headache, and gastrointestinal distress to chest pain, myocardial infarction, hepatitis, stroke and death. Of 140 reports submitted to the FDA between June 1997 and March 1999, 47% involved cardiovascular symptoms and 18% neurological symptoms. Severe hypertension was the single most frequent adverse effect followed by tachycardia, myocardial infarction, stroke, seizure. Ten events resulted in death and 13 produced permanent impairment (Haller and Benowitz, 2000).

2.3. Assessment Tools

2.3.1. Analytical Quantification

It has been reported that the type of alkaloid and the content of each alkaloid in dietary supplements vary from product to product (Gurley *et al.*, 2000). Several analytical methods have been established to determine and quantify the ephedrine-type alkaloids in Ephedra species and in dietary supplements containing Ephedra, including capillary electrophoresis (Liu *et al.*, 1992; Flurer *et al.*, 1995; Chinaka *et al.*, 2000), chiral gas chromatography (Betz *et al.*, 1997), gas chromatography with mass spectrometry (GC-MS) (Hansen, 2001), high performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Gurley *et al.*, 1998; Sheu and Huang, 2001) and with mass spectrometry detection (LC-MS) (Gay *et al.*, 2001) and proton nuclear magnetic resonance (NMR) spectroscopy (Hanna, 1995).

Among the analytical methods developed to date, HPLC methods are preferred because separation of all the six major bioactive components is achieved using HPLC (Sheu and Huang, 2000). Improved HPLC methods are available including different extraction methods (Hurlbut *et al.*, 1998; Ichikawa *et al.*, 2003), types of column (Sheu and Huang, 2000), and mobile-phase compositions (Sheu and Huang, 2000; Dong *et al.*, 2002).

2.3.2. *In Vitro* Cytotoxicity

In vitro cytotoxicity methods are important tools to enhance our understanding of hazardous effects caused by chemicals or bioactive components , which avoids using animals (Broadhead and Combes, 2001). *In vitro* cytotoxicity tests provide useful and necessary information in defining basal cytotoxicity, which is commonly used as a starting point in an integral assessment of potential *in vivo* toxicity of chemicals or active components in foods (Eisenbrand *et al.*, 2002).

The endpoints frequently used in cytotoxicity testing are based on the breakdown of the cellular permeability barrier, reduced mitochondrial function, changes in cell morphology, and changes in cell replication (Eisenbrand *et al.*, 2002). Several methods have been developed for measurement of cell proliferation; counting cells that exclude a dye (trypan blue), measuring released ^{51}Cr-labeled protein after cell lysis, measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation, and measuring colorimetric changes of tetrazolium salts in active cells (Barile, 1994). Among these methods, the colorimetric assay using tetrazolium salts are often employed as it does not involve

hazardous radio-active materials, and it is suitable for handling a large number of samples. (Lee *et al.*, 2000)

MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is reduced to yield a purple-colored water-insoluble formazan product (Altman, 1976). Since MTT is cleaved only by active mitochondria in living cells and not by dead cells or erythrocytes, MTT reduction is the most widely used method for measuring cell proliferation and viability (Mosmann, 1983; Hansen *et al.*, 1989). The formazan salt is produced in proportion to the active cell number, and accumulates within the cell since it is not membrane permeable. However when dimethyl sulfoxide (DMSO), isopropanol or other suitable solvent is added, formazan salt can be quantified calorimetrically. The MTT assay is simple and suitable for a wide variety of cell lines (Barile, 1994). However, the MTT assay requires monitoring as duration of MTT treatment, concentration of MTT used, and the number of test cells. These experimental conditions need to be taken into consideration when comparing inter-laboratory results (Lee *et al.*, 2000).

2.3.3. Biosensing Using Bioluminescent Reporter Bacteria

Bioluminescent bacteria containing *lux* reporter fusions have been extensively studied due to their potential as a sensing element in biosensors however they can respond selectively to analytes and/or environmental stresses. Their responses could then be converted into a signal (Daunert *et al.*, 2000; Köhler *et al.*, 2000).

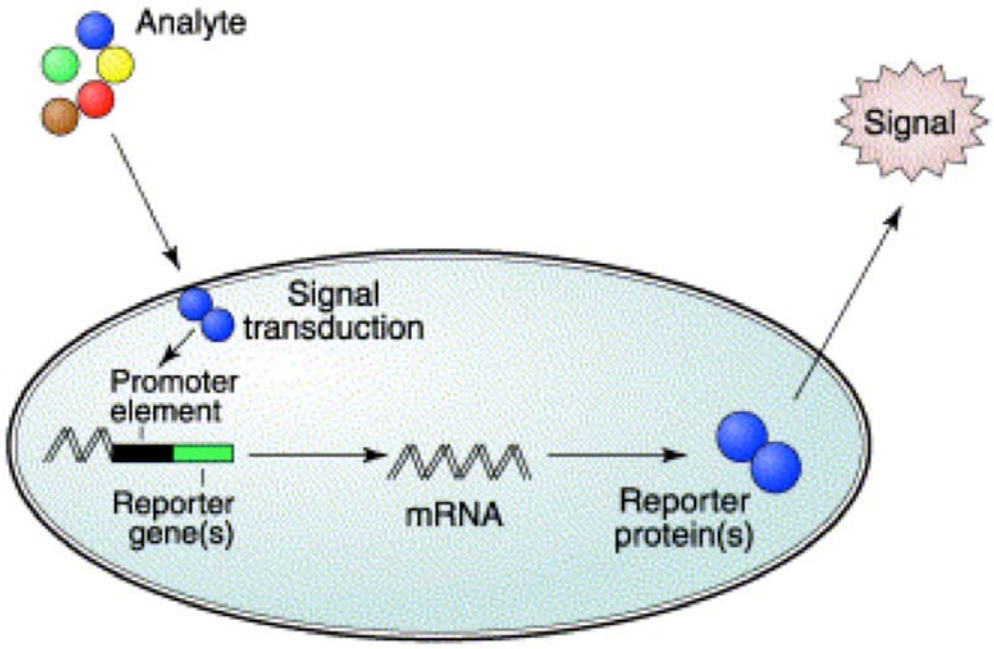


Figure 2-2. Principle of biosensing using bioluminescent reporter bacteria (adapted from Belkin, 2003)

Figure 2-2 summarizes the principle of bacterial biosensing. A molecular reporter such as *lux* and *β-gal* is fused to specific gene promoter, which is known to be activated by the specific chemicals or stress conditions (Belkin, 2003). Since bioluminescent bacteria containing *lux* reporter fusions are able to provide quantitative or semi-quantitative analytical information specific to analytes in a short period of time without additional processing steps, they have been widely used for monitoring of chemicals or hazardous substances in the environment instead of traditional methods based on chemical or physical analysis (Van Dyk *et al.*, 1994;

Belkin *et al.*, 1996, 1997; Vollmer *et al.*, 1997; Davidov *et al.*, 2000; Rosen *et al.*, 2000).

Figure 2-3 summarizes the mechanism of light emitting reaction controlled by each *lux* gene. The five structural genes encode luciferase (*luxAB*) and fatty acid reductase complex (*luxCDE*) catalyzing the biosynthesis of fatty aldehyde substrate. The bioluminescence reaction is a result of the oxidation of the fatty aldehyde and reduced flavin mononucleotide ($FMNH_2$) catalyzed by luciferase (*luxAB*) (Meighen, 1991).

In the genetically engineered bioluminescent bacteria, the *lux* reporter genes controlling luciferase are placed under the control of a promoter that is activated by the presence of specific chemicals and/or cellular activity. When bacteria are exposed to hazardous chemicals or other environmental stresses, the genetic control mechanism turns on the synthesis of luciferase, which produces a visible blue-green light emission that is easy to monitor and quantify. The *lux* reporter genes have advantages compared to *lacZ* genes and other reporter genes used in bacterial systems because the activity can be monitored in real time without cell lysis (Rozen *et al.*, 2001). Moreover, if the five-gene *luxCDABE* reporter is used, the activity of the reporter may be assayed directly without any additional substrate since all the requirements for bioluminescence are readily available in bacteria.

