2012•2013
FACULTY OF SCIENCES
Master of Statistics: Biostatistics

Masterproef

Statistical Evaluation of In Vivo and In Vitro Micronucleus Assays in Toxicology

Promotor :
Prof. dr. Helena GEYS

John Closter Olivo
Master Thesis nominated to obtain the degree of Master of Statistics , specialization Biostatistics

Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

universiteit hasselt
KNOWLEDGE IN ACTION

Universiteit Hasselt | Campus Hasselt | Martelarenlaan 42 | BE-3500 Hasselt
Universiteit Hasselt | Campus Diepenbeek | Agoralaan Gebouw D | BE-3590 Diepenbeek

universiteit hasselt | Maastricht University

2012•2013
FACULTY OF SCIENCES
Master of Statistics: Biostatistics

Masterproef

Statistical Evaluation of In Vivo and In Vitro Micronucleus Assays in Toxicology

Promotor :
Prof. dr. Helena GEYS

John Closter Olivo

Master Thesis nominated to obtain the degree of Master of Statistics , specialization Biostatistics

universiteit hasselt | Maastricht University

CERTIFICATION

This is to certify that this report was written by *John Closter Olivo* under my Supervision.

Olivo John Closter
Student

Signature

Date

Prof. dr. Helena Geys
Supervisor

Signature

Date

Acknowledgments

No man is an island. Indeed, the successful completion of this project would not have been possible without the invaluable assistance, inspiration, motivation and encouragement of several individuals who in one way or another extended their help with open arms. Their names deserve to be highly recognized in this humble acknowledgment.

Foremost, I would like to express my gratitude to Prof. dr. Helena Geys for entrusting me this topic under her auspices. I am deeply thankful for her constructive comments and remarks, valuable guidance and directives, enlightening discussions and engagement through the learning process of this master thesis. Without her unflinching support, this thesis would definitely not have been completed.

I offer my utmost gratitude to the Belgian Government through its Vlaamse InterUniversitaire Raad (VLIR) Scholarship which offered me an incredible opportunity to undertake my MSc program at Hasselt University without financial worry. Two valuable gifts you have definitely provided to me – my education and my future. It was an honor to have received such a prestigious award, and my gratitude cannot be expressed.

The whole experience of settling into Hasselt, Belgium has been very soothing. Words fail me to express my deepest appreciation to Chella, Nolen, Cesar, Ma'am Tina, Nay Gemma, Cris and Jed who have been my guardians and professional advisors spiritually, emotionally and academically. Thank you very much!

Most importantly, accomplishment would not have been half sweet without the unconditional love and unending support from my family. I wish to thank my dear Papang Noly and Mamang Nita and to my 5J's siblings – Jinky, Jones Oliver, Jestoni, Jherenza Carlee and Jwhyxz Cyver- for being the source of my strength and inspiration. You are the main reason why I am striving very hard. My success is wholeheartedly dedicated to all of you. I wish also to extend my appreciation to all my relatives in and out of the Philippines.

With God nothing is impossible. Indeed, I offer everything to God for without Him everything is nothing to me. Because of Him I am totally vindicated with all the accusations and underestimations of the realities of the world. Nothing can stop me from praising and thanking Him for all these overflowing blessings!

To all of you, for the successful realization of this endeavor, this Master Thesis is humbly dedicated.

John Closter Fronda Olivo
10 September 2013

Contents

List of Tables

List of Figure

Abstract

Several alternative statistical procedures have been suggested and published to statistically analyze the incidence of micronucleated polychromatic erythrocytes (MNPCs) among treatment groups, but no standard procedure has been singled out and exclusively recommended. In this project, Hothorn and Gerhard (2008, 2009) recommendations for the statistical evaluation of *in vivo* and *in vitro* micronucleus (MN) assays were implemented.

