

# Evaluation of The Genotoxicity of Three Food Additives using CHO-K1 Cells under *in vitro* Micronucleus Flow Cytometry Assay

Beni Lestari<sup>1</sup>, Dhanita Novitasari<sup>1</sup>, Herwandhani Putri<sup>1</sup>, Sari Haryanti<sup>3</sup>, Ediati Sasmito<sup>2</sup>,  
Edy Meiyanto<sup>1,2\*</sup>

<sup>1</sup>Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia

<sup>2</sup>Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia

<sup>3</sup>Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Kementerian Kesehatan RI, Tawangmangu, Indonesia

## Abstract

Exposure of genotoxic substances come from various sources such as food additives. The aim of this study is to evaluate the genotoxicity of food additives in CHO-K1 cells by micronucleus test flow cytometry. The food additives: sodium saccharine (SS), monosodium glutamate (MSG), and sodium benzoate (SB) were assessed by *in vitro* cytotoxicity and genotoxicity using Chinese Hamster Ovary-K1 (CHO-K1) cells. The cytotoxic effect of those compounds was evaluated by MTT Assay on CHO-K1 Cells. The genotoxic evaluation was observed by *in vitro* micronucleus test by flowcytometry with double staining method. The results showed that the three compounds did not perform cytotoxic effect, increased the frequency of micronucleus, and changed the cell cycle profiles. In general, these studies obtained that none of three food additives showed cytotoxic and genotoxic effect on CHO-K1 cells. Micronucleus test using flow cytometry is suitable for this purpose study.

**Keywords** : food additives, genotoxic, cytotoxic, micronucleus

## INTRODUCTION

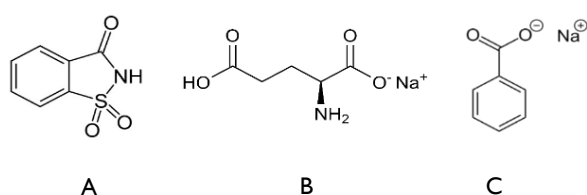
Foods and food ingredients are consisted of various of food materials, ranging from the relatively simple chemical compounds at one end to whole foods and ingredients at the other. Food additives and flavoring agents are mostly assumed safe to be consumed in daily. Across the world, food additives and flavorings are governed by legislation that includes well-established requirements for the demonstration of safety in order for them to be used as ingredients in food products (Blaauboer, *et al.* 2016). Even though the food additives are extensively used in daily basis of diet, it should be remembered that none are without some level of risk, because it may hazardous in high concentration, or accumulated in body (Dreisig, *et al.* 2013).

Genotoxicity assessment possesses as an essential safety assessment of all types of substances, ranging from pharmaceuticals, industrial chemicals, pesticides, food additives to cosmetics ingredients, relevant in the context of international legislations aiming at the protection of human and animal health. Generally, the assessment of

genotoxic hazard to humans was beginning with a basic *in vitro* tests followed by *in vivo* testing (ECVAM 2013). Genotoxicity testing requires the measurement of DNA primary damage that can be repaired and is therefore reversible. For an adequate assessment of genotoxicity need to be evaluated either in gene mutation, structural chromosome aberrations and numerical chromosome aberrations, as each of these events has been found to be implicated in carcinogenesis and degenerative diseases (Corvi and Madia, 2017). One of the developing method to asses the genotoxicity is micronucleus formation assay based on flowcytometry analysis. This method measures the micronucleus evidence in the cells through semi-automatic calculation that may promising for routine testing (Bryce, *et al.*, 2007).

\*Corresponding author email: edy\_meiyanto@ugm.ac.id

In this study, we used sodium saccharin (SS), monosodium glutamate (MSG), and sodium benzoate (SB) (Fig. 1) which have been largely used in Indonesia as sweetener, flavor enhancer, and preservative, respectively, to evaluate their genotoxicity effect by *in vitro* assay using CHO-K1 cells and analyze micronuclei (MN) that formed after treatment using these ingredients under flowcytometry. The results from this study can be used as another scientific evidence and reference regarding the validation from *in vitro* method using CHO-K1 mammalian cells.