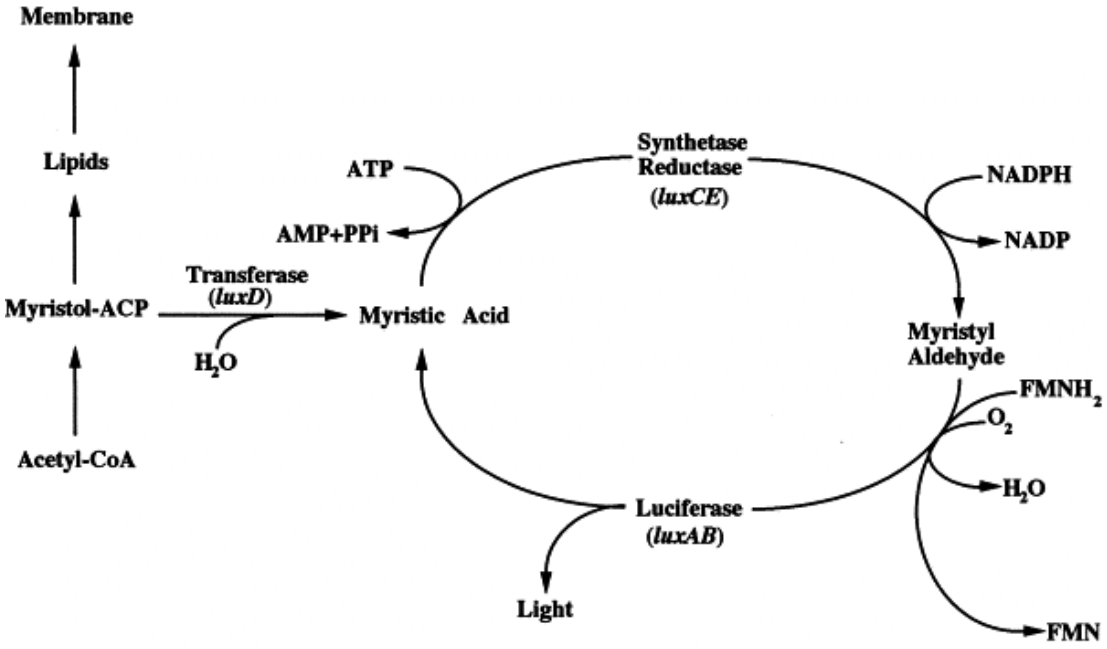


Figure 2-3. Bacterial bioluminescence pathway

(adapted from Heitzer *et al*., 1998)

Various bioluminescent strains have been constructed to enable screening for specific toxic mechanisms. However, it is impossible to cover all potential cellular stress factors with a single reporter gene. Therefore, a panel of genetically engineered strains should be used to increase their light production in response to a different type of stress such as oxidative stress (Belkin *et al.*, 1996), DNA damage (Vollmer *et al.*, 1997; Davidov *et al.*, 2000) and protein damage (Van Dyk *et al.*, 1994) caused by the presence of organic chemical pollutants such as naphthalene, toluene and isopropylbenzene. Choi and Gu (2002) developed a biosensor kit using four recombinant bioluminescent *E. coli* strains. This biosensor enables one to detect toxicity of different chemicals on-site by using freeze-dried *E. coli* cells which show an increased bioluminescence under specific stressful conditions (e.g. DNA damage, protein damage, membrane damage, and oxidative stress) (Choi and Gu, 2002).

The limitations of bioluminescent bacteria containing *lux* reporter fusions are that they may need a longer time to return to the baseline signal after use, and hence reversibility may be a problem. Also, the response tends to be slow relative to enzyme-based sensors since the substrate must first diffuse through the cell wall (D'Souza, 2001).

Bioluminescent bacteria containing *lux* reporter fusions have been mainly used for environmental monitoring: they are also applied for food bacterial detection in food, screening for bioactive component in food industry and food research, quality control, and detection of naturally occurring hazardous components. Vansal and Feller (1999) studied the direct effects of four different ephedrine isomers on human

β_1-, β_2- and β_3-adrenergic receptors in Chinese hamster ovary (CHO) cells transfected with a 6 CRE-LUC plasmid by measuring the light production.

CHAPTER 3: OBJECTIVES

The objective of this study was to evaluate bioactivity of ephedrine-type alkaloids by measuring its cytotoxicity against animal cell lines and analyzing the stress responses of a panel of genetically engineered biosensing bacterial strains capable of producing real-time responses to specific cell damages. This bioassay integrating cytotoxicity assessment with real-time biosensing is a systematic approach to screening Ephedra bioactivity, and the knowledge obtained from the damage caused by active components of Ephedra on the living cells will help to establish *in vitro* assessment tool, and to identify the damage mechanism of ephedrine-type alkaloids.

To accomplish these objectives, the experiments was divided into two parts; two cell lines, human neuroblastoma and rat myoblastoma, were used to detect and profile the cytotoxicity of major ephedrine-type alkaloids, ephedrine and pseudoephedrine, using a MTT cell proliferation assay. Secondly, six strains of bioluminescent *E. coli* were used to identify the stress fingerprints induced by the ephedrine-type alkaloids.

CHAPTER 4: MATERIALS AND METHODS

4.1. Cytotoxicity Assessment Using MTT Cell Proliferation Assay

4.1.1. Materials

Ephedrine-type alkaloids, (1R, 2S)-(-)-ephedrine (99%) and (1S, 2S)-(+)-pseudoephedrine (98%), were purchased from Aldrich (Allentown, PA). And the Minimum Essential Medium Alpha Medium and F-12 Nutrient Mixture (HAM) were from Invitrogen life technologies (Carlsbad, CA). Dulbecco's Modified Eagle's Medium (DMEM), MTT cell proliferation assay kit and other reagents for cell cultures were from the American Type Culture Collection (ATCC, Manassas, VA).

4.1.2. Cell Cultures

Human neuroblastoma cell line (SH-SY5Y) (ATCC #CRL-2266) and rat myoblast cell line (H9c2 (2-1)) (ATCC #CRL-1446) were purchased from ATCC. SH-SY5Y and H9c2 (2-1) were routinely maintained in 1:1 Minimum Essential Medium/F-12 Nutrient Mixture (HAM) and in Dulbecco's Modified Eagle's Medium (DMEM) respectively. Both culture media are supplemented with 10% fetal bovine serum. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C.

4.1.3. MTT Assays

MTT cell proliferation assay kit was used to determine the cytotoxicity of ephedrine-type alkaloids. Cells were harvested by centrifugation from the

maintenance cultures in the exponential phase, stained with trypan blue and counted by a hemocytometer. Cell suspensions (200 µl) were dispensed into 96-well flat-bottomed tissue culture plates at concentrations of 2 x 10^5 cells/ml for SH-SY5Y and 3 x 10^4 cells/ml for H9c2 (2-1). After a recovery period (72-hour for SH-SY5Y and 48-hour for H9c2 (2-1)), the media were removed from the wells with a hypodermic needle attached to a suction line, and then various concentrations of ephedrine-type alkaloids ranging from 0.1 to 1.0 mg/ml diluted in 200 µl growth media were added to each well. Control wells received only 200 µl growth media. Plates were incubated at 37°C in 5% CO_2 for 72 hours. The media was then aspirated from the wells with a hypodermic needle attached to a suction line, and 100 µl of fresh media added. Ten µl of MTT Reagent was added to each well, and the plates were incubated for 4 hours. One hundred µl of Detergent Reagent (sodium dodecyl sulfate) was added to each well, and the plates left at room temperature in the dark overnight. The absorbance of each well was read by the Opsys MR Microplate Reader (Thermo Labsystems, Chantilly, VA) at 570 nm with 690 nm as the reference wavelength.

4.1.4. Data Analysis

The relative viability of the treated cells as compared to the control cells was expressed as the % cytoviability, using the following formula:

% cytoviability = [A570 of treated cells] × 100% / [A570 of control cells].

The IC_{50} (median inhibition concentration) was determined by nonlinear regression analysis of the corresponding dose response curve utilizing the analytical software package GraphPad Prism Version 4 (GraphPad Software, San Diego, CA).

The results were presented as the mean ± standard error of the mean (S.E.M.) of four experiments, and Student's *t*-test was used to assess the significance of the data.

4.2. Real-Time Biosensing Using Bioluminescent E. coli Strains

4.2.1. Bioluminescent *E. coli* strains

Six bioluminescent *E. coli* strains obtained from DuPont Genetics Lab (DuPont Company, Wilmington, DE) was used in this study. Each strain contained different selected stress-responsive promoter fused to the *Photorhabdus luminescence luxCDABE* reporter. The panel strains were chosen to represent a range of stress responses to result in different patterns of induced gene expression. Each strain responds respectively to oxidative damage, internal acidification, DNA damage, protein damage, "super-stationary phase" and sigma S stress (Table 4-1) (Van Dyk, 1998). There are two sets of the stress-responsive *E. coli* strains, and one set introduces an outer membrane mutation, *tolC*, which enables highly sensitive detection of a variety of organic molecules because their ability to pump out undesired molecules is limited (Davidov *et al*., 2000). In this experiment, the set of six *tolC*⁻ strains was used.