The genotoxic activity of two candidate chemicals, T01 and T02, were assessed in the rodent bone marrow micronucleus test using male mice. Results show that oral administration of T01 for a day was found not to increase significantly the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in any of the test compound dose groups when compared to their concurrent vehicle control group (demineralised water). An increase in MN frequencies was noted only in the positive control group indicating the validity of the assay. On the other hand, MN frequencies were significantly elevated in mice exposed to any dose level of T02 administered orally in a single frequency of dose. Hence, the results of this study indicate that T01 and T02 were tested to be a negative and a positive compound, respectively, under the anticipated condition of the tests used.

Similarly, the possible genotoxic effect of D/L Menthol was investigated by analyzing the frequencies of micronuclei in cultured lymphocytes exposed to D/L Menthol. Under the test conditions of the assay, D/L Menthol, either in the presence or absence of metabolic activation (S-9), did not induce a clear dose-dependent increase in the micronuclei occurrence. These results suggest that D/L menthol does not have a chromosomal-damaging effect in mammalian lymphocytes.

Keywords: *D/L Menthol; In vivo; In vitro; micronucleus; MNPCs; S- ; T01; T02*

1 Introduction

Genotoxicity testing is a vital element of a product safety assessment which is highly recommended by the regulatory agencies around the globe. It is designed to assess and detect chemicals that induce genetic damage. Thus far, the fourth International Conference on Harmonization (ICH4) of Genotoxicity Guidelines recommended the micronucleus assay (MNC) as one of the standard three-test batteries for genotoxicity testing of pharmaceuticals. The two most well established tests as screening methods of new chemical entities with a widespread acceptance in industry and authorities are the *in vivo* and *in vitro* micronucleus assays.

As a measure for chromosomal aberrations *in vivo*, data on the frequency of micronucleated erythrocytes (MN) per a certain number of scored polychromatic erythrocytes (PCE) per animal are usually determined and analyzed using an appropriate statistical test. On the other hand, in the case of *in vitro,* data on the frequency of MN from a thousand of cells were evaluated. Thus, the attention of this paper was restricted only on the statistical procedure for the analysis of micronuclei particularly on the application of methodology suggested by Hothorn and Gerhard (HG, 2008; 2009).

HG considered different aspects in the statistical evaluation of the *in vivo/in vitro* micronucleus assay. One aspect concerns the choice of the experimental unit and the potential presence of overdispersion often ignored in traditional analyses. In *in vivo,* biologically speaking the standard protocol design is basically a randomized one-way layout including a negative control, several doses or treatment groups and, optionally, a positive control. Five to ten animals are randomly assigned to each treatment group. For each animal, a number of polychromatic erythrocytes (PCEs) are scored and evaluated for the presence of small micronuclei. Likewise, the predominant design for *in vitro* is a randomized one-way layout. A duplicate cell culture of mammalian origin is exposed to a series of test substance concentrations, i.e. a minimum of three dose levels, both with and without a source of metabolic activation. Concurrent vehicle and positive controls are included in all tests. During or after exposure to the test substance, the cells are grown for a period sufficient to allow chromosome damage to lead to the formation of micronuclei in interphase cells. Harvested and stained interphase cells are then analysed for the presence of micronuclei (OECD, 2009).

As proposed by Kim et al. (2000), a formal statistical approach is to assume a Binomial distribution of the number of micronuclei observed in 2,000 PCEs. This means pooling the number of MN over the animals to have one estimate of the proportion for each treatment group. These proportions are evaluated using a Dunnet-type procedure or the Cochran-Armitage trend test. However, such approach is not recommended by HG because pooling the number of MN over all animals/cultures ignores the variability between the animals as experimental units. They propose to model this between-animal variability using extra-Poisson or extra-Binomial models.

The second statistical aspect is the contradiction between statistical significance and biological relevance. Traditionally, scientists rely on the p-values from their reporting systems to draw conclusions regarding the safety of a compound. P-values represent a probability of falsification but do not provide interpretation in terms of biological relevance. HG suggest the use of confidence intervals which allow interpretation of both statistical significance and biological relevance at the same time.

The third aspect is the type of inference. HG dwell upon the type of inference that is of relevance in trying to identify an increasing dose-related trend, possibly with downturn effects at high doses (Bretz and Hothorn , 2002).