**Figure 1. Chemical structure of SS (A), MSG (B), SB (C)**

## MATERIAL AND METHODS

### Sample Preparation

The three food additives including SS, MSG, and SB (Fig. 1) from market were prepared by dissolving those compounds in aquabidest sterile. Doxorubicin (DOX) was purchased from Sigma and was dissolved in dimethyl sulfoxide (DMSO) as the solvent.

### Cell Culture

The Chinese Hamster Ovarian cells (CHO-K1) were obtained from Prof. Masashi Kawaichi, Laboratory of Gene Function in Animal, Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST), Japan. CHO-K1 cells were grown in Roswell Park Memorial Institute medium (RPMI) (Gibco), supplemented with 10% Fetal Bovine Serum (Sigma), 1.5% (w/w) penicillin-streptomycin (Gibco, Invitrogen USA), and 0.5 % fungizone (Gibco, Invitrogen USA). Cells were incubated at 37°C and 5% CO<sub>2</sub>.

### Cytotoxicity Test

CHO-K1 cell lines were harvested by trypsinization and subsequently collected in a 15 mL conical tube and centrifuge at 1000 rpm for 5-10 minutes. The supernatant was discarded and the cells were washed once with 1 ml PBS and spinned at 1000 rpm for 5 minutes. The supernatant was again discarded and medium was added up to 1 mL. CHO-K1 cell lines were seeded in 96-wellplates (8.000 cells in each well). After confluent, cells were treated with various concentrations of compound. After 24-h incubation, culture medium was removed and cells werewashed in PBS (Sigma). Then, cells were incubated with 0.5 mg/mL MTT (Biovision) in 100µL culture medium each well for 4h. MTT reaction was stopped by SDS reagent (10% sodium dodecyl sulphate) (Merck) in HCl 0.01 N (Merck) and was incubated overnight. The absorbance was measured by ELISA reader (Bio-Rad) at wave length of 595 nm (Mosmann, 1983).

### *In vitro* Micronucleus Test (Flow Cytometry scoring)

CHO-K1 cells were grown in the 24-well plate for a 24-h before the treatments. The treatments were carried out as follows: (a) untreated, (b) DNA damage-inducing agents (DOXO), (c) treatment of SS, MSG, and NB and were incubated for 24-h. Analysis of micronucleus (MN) frequency was revealed by flow cytometric analysis based on the Microflow assay (Kirsch-Volder, *et al.*, 2003; Phelps, *et al.*, 2002). Briefly, CHO-K1 cells were first stained with a photoactivated dye 1 EMA; Molecular probes) and then washed, lysed, and stained with lysis buffer containing RNase, nucleic acid dye 2 (SYTOX Green; Molecular probes). DNA from apoptotic/necrotic cells with compromised cell membranes was labeled with both EMA and SYTOX Green, which can be distinguished from EMA-negative and SYTOX Green-positive MN. Samples were analyzed with BD Accuri C6 flow cytometer (BD Biosciences). The incidence of MN was determined through the acquisition of 10,000 healthy nuclei (EMA<sup>-</sup>/SYTOX<sup>+</sup>) per well. In addition, the SYTOX fluorescence histogram showed the cell cycle progression profile and the hypodiploid gate was used to determine whether aneuploidy was induced.

## RESULTS

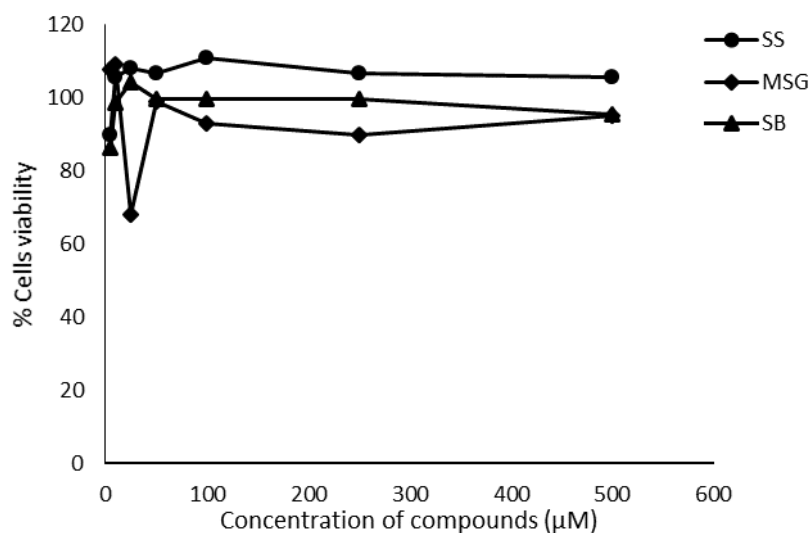
### Cytotoxic Effect of SS, MSG, and SB on CHO-K1 Cells