Table 4-1. Stress-responsive *E. coli lux* fusion strains (adapted from Van Dyk, 1998)

Stress Response	Regulatory Circuit	Promoter Fused to *lux*	Strain Name	t*olC* allele	Plasmid-containing
DNA damage	SOS	*recA*	DPD1710	+	No
			DPD2222	-	No
"super-stationary phase"	?	*o513*	DPD2173	+	Yes
			DPD2232	-	Yes
sigma S stress response	Stationary phase (σ^S)	*yciG*	DPD2161	+	Yes
			DPD2233	-	Yes
protein damage	Heatshock (σ^{32})	*grpE*	DPD3084	+	No
			DPD2234	-	No
oxidative damage	OxyR & σ^S	*katG*	DPD2227	+	Yes
			DPD2238	-	Yes
internal acidification	Mar/sox/Rob	*inaA*	DPD2226	+	Yes
			DPD2240	-	Yes

The *E. coli* strains were maintained in a 70% glycerol suspension at -78°C. Prior to the assay, the stock cultures were transferred to 250 ml flasks with 50 ml sterilized Luria Bertani (LB) medium and incubated for 12 hours at 37°C on a model 1575 orbital shaking incubator (VWR Scientific, Cornelius, OR) at 300 rpm. To ensure stability of the plasmids containing *lux* fusion genes, ampicillin (100 μg/ml) was added in the growth media of all the strains except for strains DPD2222 and DPD2234, which have the *lux* gene fusion in their chromosomes.

4.2.2. *E. coli* stress fingerprinting

Cultures of each *E. coli* strain were diluted with sterile distilled water at a ratio of 1:10. Nine hundred μl of culture solution was placed in a transparent glass cuvette. Luminescence from *E. coli* of each cuvette was measured by a TD-20e luminometer (Turner Designs, Sunnyvale, CA) and the luminescence values were presented as instrument's arbitrary relative light unit (RLU). The RLU value was recorded again after 100 μl of ephedrine-type alkaloids. The difference and ratio of the two RLU values ("before RLU" and "after RLU") were both calculated to indicate the stress responses from the bioluminescent *E. coli* strains.

ΔRLU = "after RLU" – "before RLU"

RLU ratio = "after RLU"/"before RLU"

If the RLU rations are greater than 1.0 ("lights-on"), it indicates that the *lux* gene fusion is expressed because of the ephedrine-type alkaloids. If the RLU rations

are less than 1.0 (“lights-off”), it suggests a dampening of bioluminescent *E. coli* strains in the presence of ephedrine-type alkaloids.

4.2.3. Data Analysis

Data are presented as mean ± the standard errors of the mean (S. E. M.). Statistical analysis was performed using either Student’s *t*-test or analysis of variance (ANOVA) utilizing the analytical software package GraphPad Prism Version 4 (GraphPad Software, San Diego, CA). For ANOVA, pairwise comparisons between treatments were made using Tukey’s Multiple Test Comparison.

CHAPTER 5: RESULTS AND DISCUSSION

5.1. Cytotoxicity Assessment Using MTT Cell Proliferation Assay

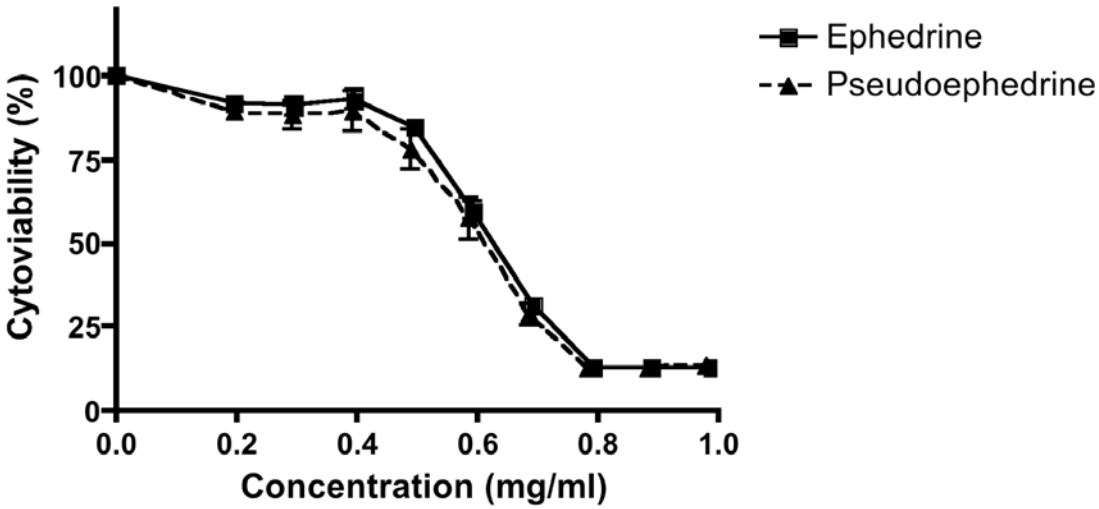
5.1.1. Effects of Ephedrine and Pseudoephedrine on Cytoviability

Cytotoxicity against two different cell lines was evaluated by determining the IC_{50} values of (-)-ephedrine and (+)-pseudoephedrine. The cytoviability of SH-SY5Y and H9c2 (2-1) cells in the presence of ephedrine-type alkaloids is given in Figure 5-1. In each case, the percentage of viable cells decreased significantly in a dose-dependent manner.

For each replicate, the concentration resulting in 50% inhibition (IC_{50}) was determined using nonlinear regression analysis. Figure 5-2 shows an example dose-response curve obtained from MTT cytotoxicity assessment of (-)-ephedrine using SH-SY5Y. Percentage cytoviability ([absorbance of test cells/absorbance of control wells] × 100) was plotted against the log concentration of (-)-ephedrine.

The IC_{50} values for (-)-ephedrine and (+)-pseudoephedrine on SH-SY5Y and H9c2 (2-1) are presented in Table 5.1. Both (-)-ephedrine and (+)-pseudoephedrine were inhibitory to cell growth of SH-SY5Y and H9c2 (2-1). For SH-SY5Y, the IC_{50} value of (-)-ephedrine was 0.619 ± 0.004 mg/ml and (+)-pseudoephedrine was 0.605 ± 0.011 mg/ml. These values were not significantly different. For H9c2 (2-1), the IC_{50} value of (-)-ephedrine was 0.617 ± 0.005 mg/ml and (+)-pseudoephedrine was 0.666 ± 0.012 mg/ml. A Student's *t*-test showed that the IC_{50} value of (-)-ephedrine to H9c2 (2-1) was significantly lower than that of (+)-pseudoephedrine ($p<0.01$). The

a) SH-SY5Y



b) H9c2 (2-1)

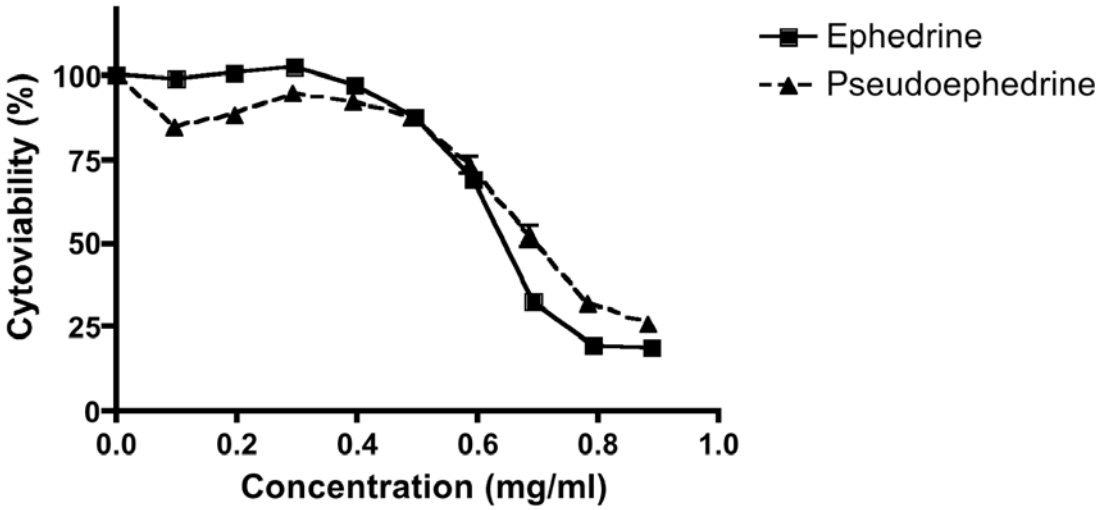


Figure 5-1. Effects of ephedrine-type alkaloids on SH-SY5Y and H9c2 (2-1) cell viability. Values (means ± S.E.M.) represent percent cytoviability relative to control; n=4.