Finally, HG propose a proof of safety approach for a possible claim that a compound is not genotoxic.

The purpose of this project was to implement, apply and discuss the recommendations of HG using the genotoxicity database of the test compounds T01 and T02 from *in vivo* and D/L Menthol from *in vitro* micronucleus assays. Furthermore, the results were compared with the widespread "traditional" ANOVA-like approaches that completely ignore the between-animal variability, focus on p-values and linear trend tests only, and are set up in terms of proof of hazard.

The paper was organized as follows: in the subsequent section motivating examples are introduced from the *in vivo* as well as the *in vitro* settings. Section 3 describes the statistical methodology. Results are discussed in Section 4. And finally, Section 5 presents the discussion of the findings, conclusions and recommendations.

2 Motivating Examples

2.1 *In Vivo* Micronucleus Assay

In this project data were considered from two independent *in vivo* assays– one from an assay with compound T02 and another (with different harvesting times at 48 and 72 h), from an assay with compound T01. In both assays, male mice were randomly allocated into three different dose levels of testing groups – low, medium, high - , a concurrent negative and, optionally, a positive control group. Five to six mice were selected randomly in each group. In total, 15 male mice in each harvesting time was used and analyzed separately for T01 while a total of 20 mice was sacrificed for T02. Each mouse in the vehicle control was given demineralised water while each mouse in the remaining groups was treated with a certain dose of T01 or T02. Next, blood samples were obtained for each mouse based on the harvesting time after dosing specified in the protocol (*see* Table 1). Micronucleus frequencies were determined for each animal by scoring 20,000 polychromatic erythrocytes (PCEs) and the micronucleus occurrence per 20,000 PCEs was recorded. Results from animals that died, or for which no information could be obtained, were excluded from the data. The dosing schemes for the two different compounds are summarized in Table 1 below.

Table 1
Dosing scheme for the micronucleus assay with T01 and T02 compounds

	T01			T02		
Dose Group	No. of Rat	Harvest Time (h)	Frequency of Dosing	No. of Rat	Harvest Time (h)	Frequency of Dosing
VC	6	48, 72	Single dose	5	27	3-day repeat dose
Low	6	48, 72		5	27	
Medium	6	48, 72		5	27	
High	6	48, 72		5	27	
PC	6	48		-	-	

h: hour

2.2 *In Vitro* Micronucleus Assay

D/L Menthol was tested in an *in vitro* micronucleus assay using duplicate mammalian lymphocyte cultures prepared from two mice donors in two independent experiments. To show reproducibility of the results, the two replicate cultures were performed both in the absence and presence of metabolic activation (S9-mix). Mitomycin-C (MMC), at 0.02, 0.03, 0.07 and 0.10 µg. /mL and Cyclophosphamide (CP), at 1.5 and 2.5 µg. /mL, were used as the positive controls in the non-activated and activated systems, respectively. Sterile dimethyl sulphoxide (DMS) was added to cultures designated as negative control. The cells were treated at concentrations ranging from 40 to 250 µg. /mL, both in the presence and absence of the metabolic activator S-9, until cell harvest. The harvest time was either 4h or 24h after the initiation of the treatment. The treatment scheme is summarized in Table 2 below. Finally, around 1000 cells from each culture (around 2,000 per concentration) were analysed for the presence of micronuclei. Information such as the number of micronucleated cytokinesis blocked cells (MN), the treatment groups, a negative control, the different doses and the positive controls were extracted from the raw data for the analysis (*see* Appendix, Tables 1-3).