For preliminary study, we evaluated cytotoxic effect of SS, MSG, and SB against CHO-K1 cells (Fig. 2). Single treatment of SS, MSG, and SB on CHO-K1 with the concentration at 1-500  $\mu$ M did not show growth inhibitory effect after 24 h incubation under MTT assay (Fig. 1). The compounds did not give  $IC_{50}$  values until concentration of 500  $\mu$ g/mL, indicating that those compounds were not toxic on CHO-K1. Therefore, concentrations of 100 and 500  $\mu$ M of SS, MSG, and NB were chosen for the

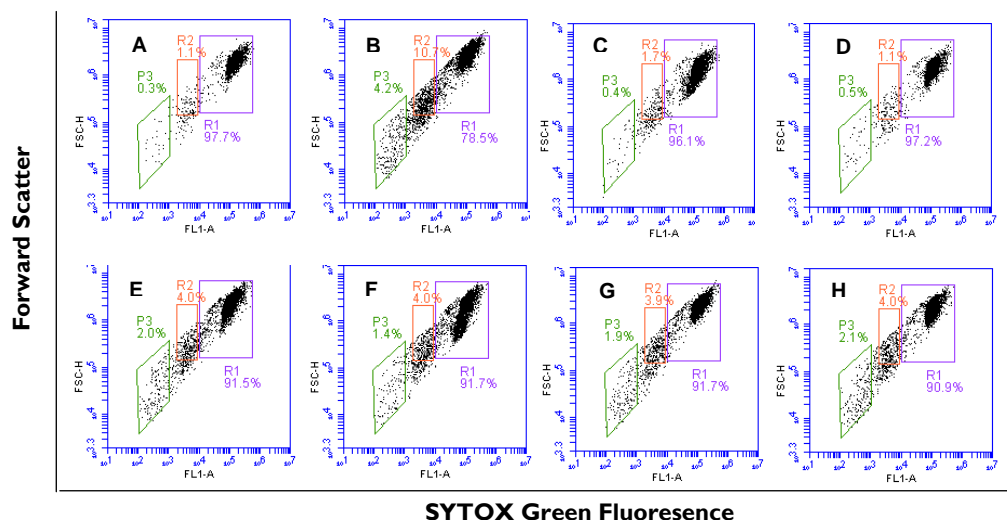
following experiment in the genotoxicity and antigenotoxicity protocols.

### Genotoxic Effect of SS, MSG, SB on CHO-K1 Cells

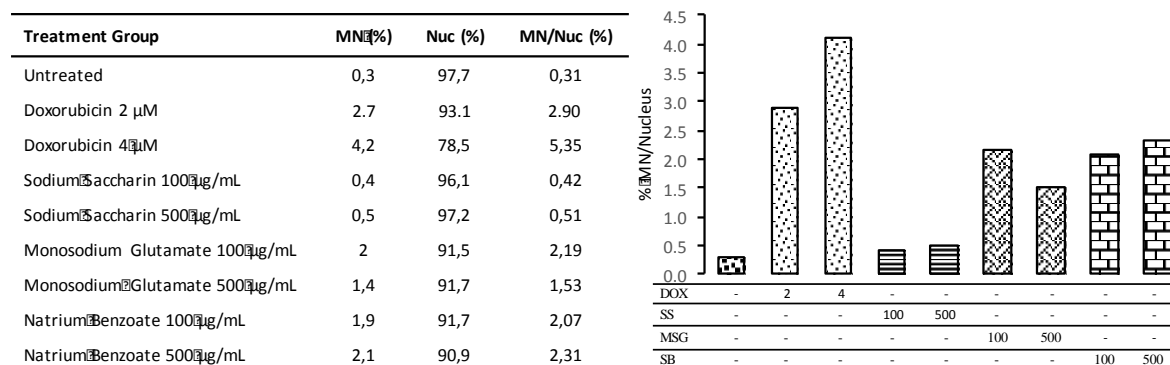
Genotoxicity evaluation of SS, MSG, and SB was carried out through CBMN assay using CHO-K1 cells, as summarized in Fig. 3 and 4. The three compounds did not show genotoxic activity on CHO-K1 cells, since the number of micronucleus observed after treatments of cells at concentrations 100 and 500  $\mu$ g/mL of three compounds was not increased statistically than that negative control.