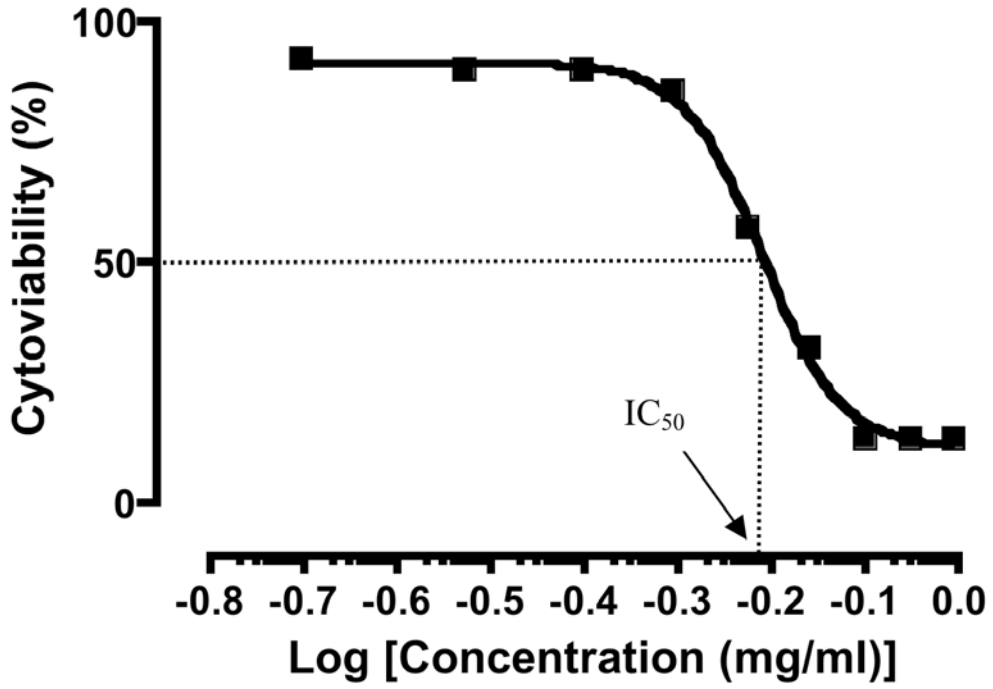


Figure 5-2. Sample dose-response curve. Percentage cytoviability ([absorbance of treated cells]/[absorbance of control cells] × 100) plotted against the concentration of ephedrine-type alkaloids.

results indicate that H9c2 (2-1) could differentiate the cytotoxicity of (-)-ephedrine and (+)-pseudoephedrine while SH-SY5Y showed similar sensitivity to both ephedrine-type alkaloids. Figure 5-3 shows morphological changes of H9c2 (2-1) cell line when it was treated with (-)-ephedrine for 72 hours. As the concentration of (-)-ephedrine increases, swelling of the cells became prominent and vacuolar degeneration was observed microscopically.

5.1.2. Effects of Combined Treatments by Ephedrine and Pseudoephedrine

The cumulative effects of (-)-ephedrine and (+)-pseudoephedrine on the cytoviability of SH-SY5Y and H9c2 (2-1) cells were illustrated in Figure 5-4. At low concentrations of (-)-ephedrine (0.2 mg/ml) and (+)-pseudoephedrine (0.2 mg/ml), the cytoviability of SH-SY5Y cells were suppressed 11 and 12%, respectively. The combined treatment suppressed cytoviability 18%, slightly lowering the 23% predicted by the sum of the individual suppression. Other combination of (-)-ephedrine (0.4 mg/ml) and (+)-pseudoephedrine (0.2 mg/ml) suppressed the cytoviability to the extent predicted by the individual effects while the combination of (-)-ephedrine (0.2 mg/ml) and (+)-pseudoephedrine (0.4 mg/ml) suppressed the cytoviability to the larger extent than additive effect. For H9c2 (2-1), on the other hand, all the combinations of (-)-ephedrine and (+)-pseudoephedrine showed lesser suppression of cytoviability than predicted value from the individual effects.

Table 5-1. The IC_{50} values of (-)-ephedrine and (+)-pseudoephedrine for two cell lines.

	IC_{50} (mg/ml)	
Cell Lines	ephedrine	pseudoephedrine
SH-SY5Y	0.619 ± 0.004[a]	0.605 ± 0.011[a]
H9c2 (2-1)	0.617 ± 0.005[a]	0.666 ± 0.012[b]

Each value is the mean ± S.E.M., n=4.
IC_{50}: concentration of test sample to inhibit cytoviability by 50%
Values followed with an identical letter are not significantly different (p<0.01).

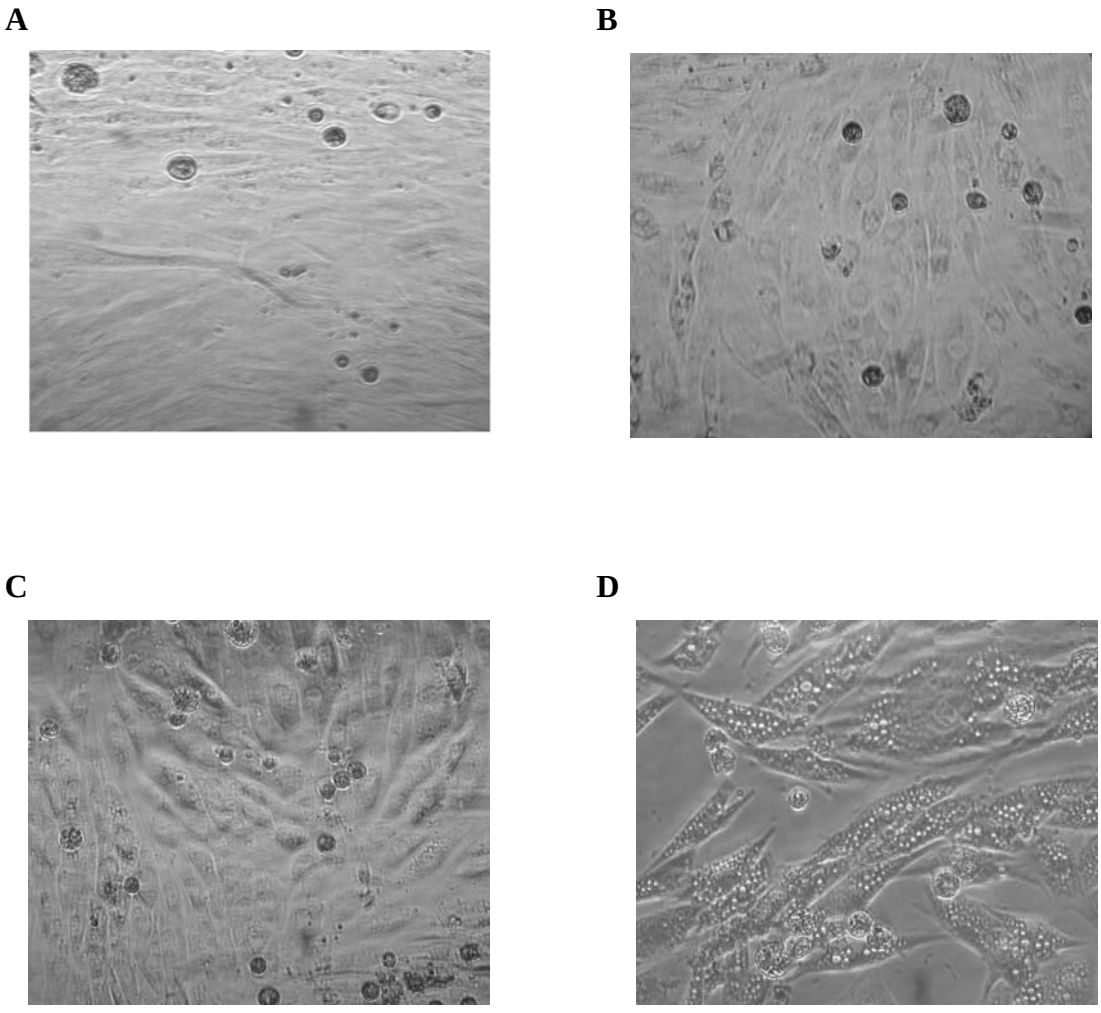
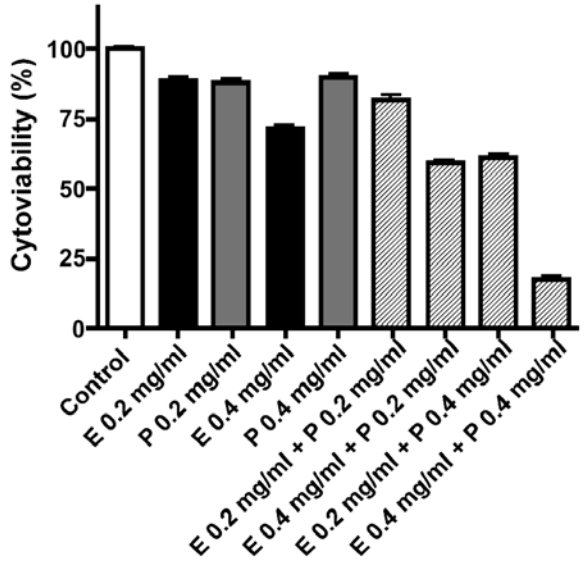