Table 2

Treatment scheme for the in vitro micronucleus assay on D/L Menthol

Treatment Condition					
24/-S9		4h/+S9		4h/-S9	
Group	Concentration *µg. /mL*	Group	Concentration *µg. /mL*	Group	Concentration *µg. /mL*
Conc. 1	40	Conc. 1	50	Conc. 1	50
Conc. 2	60	Conc. 2	100	Conc. 2	100
Conc. 3	80	Conc. 3	150	Conc. 3	125
Conc. 4	100	Conc. 4	200	Conc. 4	150
Conc. 5	150	Conc. 5	215	Conc. 5	175
Conc. 6	175	Conc. 6	225	Conc. 6	200
Conc. 7	200	Conc. 7	230	Conc. 7	250
MMC	0.02	CP	1.50	DMS	0.00
MMC	0.04	CP	2.50	MMC	0.07
DMS	0.00	DMS	0.00	MMC	0.10

3 Statistical methodology

3.1 *In vivo* micronucleus assay

In this section the number of micronucleated erythrocytes (MN) in an *in vivo* micronucleus assay constitutes the primary endpoint. The micronucleus frequencies were determined by analyzing the number of micronuclei from at least 20,000 PCEs per animal. Preliminary analyses were done by presenting box plots to get an initial impression about the data at hand. In this section we further present the traditional Poisson model, the quasi-Poisson model, a trend test approach and contrast the classical 'proof of hazard' approach to a 'proof of safety' approach.

3.1.1 Poisson model

The statistical evaluation of the number of micronuclei was primarily focused on multiple contrast tests for comparisons versus the vehicular control. This number of micronuclei as counts is analyzed using the classical approach Poisson model. This means that for each treatment group only one count is estimated by pooling the number of MN over animals. According to Parodi and Bottarelli (2006), the Poisson regression model is often applied to study the occurrence of small number of counts or events as a function of a set of predictor variables.

Let Y_{ij} be the number of micronuclei observed in the i^{th} animal at dose d_j, j=0, 1, 2,…, m. If Y_{ij} can be assumed to be distributed independently with

$$Y_{ij} \sim \text{Poisson}(\mu(x_j)) \tag{1}$$

Then, the log-linear Poisson regression can be applied such that

$$\mu(x_j) = \exp(\alpha + \beta^T x_j) \tag{2}$$

where α is the log of mean of the reference group, i.e. vehicle control, β^T is a vector of the parameter estimates and x_j is a vector of the covariates. In this case the covariate was the indicator variable for the dose group.

Accordingly, to observe any significant differences amongst the dosage sets in assessing the genotoxicity effects, the Dunnet's-type procedure was used to determine if any differed from the vehicle control.

The key assumption of this model is that mean and variance are equal, i.e. $V(\mu)=\mu$. This approach, however, does not recognize animals as experimental units and is therefore not recommended since it results in too liberal decisions (HG, 2009). HG propose to take into account the between-animal variability which is further discussed in the next subsection.

3.1.2 Quasi-Poisson model

HG highlight the importance of taking into account the between-animal variability since the individual animal is the experimental unit. Without accounting for extra-variability, the simple Poisson approach becomes more liberal with increasing overdispersion. Its implications include underestimation of standard errors and thus wrongly inflating the level of significance (Lee *et al.*, 2012). They recommend fitting a quasi-Poisson model for counts. A quasi-Poisson model is a type of generalized linear models where instead of the maximization of the Poisson likelihood a more relaxed relationship of the mean-to-variance dependency is assumed. Ramon *et al.* (2002) suggested that, with generalized linear models, overdispersion can be accounted for by fitting the Poisson model but adjust the standard errors and test statistics. This can be done by introducing an additional parameter to the model, representing the deviation from the Poisson variance assumption. To account for extra variation, the quasi-likelihood methods can be fitted under the assumption $V(\mu) = \rho\mu$ where a value of ρ larger than one indicates overdispersion (HG, 2009).

3.1.3 The Trend Test

Traditionally, to evaluate the number of micronuclei it is suggested by Margolin and Risko (1988), cited by Hayashi *et al.* (1989), to use the Cochran-Armitage trend test (Cochran, 1954; Armitage, 1955) to verify the dose-response trends of MNPCEs. This tests the null hypothesis of no trend, i.e. the number of MNPCEs is the same for all dose levels versus the alternative that there is a linear trend across increasing levels of dosage. However, the CA test should not be recommended because it is underpowered when the true trend is not linear, i.e. when the dose and response exhibit a convex and concave relationship (Bretz and Hothorn, 2002). Moreover, this should not be recommended because of its ignorance of the between-animal variability (HG, 2009). For this reason HG propose to use a Williams-type

procedure for trend test over Cochran-Armitage (CA) trend test. The CA trend test is particularly sensitive for near-linear shape, whereas the Williams trend test is sensitive to several shapes. This procedure tests the null hypothesis of no difference among the counts against the alternative that the counts are increasing with increasing dosage compared to the control group. To achieve this comparison, higher concentrations groups are successively pooled and compared to the control (Herberich and Horthorn, 2012).