**Figure 2. Cytotoxic effect of food additives on CHO-K1 cells.** CHO-K1 cells ( $1 \times 10^4$  cells/well) were seeded in 96-wellplate and incubated for 24h, then treated with MSG, Na Benzoate and Saccharine (5-500  $\mu$ g/mL). Cells viability was determined by using MTT assay as described in the method.



**Figure 3. Histogram of micronuclei and nuclei of CHO-K1 cells.** The CHO-K1 cells were incubated in sample of food additives and doxorubicin as positive control for 24 h. Untreated (A); Doxorubicin 4 μM (B); SS 100 μg/mL (C); SS 500 μg/mL (D); MSG 100 μg/mL (E); MSG 500 μg/mL (F); SB 100 μg/mL (G); SB 500 μg/mL (H).

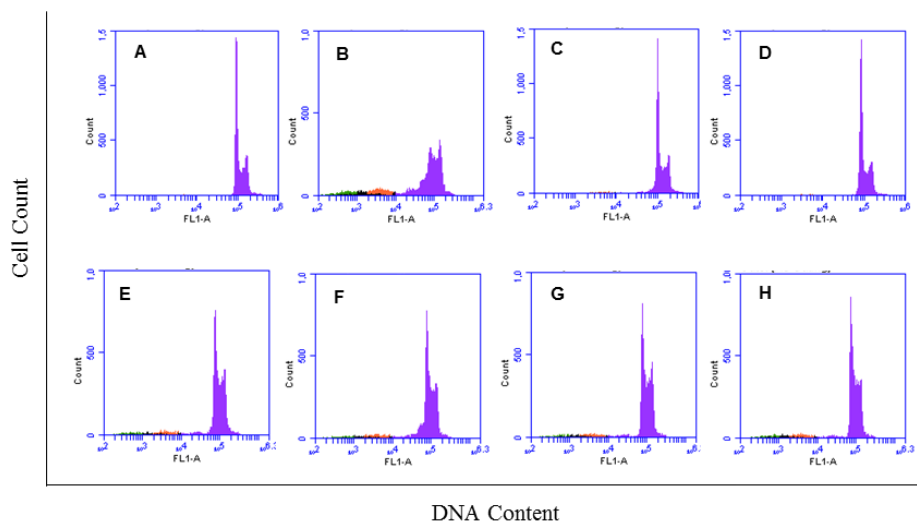


**Figure 4. Genotoxicity evaluation of food additives on the micronuclei CBMN assay on CHO-K1 cells.** For the quantitative analysis, CHO-K1 cells were treated with compounds at various concentrations for 24h. The frequencies of micronuclei of all treatments were analyzed by using flow cytometric.

### Cell Cycle Profile of SS, MSG, SB on CHO-K1 Cell

In addition to provide MN frequency data, we found that flow cytometry analysis using SYTOX-green staining could also cell cycle data for qualitative assessment. The information regarding cell cycle effects displayed in the SYTOX-

associated signal as fluorescence area (FLA-1). The treatment of DOXO induced cells accumulation in Sub-G1 as apoptotic population (Fig. 5). Whereas the treatment of SS, MSG, and SB did not change the cell cycle profile. This data indicated that SS, MSG, and SB not enhanced the cytotoxic effect through cell cycle modulation.



**Figure 5. Histogram of the cell cycle profiles on CHO-K1 cells.** The CHO-K1 cells were incubated in sample of food additives and doxorubicin as positive control for 24-h. Control (A); Doxorubicin 4  $\mu$ M (B); SS 100  $\mu$ M (C); SS 500  $\mu$ M (D); MSG 100  $\mu$ M (E); MSG 500  $\mu$ M (F); SBI00  $\mu$ M (G); SB 500  $\mu$ M (H).