Figure 5-3. H9c2 (2-1) cells treated with (-)-ephedrine. ×200 magnifications. A: control; B, C, and D: cells treated with (-)-ephedrine 0.2 mg/ml, 0.4 mg/ml, and 0.6 mg/ml respectively.

a) SH-SY5Y



b) H9c2 (2-1)

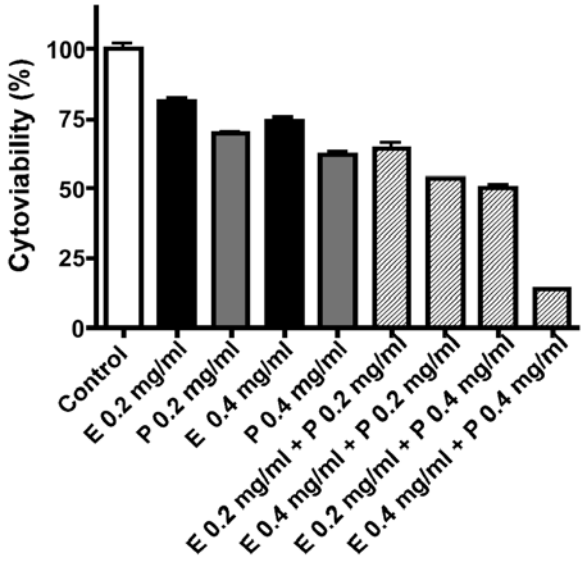


Figure 5-4. Cumulative effects of two ephedrine-type alkaloids on the cytoviability of SH-SY5Y and H92c (2-1). Shown are mean ± S. E. M. for n=6. E: (-)-ephedrine, P: (+)-pseudoephedrine.

5.1.3. Discussion

Among the adverse events potentially associated with dietary supplements containing ephedrine-type alkaloids reported to FDA from 1997 through 1999, approximately 60% of the total reports were characterized as clinically serious such as deaths, cardiovascular events, and serious nervous system effects. Serious nervous system effects including seizures, psychosis and depression accounted for 16% of the total adverse events (FDA, 2000). Lee *et al.* (2000) assayed the cytotoxicity of pure ephedrine and extracts of Ephedra using a mouse neuroblastoma cell line (Neuro-2a) and reported that Neuro-2a was more sensitive to Ephedra extracts compared to other cell lines including human hepatoblastoma (HepG2). In this study, a human neuroblastoma cell line SH-SY5Y, which is frequently used in the assessments of cytotoxicity or protective effects for other chemicals (Slaughter *et al.*, 2002; Legros *et al.*, 2004; Miglio *et al.*, 2004), was employed instead of mouse neuroblastoma cell line, to investigate whether it would show similar sensitivity to ephedrine-type alkaloids.

Serious cardiovascular events that included myocardial infraction (MI), unstable angina, dysrhythmias and transient ischemic attacks, accounted for 31% of reported adverse events (FDA, 2000). Therefore, rat myocardium cell line (H9c2 (2-1)) was used in this MTT cytotoxicity assessment in addition to SH-SY5Y. The H9c2 (2-1) cell line is a permanent cell line derived from rat heart tissue, that shows morphological characteristics similar to those of immature embryonic cardiocytes and has preserved several elements of the electrical and hormonal signaling pathways

found in adult rat cardiac myocytes (Hescheler *et al.*, 1991). Also, it has been reported that H9c2 (2-1) expresses both β_1- and β_2-adrenergic receptors on which ephedrine acts (Dangel *et al.*, 1996). Therefore, H9c2 (2-1) cell line was expected to be a useful model for cytotoxicity assessment of ephedrine-type alkaloids.

With regard to the cytotoxicity of (-)-ephedrine, the IC_{50} values achieved from the current study (0.615 ~ 0.616 mg/ml) were close to the results previously reported (Lee *et al.*, 2000). However, Lee *et al.* also reported that the cytotoxicity of (-)-ephedrine was significantly higher than that of (+)-pseudoephedrine when they determined the % cytoviability of equimolar concentrations (3.0 mM = approximately 0.50 mg/ml) of (-)-ephedrine and (+)-pseudoephedrine for HepG2 cell line (30.8% for (-)-ephedrine and 89.4% for (+)-pseudoephedrine). In the current study, the % cytoviability of 0.50 mg/ml of (-)-ephedrine and (+)-pseudoephedrine was 83.7% and 76.5% for SH-SY5Y and 91.4% and 86.1% for H9c2 (2-1) respectively, and Student's *t*-test showed no significant difference in either cell line at this level of concentration. The same can be said for the IC_{50} values. There was no significant difference between (-)-ephedrine and (+)-pseudoephedrine for SH-SY5Y. Although (-)-ephedrine is considered to be more potent and toxic than (+)-pseudoephedrine clinically (Tang, 1996), it is possible that the sensitivity to (-)-ephedrine and (+)-pseudoephedrine is different from cell line to cell line, and it may not be necessarily appropriate to suggest that (-)-ephedrine is more cytotoxic than (+)-pseudoephedrine. For H9c2 (2-1), (-)-ephedrine was proved to be more cytotoxic but the difference was less prominent.

Although there is a little data on blood and tissue levels of (-)-ephedrine and other ephedrine-type alkaloids in humans, Haller *et al.* (2002) reported that maximum plasma concentrations were 63.5 ng/ml for ephedrine and 24.1 ng/ml for pseudoephedrine 2.4 hours after the ingestion of dietary supplements containing ephedrine-type alkaloids (17.3 mg ephedrine, 5.3 mg pseudoephedrine and insignificant amounts of the other alkaloids on an average). Other studies also showed that the therapeutic plasma levels of ephedrine in humans following ingestion of ephedrine in the form *Ephedra sinica* capsules (19 mg ephedrine), an ephedrine tablet (20 mg ephedrine), or an ephedrine solution (22 mg) are 81 ng/ml, 74 ng/ml, and 79 ng/ml respectively (Vansal and Feller, 1999). The IC_{50} values achieved from the present study were 0.605 ~ 0.666 mg/ml, which were several orders of magnitude higher than the therapeutic plasma levels. Therefore, it is difficult to directly associate the *in vitro* cytotoxic effects with the actual adverse responses of human.

Ephedrine is both a direct and indirect adrenergic agonist (Abourashed *et al.*, 2003). That is, ephedrine activates adrenergic receptors both by direct agonist activity as well as by releasing norepinephrine via carrier-mediated exchange mechanism. Ephedrine possesses two asymmetrical carbon atoms and exists as four isomers. Among these four ephedrine isomers, (-)-ephedrine and (+)- pseudoephedrine are naturally contained in some of the Ephedra species (Vansal and Feller, 1999). Vansal and Feller (1999) reported (-)-ephedrine was the most potent of the four ephedrine isomers on human β-adrenergic receptors expressed in Chinese hamster ovary cells. However, Rothman *et al.* (2003) reported that ephedrine-type alkaloids showed no agonist effects at α_1- and β-adrenergic receptors and they suggested that

pharmacological actions of ephedrine and its derivatives resulted primarily from release of norepinephrine rather than direct activation of adrenergic receptors. Although the cell lines employed in this study are reported to express several subtypes of the adrenergic receptors (Dangel *et al.*, 1996), it is not known if their response resulted from the direct effects on a specific adrenergic receptor or from indirect effects. However, the MTT assay using these cell lines may serve as an efficient model to detect general cytotoxicity of compounds included in dietary supplements.

Although little is known about the cytotoxicity of other ephedrine-type alkaloids and compounds in *Ephedra*, norpseudoephedrine is known to be more potent than (-)-ephedrine with regard to CNS stimulation (Kalix, 1991). Moreover, the presence of toxins in Ephedra other than (-)-ephedrine is suggested since IC_{50} values of Ephedra extracts normalized by their (-)-ephedrine contents were significantly lower than pure (-)-ephedrine (Lee *et al*., 2000). Dietary supplements containing ephedrine-type alkaloids often include other agents including stimulants, diuretics and cathartics (CANTOX, 2000). The synergistic interaction between ephedrine-type alkaloids and caffeine is well known (Haller *et al.*, 2004). Therefore, it may also be useful to evaluate the interaction between ephedrine-type alkaloids and other functional compounds using this MTT cytotoxicity assessment.