3.1.4 Proof of hazard vs. proof of safety

The purpose of toxicology testing is to assess the safety of a new test substance relative to a control whether it is harmless up to a specified dose, or harmful. Based on this aim, statistical test of the classical null hypothesis of no difference are usually performed. Failing to reject the hypothesis, i.e. either a non-significant p-value or when the point-one hypothesized value is greater than the lower bound confidence limit of relative risk, often leads to the conclusion that the compound has no harmful effect. This is the traditionally used criterion for harmlessness which demonstrates proof of hazard. The major drawback of this approach is the fact that what is controlled by a pre-specified level is the probability of erroneously concluding hazard. In fact the primary control of the false decision rate, i.e. confidence in negative results, should be preferred in toxicology (Herberich and Horthorn, 2012). Summing up, proof of hazard is an indirect approach and often leads to the problem that statistical significance does not necessarily mean toxicological relevance and statistical non-significance does not necessarily mean toxicological irrelevance (Hauschke *et al.*, 1999). In short, be confident with negative results (Kirkland, 2000). Thus, the so-called proof of hazard is inappropriate simply because absence of evidence is not evidence of absence (Altman and Bland, 1995). For this reason HG recommend proof of safety to demonstrate harmlessness where the probability of erroneously concluding safety is directly controlled. The differences to the proof of hazard with the control of the familywise error rate are (1) the estimation of the upper confidence limits instead of the lower limits, and (2) interpreting interval inclusion instead of superiority interpretation by means of point estimator and confidence limit (HG, 2009).

In this study, for proof of hazard, the common decision was to conclude harmlessness if the p-value of the test for any dose vs. control was non-significant (p-value>0.05), otherwise harmfulness is concluded. This classical test problem was formulated as follows:

$$Ho: \pi_{dose} / \pi_{control} \leq 1 \text{ harmless}$$
$$Ha: \pi_{dose} / \pi_{conrol} > 1 \text{ harmful}$$

For the proof of safety approach, the specified direction of harmlessness was defined, i.e. only an increasing number of micronuclei were considered to be harmful. For this reason, the non-inferiority test which is a one-sided hypotheses test was primarily used for proof of safety to demonstrate the possible harmfulness of a certain dose assuming a three-fold threshold of tolerability. Harmlessness was declared for at least one dose if the upper limit of the relative risk is below the three-fold threshold. The following hypotheses were evaluated:

$$Ho: \pi_{dose} / \pi_{control} \geq 3 \text{harmful}$$
$$Ha: \pi_{dose} / \pi_{conrol} < 3 \text{ harmless}$$

3.2 *In vitro* micronucleus assay

Similar to *in vivo*, we can also analyze the MN as counts by pooling the number of MN over culture using a Poisson model. Let Y_{ij} be the number of micronuclei observed in the i^{th} culture under concentration c_j, j=0,1,2,…,m. If Y_{ij} can be assumed to be distributed independently with

$$Y_{ij} \sim \text{Poisson}(\mu(x_j)) \quad (3)$$

Then, the log-linear Poisson regression can be applied such that

$$\mu(x_j) = \exp(\alpha + \beta^T x_j) \quad (4)$$

where α is the log of mean of the reference group, i.e. vehicle control, β^T is a vector of the parameter estimates and x_j is a vector of the covariates. In this case the covariate was the indicator variable for the concentration group.

Moreover, trend test, proof of safety and proof of hazard were performed as discussed previously.