## DISCUSSION

The mainly purpose of this research is to assess the potential toxicity effect of food additives especially in the genotoxicity effect through *in vitro* assays. We used CHO-K1 cells in this model due to its susceptibility in genomic instability under genotoxic exposure. In this study, we also used DOX as control positive of genotoxicity through increasing of intracellular reactive oxygen species (ROS) such as superoxide radical anion, hydroxyl radical, and singlet oxygen (Lehmann, *et al.*, 2003). This mechanism leads to the cell death and the micronucleus formation (Bryce, 2010; Tripathi, 2009). Doxorubicin also inhibits DNA synthesis and DNA polymerase activity (Santiago, 2010). As previously reported elsewhere under different methods, our findings also showed that all of the three tested food additives including SS, MSG and SB performed no micronucleus formation as well as there was no cell cycle profile changes. Those data means that these food additives had no genotoxic effect in CHO-K1 cells. Since this genotoxicity test was carried out under *in vitro* experiment with directly exposed to target cells, we can conclude that the non-genotoxicity properties may due to the non-directly interaction between these compounds with DNA as well as the molecules involved in cellular physiological processes.

Genotoxicity properties of some compounds usually mediated through metabolism under oxidation reaction resulting in toxic substances. This toxic metabolism usually occurs in some organs not in directly affected cells (Damian, 2012). Due to their chemical structure containing the double bonds, many food additives might be could undergone cellular oxidation in the cell metabolism (Sharma, *et al.*, 2012). The previous *in vitro* studies reported that SS was found weakly positive of genotoxicity under chromosomal aberration (Ashby and Ishidate, 1986; Kristofferson, 1977). On the other hand, the studies from Ataseven, *et al.* (2016) demonstrate that MSG is genotoxic to the human peripheral blood lymphocytes *in vitro* in high concentration. For sodium benzoate, negative results were obtained in several *in vitro* tests and *in vivo* cytogenetic assay with rats. However, a dominant lethal assay with rats gave a positive result (Wibbertmann, 2000). Therefore, the non-cytotoxic properties of these additives performed in the *in vitro* experiments should be confirmed further using *in vivo* experiments.

In summary, these results suggest that *in vitro* micronucleus test is recommended for assessing genotoxicity of many compounds such as food additives because this method is simple, rapid and representing the effect directly to the cells. More over, micronucleus (MN) formation is a hallmark of

genetic toxicity, thus the micronucleus assay is an important component of genetic toxicity screening (Lal and Ames, 2011). Thus, this micronucleus assay with flowcytometry is suitable for routine testing of food additives and other materials that commonly consumed among people. The present studies also suggest that the three of food additives such as SS, MSG, and SB can be used in products manufactured for human consumption at concentrations higher than currently acceptable limit without posing increased risk of genotoxic effects.

## CONCLUSION

The three food additives such as sodium saccharine (SS), monosodium glutamate (MSG), sodium benzoate (SB) does not perform cytotoxic effect on proliferative cells and does not induced genotoxic effect on *in vitro* model system. Micronucleus testing under flowcytometry is suitable for assessing genotoxicity of food additives and may applicable for routine testing.

## ACKNOWLEDGMENT

We would express our gratitude to Prof. Masashi Kawaichi, Laboratory of Gene Function in Animal, Graduate School of Biological Science, Nara Institute of Science and Technology (NAIST) for providing the CHO-K1 cells. This research was financially supported by Research Grant from The Ministry of Research, Technology and Higher Education of the Republic of Indonesia under PUPT scheme.

## REFERENCES

Ashby, J., and Ishidate, M.Jr., Clastogenicity in vitro of the NA, K, CA and Mg Salts of Saccharin; and of Magnesium Chloride; Consideration of Significance, *Mutat. Res.*, **163**, 63-73.

Ataseven, N., Yuzbasioglu, D., Keskin, A.C., Unal, F., 2016, Genotoxicity of Monosodium Glutamate, *Food Chem. Toxicol.*, **91**, 8-18.