In response to the FDA's decision to ban the sales of Ephedra-containing dietary supplements, manufacturers and companies have developed new "Ephedra-free" dietary supplements (Marcus and Grollman, 2003). One of the most popular substitutes for Ephedra is *Citrus aurantium* (bitter orange). Its major active

component “synephrine” is an ephedrine-type alkaloid and the combination of synephrine and caffeine has the similar potential to induce cardiac arrhythmias, hypertension, heart attacks, and strokes as the combination of Ephedra and caffeine (Marcus and Grollman, 2003). The association of dietary supplements containing bitter oranges with myocardial infarction has been reported (Nykamp *et al.*, 2004). Therefore, there is a concern that the misuse of “emerging” dietary supplements containing botanical substances might cause other health problems. The MTT cytotoxicity assay used in the present study could serve as a useful assessment tool to analyze the cytotoxic pattern of other botanical substances contained in dietary supplements and to predict potential adverse effects.

5.2. Real-Time Biosensing Using Bioluminescent E. coli Strains

The RLU ratio of the *E. coli* strains exposed to (-)-ephedrine and (+)-pseudoephedrine at differing concentrations (0.03 mg/ml to 0.06mg/ml) is summarized in Figures 5-5 - 5-8. All strains showed increased bioluminescence in response to the stress caused by (-)-ephedrine and (+)-pseudoephedrine at the concentrations as low as 0.03 mg/ml. This concentration was much lower than the ID_{50} (0.605 ~ 0.666 mg/ml) obtained from the cytotoxicity assessment using human and rat cell lines.

The RLU ratio of a panel of bioluminescent *E. coli* strains exposed to (-)-ephedrine and (+)-pseudoephedrine at a concentration of 0.3 mg/ml is presented in Figure 5-9. Both (-)-ephedrine and (+)-pseudoephedrine decreased the RLU ratios and was toxic to all strains. The RLU ratios in four strains (DPD2232, DPD2233, DPD2238 and

DPD2240) for (-)-ephedrine were less than 1.0 (“Lights-off” response) which could be attributed to inhibition of cellular metabolism required for production of energy or reduction power (Chatterjee and Meighen, 1993), and the RLU ratios were significantly lower than those of (+)-pseudoephedrine, whereas there was no significant difference in strains DPD2222 and DPD2234. These results indicate that this biosensing panel was sensitive to the bioactive effects caused by ephedrine-type alkaloids and it distinguished (-)-ephedrine and (+)-pseudoephedrine. The strains DPD2222 and DPD2234 showed less intense response to (-)-ephedrine compared to other 4 strains. A possible reason for this is that the host strain of DPD2222 and DPD2234 is MM28, which is different from the parental strain of other 4 strains (GC4468). Moreover, DPD2222 and DPD2234 have their *lux*-fusions in their chromosome while the other strains have plasmids containing *lux*-fusions. These might affect the sensitivity to detect the toxicity of ephedrine-type alkaloids in short time.

At a concentration of 0.03 mg/ml, (-)-ephedrine induced increased bioluminescence in DPD2222, DPD2232 and DPD2234. In contrast, (+)-pseudoephedrine induced bioluminescence in DPD2222, DPD2232 and DPD2233. Strains DPD2238 and DPD2240 showed no significant response to either (-)-ephedrine or (+)-pseudoephedrine. Also, RLU ratios of (-)-ephedrine and (+)-pseudoephedrine were not significantly different in all strains.

At a concentration of 0.04 mg/ml, (-)-ephedrine induced increased bioluminescence in DPD2232, DPD2233 and DPD2234, and the RLU ratio of DPD2233 was significantly higher than (+)-pseudoephedrine. In contrast, (+)-

pseudoephedrine only increased the RLU ratio in strain DPD2232. Strains DPD2222, DPD2238 and DPD2240 showed no significant responses either to (-)-ephedrine or (+)-pseudoephedrine.

At the concentration of 0.05 mg/ml, (-)-ephedrine induced increased bioluminescence in DPD2222, DPD2232 and DPD2233 compared to the control although they were not significantly different from those of (+)-pseudoephedrine. On the other hand, (+)-pseudoephedrine also increased the RLU ratio of strains DPD2222 and DPD2232. Strains DPD2234, DPD2238 and DPD2240 showed no significant responses either to (-)-ephedrine or (+)-pseudoephedrine.

At a concentration of 0.06 mg/ml, (-)-ephedrine induced bioluminescence in DPD2222, DPD2232, DPD2233 and DPD2240 compared to the control although they were not significantly different from those of (+)-pseudoephedrine. (+)-Pseudoephedrine also increased the RLU ratio in strains DPD2222 and DPD2232. Strains DPD2234 and DPD2238 showed no significant responses either to (-)-ephedrine or (+)-pseudoephedrine.

Strain DPD2233 showed increased response to (-)-ephedrine in the concentration range of 0.04 mg/ml ~ 0.06 mg/ml. Strain DPD2233 is constructed to contain the plasmid in which the *E. coli yciG* promoter is fused to *luxCDABE* genes. As expression of *yciG* gene is under control of the stationary phase sigma factor σ^s, the *yciG-lux* fusion is expected to report on the activation of the σ^s-dependent stress response (Van Dyk, 1998).

In the present study, (-)-ephedrine slightly increased the bioluminescence of strain DPD2234 at the lower concentration (0.03 mg/ml and 0.04 mg/ml), but showed

no significant increase of RLU ratio when exposed to (+)-pseudoephedrine. *Escherichia coli* strain DPD2234 contains chromosomal insertion of a *grpE* promoter fused to the *P. luminescens luxCDABE*. Since *grpE* gene is in the heat shock regulon controlled by σ^{32}, the *grpE-lux* fusion responds to stresses that induce this protein-damage responsive regulon (Van Dyk, 1998), and it is also known to respond to a variety of stresses and chemicals (Van Dyk *et al*., 1995). Increased RLU ratio of the strain DPD2234 indicated the induction of the general stress-response by (-)-ephedrine.

Both (-)-ephedrine and (+)-pseudoephedrine appeared to increase the RLU ratio of strain DPD2222, which is designed to respond to DNA damage through SOS regulatory circuit. The increased RLU ratio of DPD2222 indicated the possibility that DNA damage-sensing SOS response was induced by both (-)-ephedrine and (+)-pseudoephedrine. Strain DPD2222 is the one that contains chromosomal insertion of *E. coli recA* promoter fused to the *P. luminescens luxCDABE*. When DNA damage is present, a resultant single stranded DNA acts as a signal for induction of the SOS response (Daunert *et al*., 2000). According to the studies on the genotoxicity of ephedrine and Ephedra extract, both *in vitro* and *in vivo,* ephedrine sulfate had no genotoxicity in three strains of *Salmonella typhimurium* (TA97, TA98, TA100 and TA1535), nor in cultured Chinese hamster ovary (CHO) cells (NTP, 1986). Hillard *et al*. (1998) reported that ephedrine sulfate was negative in two chromosome aberration tests using CHO cells. These studies demonstrated that ephedrine and Ephedra are not genotoxic. Although SOS activation in itself does not imply genotoxicity, the two activities are reported to be correlated (Davidov *et al.*, 2000). Further information is

thus needed to determine whether mechanisms other than DNA damages are involved in the stress response obtained in the present study, or ephedrine-type alkaloids cause DNA damage but not mutation.

Strain DPD2238 showed no significant increase of RLU ratio when exposed to either (-)-ephedrine or (+)-pseudoephedrine in different concentrations. The strain DPD2238 is constructed to contain the plasmid in which the *E. coli katG* (catalase) promoter is fused to *luxCDABE* genes. An *E. coli* strain harboring this plasmid is known to exhibit low basal levels of luminescence, which increased up to 1,000-fold in the presence of oxidative stress such as hydrogen peroxide, organic peroxides, alcohols and cigarette smoke (Belkin *et al*., 1996). Kang *et al.* (1998) reported that ephedrine exerted mild antioxidant activity *in vitro.* The result from the present study that oxidative stress response was absent supports their observations. If direct correlations could be established between antioxidant activity and oxidative stress response, strain DPD2238 could be used as a tool to assay antioxidant property of ephedrine-type alkaloids.

Strain DPD2232 is constructed to contain the plasmid in which the *E. coli o513* promoter is fused to *luxCDABE* genes. Although the regulation of *o513* has not been well characterized, it was reported that expression of a *lux* fusion to an open reading frame *o513* is highly induced as the culture ages, suggesting that stationary phase induces the expression of *o513*; however such expression is not controlled by σ^s (Van Dyk, 1998). Van Dyk (1998) also observed that *o513-lux* fusion did not yield increased bioluminescence to the wide range of chemicals (e.g., hydrogen peroxide, nalidixic acid, ethanol, sodium salicylate, and paraquat). Rather, it gave a response

ratio of less than 1.0 (“lights off”). Therefore, Van Dyk (1998) proposed to use the strain containing *o513*-*lux* fusion as a general indicator of toxicity. Interestingly, both (-)-ephedrine and (+)-pseudoephedrine significantly induced bioluminescence of the *o513*-*lux* fusion strain (DPD2232) compared to the control, and the “lights off” response was only observed for (-)-ephedrine at the high concentration of 0.3 mg/ml. This result suggests the possibility that *o513* promoter is activated in response to the stress caused by ephedrine-type alkaloids.