3.3 Statistical Software

All statistical procedures were implemented in R. The codes used for the analysis were counterchecked by using a user-friendly graphical user interface (GUI) *stat4tox* which can be downloaded online for free. All analysis were done at 5% level of significance.

4 Results

The results of the potential genotoxicity of TO1, TO2 and D/L Menthol were presented in this part of the paper.

4.1 Statistical evaluation of *in vivo* micronucleus assay

In this section, the genotoxicity effects of T01 and T02 were evaluated using *in vivo* micronucleus assays. Figure 1 illustrates the results of the mice bone marrow erythrocyte micronucleus assay. The assay with T01 with harvesting time of 48 and 72 h depicted no effect in locations while T02 posted a positive assay. An increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in treated mice is an indication of induced chromosome damages (Krishna and Hayashi, 2000).

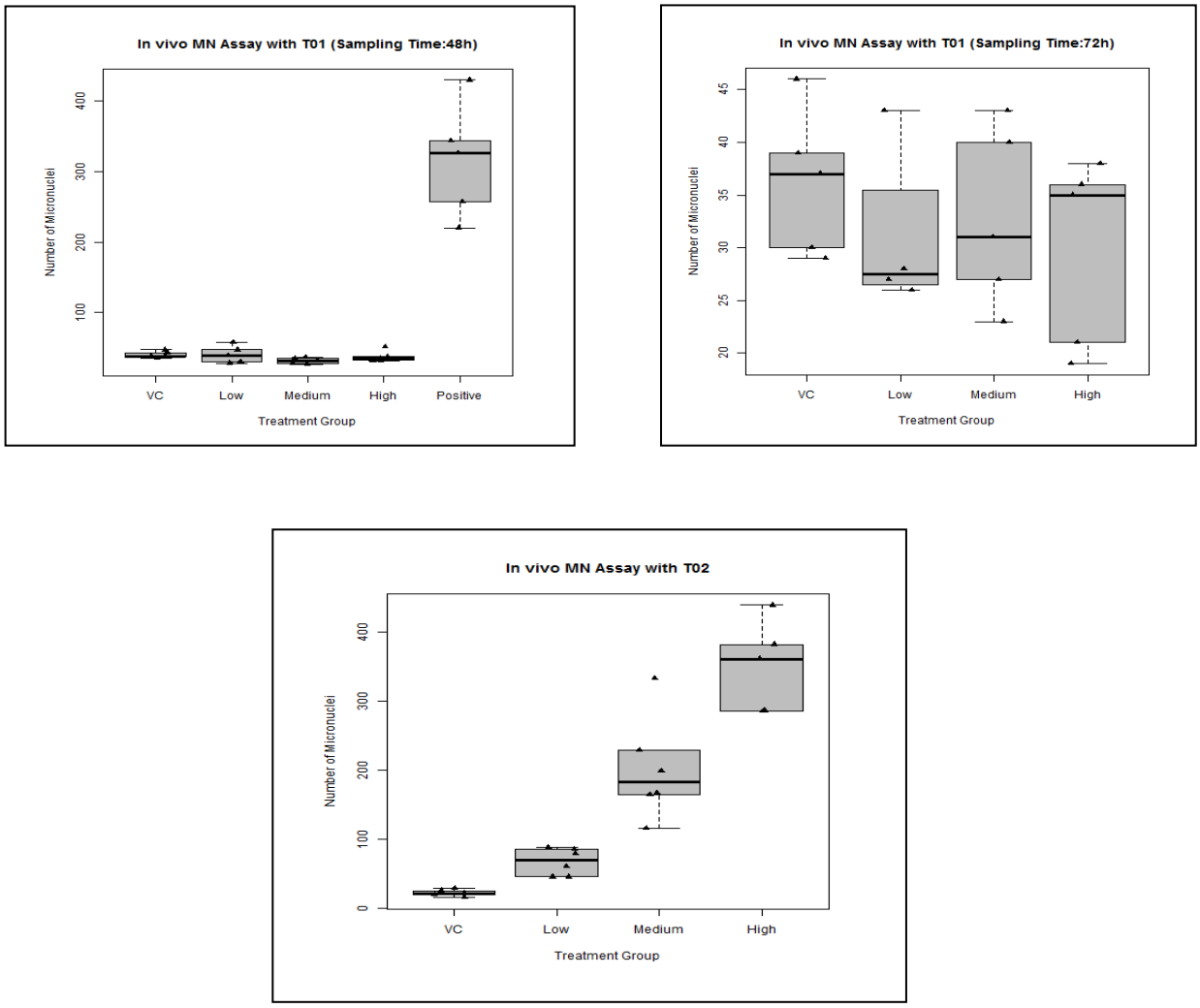

Figure 1. *Boxplots for T01 and T02*

Traditional Approach: Poisson Model

Let us first apply the traditional Poisson model, described in Section 3.1.1 on our *in vivo* data. Tables 3 to 5 summarize the results of the *in vivo* micronucleus test for each dosage group per sacrifice interval. Given from the tables are the relative risk (RR) and the corresponding lower confidence limits estimated by the Poisson model (lower RR).

Genotoxicity activity is indicated by statistically significant dose-related incidence of MNPCEs in the treatment group. A compound is traditionally considered mutagenic in the test system if at any of the preparation intervals, a statistically significant increase in the number of MNPCEs is found in comparison to the negative control (Shahrim *et al*., 2006). Looking at the p-value, the comparisons of the three doses of T01 versus control were not significant indicating no difference in the risk of having micronuclei (*see* Tables 3 and 4). However, looking just at the p-value would not allow us in claiming the biological relevance by its distance to the null hypothesis point of one. Thus, the 95% lower confidence limit for relative risk, denoted as LowerRR in the table, was provided to