Blaauboer, B. J., Boobis, A.R., Bradford, B., Cockburn, A., Constable, A., and Daneshian, M., et al., 2016, Considering New Methodologies in Strategies for Safety

Assessment of Foods and Food Ingredients, *Food Chem. Toxicol.*, **91**, 19-35.

Bryce, S.M., Bemis, J.C., Avlasevich, S.L., Dertinger, S.D., 2007, In vitro Micronucleus Assay Scored by Flow Cytometry Provides a Comprehensive Evaluation of Cytogenetic Damage and Cytotoxicity, *Mutat. Res.*, **630**(1-2), 78-91.

Bryce, S.M., Shi, J., Nicolette, J., Diehl, M., Sonders, P., Avlasevich, S., et al., 2010, High Content Flow Cytometric Micronucleus Scoring Method is Applicable to Attachment Cell Lines, *Environ. Mol. Mutagen.*, **51**(3), 260-266.

Corvi, R. and Madia, F., 2017, In vitro Genotoxicity Testinge. Can the Performance be Enhanced?, *Food Chem. Toxicol.*, **106**, 600-608.

Damian, G., Deavall, Elizabeth, A., Martin, Judith, M., Horner, and Roberts, R., 2012, Drug-Induced Oxidative Stress and Toxicity, *J. Toxicol.*, **2012**(2012), 1-13.

Dreisig, K., Taxvig, C., Birkhøj Kjørstad, M., Nellemann, C., Hass, U. and Vinggaard, A.M., 2013, Predictive Value of Cell Assays for Developmental Toxicity and Embryotoxicity, *ALTEX*, **30**(3), 319-330.

ECVAM, 2013, *EURL ECVAM Strategy to Avoid and Reduce Animal use in Genotoxicity*, JRC Scientific and Policy Report, 2013.

Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., et al., 2003, Report from the In vitro Micronucleus Assay Working Group, *Mutat. Res.*, **540**(2), 153-63.

Kristofferson, F., 2009, The Effects of Cyclamate and Saccharin on the Chromosome of a Chinese Hamster Cell Line, *Hereditas*, **70**(2), 271-282.

Lal, A. and Ames, B.N., 2011, Association of Chromosome Damage Detected as Micronuclei with Hematological Diseases and Micronutrient Status, *Mutagenesis*, **26**(1), 57-62.

Lehmann, M., Franco, A., de Souza Prudente Vilar, K., Lukza Reguly, M. and de Andrade, H. H., 2003, Doxorubicin and Two of its Analogues are Preferential Inducers of Homologous Recombination Compared with Mutational Events in Somatic Cells of *Drosophila melanogaster*. *Mutat. Res.*, **539**(1-2), 167-175.

Mosmann, T., 1983, Rapid Colorimetric Assay for Cellular Growth and Survival: Application to

- Proliferation and Cytotoxicity Assays, *J. Immunol. Methods*, **65**(1-2), 55–63.
- Phelps, J.B., Garriott, M.L. and Hoffman, W.P., 2002, A Protocol for the In vitro Micronucleus Test. II. Contributions to the Validation of a Protocol Suitable for Regulatory Submissions from an Examination of 10 Chemicals with Different Mechanisms of Action and Different Levels of Activity, *Mutat. Res.*, **521**(1–2), 103–12.
- Quiles, M.R.S., Jimenez, I.O., Saenz, D. H. and Antoun, M.D., 2010, Genotoxicity of Alkaloid-Rich Extract from *Lupinus termis* Seeds, *Pharm. Crops.*, **1**(1), 18–23.
- Sharma, Pallavi, Ambuj Bhushan Jha, Rama Shanker Dubey, and Mohammad Pessarakli, 2012, Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions, *J. Botany*, **2012**(2012), 1–26.
- Tripathi, D.N., Jena, G.B., 2009, Intervention of Astaxanthin Against Cyclophosphamide-induced Oxidative Stress and DNA Damage: a Study in Mice, *Chem. Biol. Interact.*, **180**(3):398–406.
- Wibbertmann, A., Kielhorn, J., Koennecker, G., Mangelsdorf, I. and Melber, C., 2000, *Benzoic Acid and Sodium Benzoate*, *Concise International Chemical Assessment Document 26*, World Health Organization, <http://www.inchem.org/documents/cicads/cicad26>.