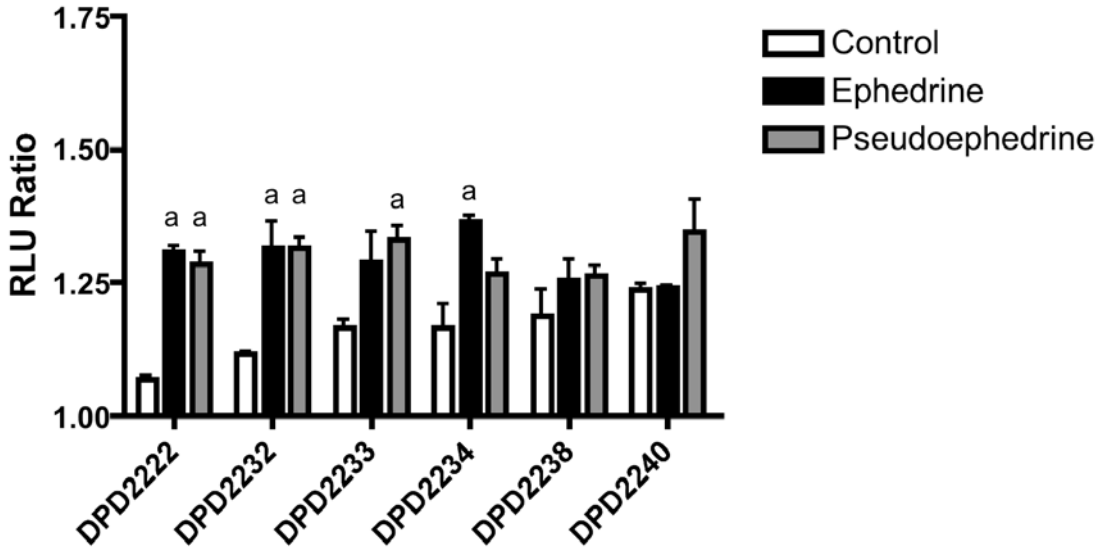


Figure 5-5. RLU ratio obtained from six *E. coli* strains exposed to 0.03 mg/ml of (-)-ephedrine and (+)-pseudoephedrine. Shown are mean ± S. E. M. for n=3. [a] p< 0.05 vs. control.

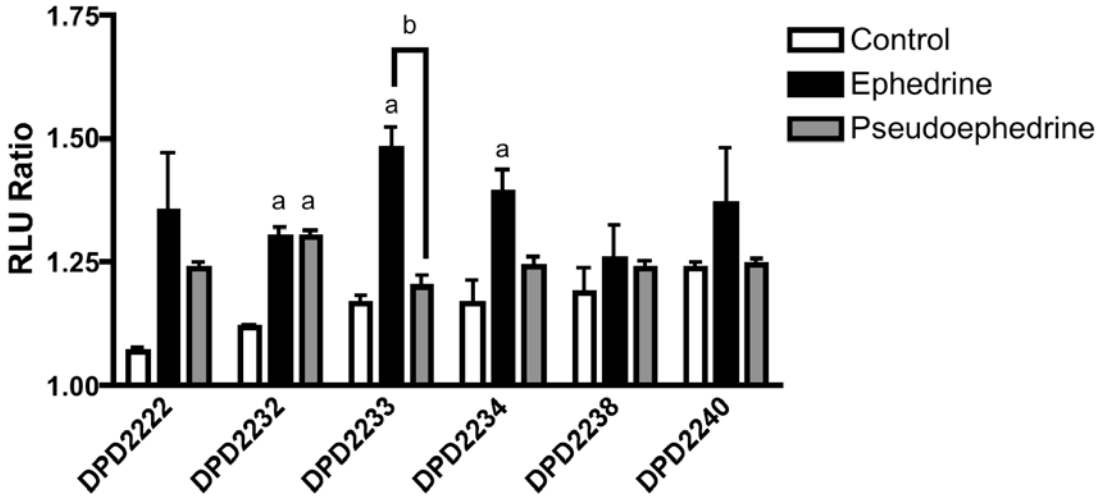


Figure 5-6. RLU ratio obtained from six *E. coli* strains exposed to 0.04 mg/ml of (-)-ephedrine and (+)-pseudoephedrine. Shown are mean ± S. E. M. for n=3. [a] $p < 0.05$ vs. control, [b] $p < 0.05$ ephedrine vs. pseudoephedrine.

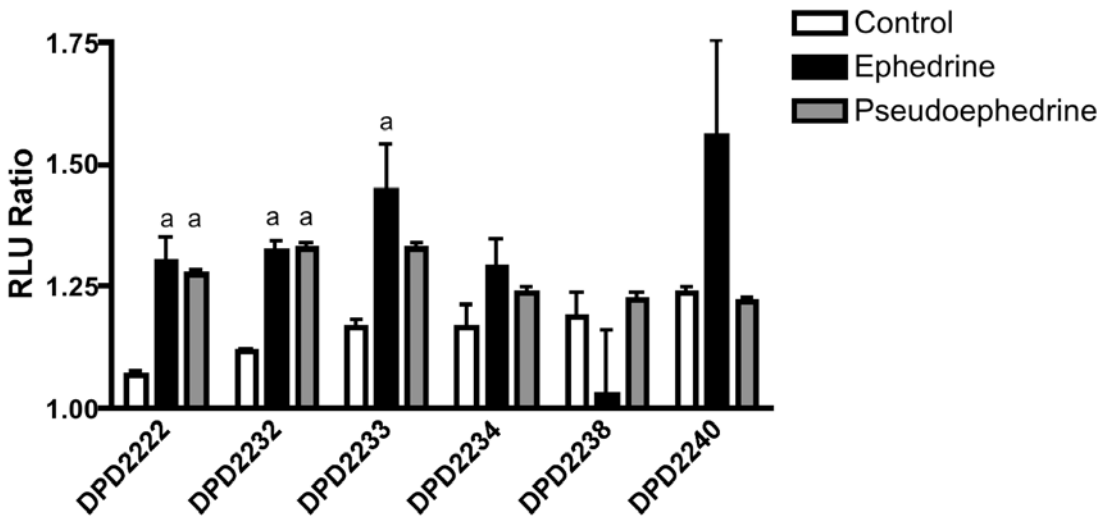


Figure 5-7. RLU ratio obtained from six *E. coli* strains exposed to 0.05 mg/ml of (-)-ephedrine and (+)-pseudoephedrine. Shown are mean ± S. E. M. for n=3. [a] p< 0.05 vs. control.

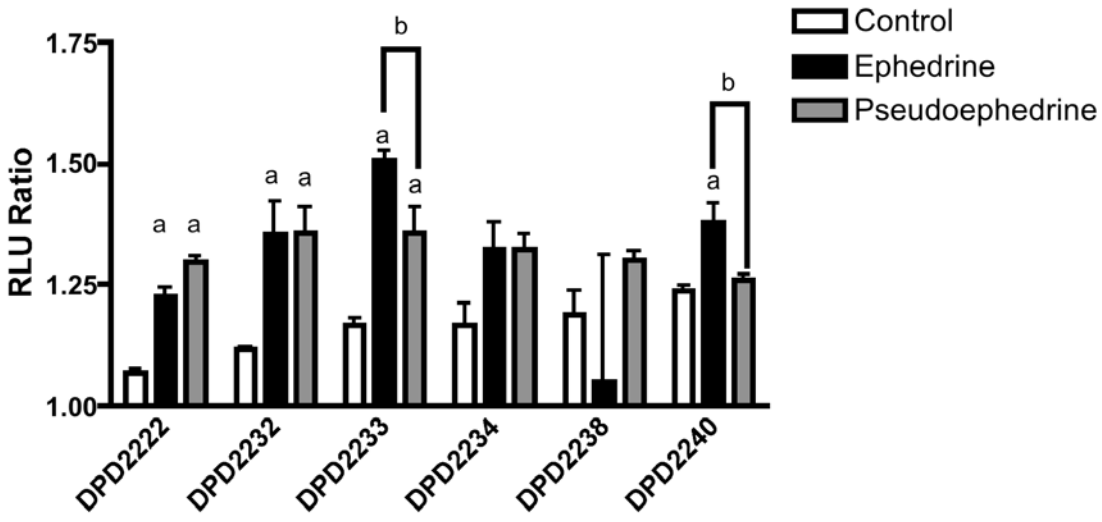


Figure 5-8. RLU ratio obtained from six *E. coli* strains exposed to 0.06 mg/ml of (-)-ephedrine and (+)-pseudoephedrine. Shown are mean ± S. E. M. for n=3. [a] $p < 0.05$ vs. control, [b] $p < 0.05$ ephedrine vs. pseudoephedrine.