show not only the statistical significance but the biological relevance as well. Results from Tables 3 and 4 show no significant increase 48 and 72h after application of the three doses of the T01 as compared with the negative control since the point one hypothesized value was greater than the estimated lower limit of relative risk. This compound had no indication to induce clastogenic effects and therefore was evaluated as negative at any level of dose administered in both periods of harvest. However, the positive control induced significant and biologically relevant increase in the number of MNPCEs at 48h administration, confirming the validity of the assay. Conversely, Table 5 shows there was a significant increase in MNPCE observed in all level of doses of T02 over the vehicle control. Lastly, the final choice in claiming drug safety or harmfulness can be done with the help or verdict from a toxicologist.

To further elucidate the genotoxic properties of T01 and T02, it is imperative to assess the dose-response relation to confirm the toxicity of chemicals (Hayashi *et al.*, 1989). The Cochran-Armitage trend test revealed no significant trend for T01 with 48h and 72h as sampling hours yielding p-values 0.11 and 1.00, respectively. For T02, however, the CA test supports a significant trend (p-value < 0.000) that implies the MN occurrence increases with increasing dose score. The trend test p-values, however, did not provide any information about the biologically relevance of the results. In the following section, we implemented the HG proposal for a Williams-type contrast on a quasi-Poisson.

Table 3
Dunnett-type contrasts for relative risks (RR) on MN for T01 (Sampling hour: 48h)

		Poisson		Quasi-Poisson	
Comparison	RR	Lower RR	Pvalue	Lower RR	Pvalue
Low-Vehicle	1.00	0.81	0.76	0.61	0.77
Med-Vehicle	0.77	0.61	1.00	0.45	0.98
High-Vehicle	0.93	0.74	0.95	0.55	0.87
Positive-Vehicle	7.85	6.69	0.00	5.38	0.00

Table 4
Dunnett-type contrasts for relative risks (RR) on MN for T01 (Sampling hour: 72h)

		Poisson		Quasi-Poisson	
Comparison	RR	Lower RR	Pvalue	Lower RR	Pvalue
Low-Vehicle	0.86	0.67	0.99	0.60	0.96
Med-Vehicle	0.91	0.72	1.00	0.66	0.93
High-Vehicle	0.82	0.65	0.96	0.59	0.98