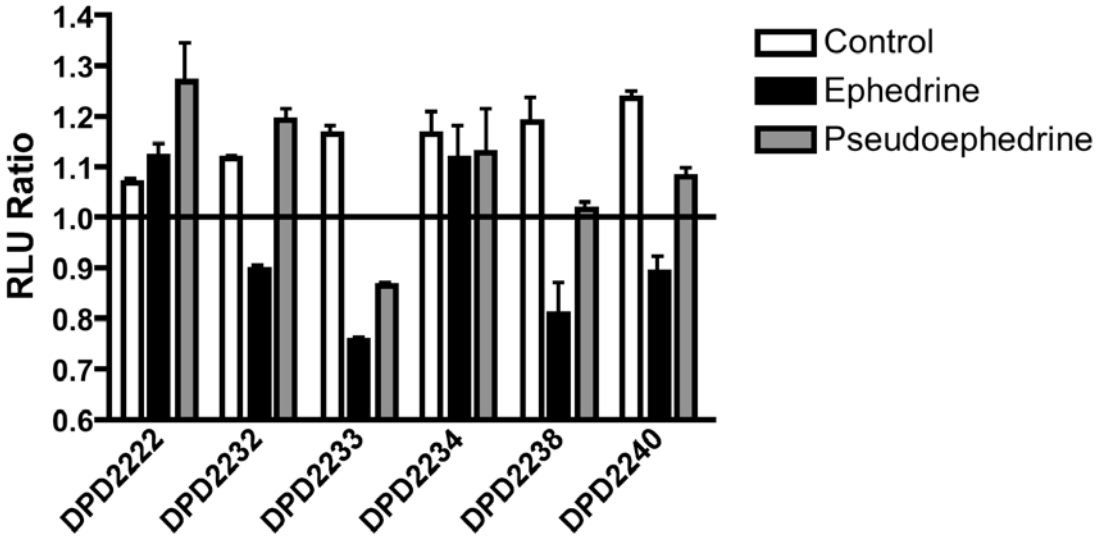


Figure 5-9. RLU ratio obtained from six *E. coli* strains exposed to 0.3 mg/ml of (-)-ephedrine and (+)-pseudoephedrine. Shown are mean ± S. E. M. for n=3.

CHAPTER 6: CONCLUSIONS

In the present study, the bioactivity of two major ephedrine-type alkaloids were evaluated by measuring its cytotoxicity against the cell lines and analyzing the stress response of a panel of genetically engineered biosensing *E. coli* strains capable of producing real-time responses to specific cell damages.

The two ephedrine-type alkaloids, (-)-ephedrine and (+)-pseudoephedrine, showed cytotoxicity to the human neuroblastoma SH-SY5Y and rat myoblastoma H9c2 (2-1) cell lines. SH-SY5Y cell lines showed similar sensitivity to (-)-ephedrine and (+)-pseudoephedrine while H9c2 (2-1) cell line was able to differentiate the cytotoxicity of (-)-ephedrine and (+)-pseudoephedrine. MTT assay using established cell lines could provide information on general information on cytotoxicity of ephedrine-type alkaloids, but a variety of cell lines should be used to assess organ/tissue toxicity.

Biosensing using a panel of bioluminescent *E. coli* strains was highly sensitive to ephedrine-type alkaloids and could produce a rapid response at the concentration as low as 0.03 mg/ml. Moreover, these *E. coli* strains distinguished the toxicity of (-)-ephedrine and (+)-pseudoephedrine; the bioluminescence from the *E. coli* strains was significantly suppressed by (-)-ephedrine compared to (+)-pseudoephedrine at the high concentration 0.3 mg/ml. At the lower concentrations, (-)-ephedrine generally induced higher bioluminescence than (+)-pseudoephedrine did throughout the experiments. As the RLU ratios dependent on the construct of strains gave unique

fingerprinting pattern of ephedrine-type alkaloids, this biosensing panel has a potential to be a very effective clue for clarifying the toxicity mechanism of ephedrine-type alkaloids. However, this biosensing panel did not give clear dose-dependent response in the range of concentrations used in the present study. In order to obtain quantitative information, it might be needed to assess the stress response using wider range of concentration.

Appendices

Table A-1. RLU ratio obtained from six *E. coli* strains exposed to 0.03 mg/ml of (-)-ephedrine, (+)-pseudoephedrine or mixture of (-)-ephedrine and (+)-pseudoephedrine in different concentrations.

	RLU Ratio		
Strain	ephedrine	pseudoephedrine	e + p*
DPD2222	1.31 ± 0.01	1.28 ± 0.02	1.18 ± 0.02 [a]
DPD2232	1.32 ± 0.05	1.31 ± 0.02	1.20 ± 0.03
DPD2233	1.29 ± 0.06	1.33 ± 0.03	1.31 ± 0.02
DPD2234	1.36 ± 0.01	1.27 ± 0.03	1.31 ± 0.07
DPD2238	1.26 ± 0.04	1.26 ± 0.02	1.29 ± 0.01
DPD2240	1.24 ± 0.00	1.34 ± 0.06	1.37 ± 0.06

* e+p: 0.015 mg/ml (-)-ephedrine + 0.015 mg/ml (+)-pseudoephedrine.
Each value is mean ± S. E. M., n=3 ([a] $p<0.05$).

Table A-2. RLU ratio obtained from six *E. coli* strains exposed to 0.04 mg/ml of (-)-ephedrine, (+)-pseudoephedrine or mixture of (-)-ephedrine and (+)-pseudoephedrine in different concentrations.

	RLU Ratio		
Strain	ephedrine	pseudoephedrine	e + p*
DPD2222	1.35 ± 0.11	1.24 ± 0.01	1.33 ± 0.04
DPD2232	1.30 ± 0.02	1.30 ± 0.01	1.27 ± 0.02
DPD2233	1.48 ± 0.04	1.20 ± 0.02 [a]	1.40 ± 0.05
DPD2234	1.39 ± 0.04	1.24 ± 0.02	1.32 ± 0.06
DPD2238	1.26 ± 0.06	1.24 ± 0.01	1.23 ± 0.00
DPD2240	1.37 ± 0.11	1.24 ± 0.01	1.35 ± 0.01

* e+p: 0.02 mg/ml (-)-ephedrine + 0.02 mg/ml (+)-pseudoephedrine.
Each value is mean ± S. E. M., n=3 ([a] $p<0.05$).

Table A-3. RLU ratio obtained from six *E. coli* strains exposed to 0.05 mg/ml (-)-ephedrine, (+)-pseudoephedrine or mixture of (-)-ephedrine and (+)-pseudoephedrine in different concentrations.

	RLU Ratio		
Strain	ephedrine	pseudoephedrine	e + p*
DPD2222	1.30 ± 0.05	1.27 ± 0.01	1.30 ± 0.01
DPD2232	1.32 ± 0.02	1.33 ± 0.01	1.25 ± 0.04
DPD2233	1.45 ± 0.10	1.22 ± 0.02	1.32 ± 0.01
DPD2234	1.29 ± 0.06	1.24 ± 0.01	1.30 ± 0.01
DPD2238	1.03 ± 0.13	1.22 ± 0.02	1.35 ± 0.05
DPD2240	1.56 ± 0.20	1.22 ± 0.01	1.45 ± 0.07

* e+p: 0.025 mg/ml (-)-ephedrine + 0.025 mg/ml (+)-pseudoephedrine.
Each value is mean ± S. E. M., n=3.

Table A-4. RLU ratio obtained from six *E. coli* strains exposed to 0.06 mg/ml (-)-ephedrine, (+)-pseudoephedrine or mixture of (-)-ephedrine and (+)-pseudoephedrine in different concentrations.

	RLU Ratio		
Strain	ephedrine	pseudoephedrine	e + p
DPD2222	1.23 ± 0.02 [a]	1.30 ± 0.01 [b]	1.29 ± 0.01 [ab]
DPD2232	1.36 ± 0.07	1.36 ± 0.05	1.15 ± 0.01
DPD2233	1.51 ± 0.02 [a]	1.30 ± 0.04 [ab]	1.24 ± 0.04 [b]
DPD2234	1.32 ± 0.06	1.32 ± 0.03	1.20 ± 0.01
DPD2238	1.05 ± 0.26	1.30 ± 0.02	1.17 ± 0.02
DPD2240	1.38 ± 0.04	1.26 ± 0.01	1.32 ± 0.03

* e+p: 0.03 mg/ml (-)-ephedrine + 0.03 mg/ml (+)-pseudoephedrine.
Each value is mean ± S. E. M., n=3.
Values followed with an identical letter are not significantly different ($p<0.05$).

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