Table 5
Dunnett-type contrasts for relative risks (RR) on MN for T02

		Poisson		Quasi-Poisson	
Comparison	RR	Lower RR	Pvalue	Lower RR	Pvalue
Low-Vehicle	3.13	2.58	0.00*	1.61	0.00*
Med-Vehicle	9.42	7.89	0.00*	5.13	0.00*
High-Vehicle	16.43	13.80	0.00*	9.04	0.00*

* significant at 5% level of significance

Hothorn-Gerhard Recommended Approach: Quasi-Poisson model

Let us now redo the analyses while properly accounting for the between-animal variations according to the proposals from HG. To that end a quasi-Poisson model was fitted. The estimated dispersion parameters were 12.0, 2.1, and 5.6 for the analysis in the assays with T02, T01 (sampling time: 48h) and T01 (sampling time: 72h), respectively. This indicates the occurrence of extra-variability.

Tables 3 to 5 present the estimated relative risk alongside with the one-sided lower confidence limit. Apparently, this model yielded similar conclusions as that previously discussed from using the Poisson model. No significant increase in the micronucleus

frequency compared with the vehicle control could be determined for T01 while T02 did show increased level of micronucleated erythrocytes.

Furthermore, HG propose Williams-type contrasts for the relative risk as an alternative to Cochran-Armitage test since the main objective was to demonstrate a possible dose-related trend. This test is sensitive to several shapes as compared to CA test which is sensitive for near linear shape. The direction of interest, i.e. increasing micronuclei induces chromosomal damage, and only the lower limit is required for a conclusion regarding the trend. Therefore we are only after in the performance of the lower bound confidence limits. The multiple comparisons was done by successively pooling the higher dose groups and compared to the control group. In Tables 6-8 the relative risk estimates and their lower simultaneous confidence limits for Williams-type contrasts for the quasi-Poisson model are given. Since all three comparisons were not significantly larger than 1, we concluded that there is no significant increase in the number of MN with increasing dosage. The reverse was observed for T02 wherein the High dose group led to the most pronounced change in the number of MN compared to the control group.

Table 6
Williams-type contrasts for relative risks (RR) on MN for T01 (Sampling hour: 48h)

Comparison	RR	LowerRR
C1: Vehicle vs. High	0.93	0.72
C2: Vehicle vs. Medium and High	0.84	0.68
C3: Vehicle vs. All doses	0.90	0.73

Table 7
Williams-type contrasts for relative risks (RR) on MN for T01 (Sampling hour: 72h)

Comparison	RR	LowerRR
C1: Vehicle vs. High	0.86	0.62
C2: Vehicle vs. Medium and High	0.84	0.64
C3: Vehicle vs. All doses	0.86	0.67

Table 8
Williams-type contrasts for relative risks (RR) on MN for T02

Comparison	RR	LowerRR
C1: Vehicle vs. High	16.43	9.41
C2: Vehicle vs. Medium and High	12.13	6.99
C3: Vehicle vs. All doses	7.52	4.32

Proof of Hazard vs. Proof of Safety

Using the traditional approach, the evaluation of the number of micronuclei using proof of hazard revealed that none of the doses of T02 found to be harmless, i.e. signicant p-values was observed in all tests (*see* Table 5). In the case of T01, however, all p-values were insignicant leading to the conclusion of harmlessness on all dose groups (*see* Tables 3 and 4). In the present study, noninferiority can be determined by one-sided upper confidence limits because increasing micronuclei was of interest. Assuming a-priori definition of an acceptance threshold θ equals to three for proof of safety, a non-inferiority can be claimed in all dosage groups of T01 but not for T02 (*see* Tables 9 and 10). This coincides with the previous findings that T01 and T02, respectively, are negative and positive compounds.

Table 9
Proof of safety: one-sided confidence limit on non-inferiority (T01)

Comparison	48 h		72 h	
	RR	Upper RR	RR	Upper RR
Low-Vehicle	1.00	1.66	0.86	1.22
Med-Vehicle	0.77	1.32	0.91	1.25
High-Vehicle	0.93	1.55	0.82	1.15
Positive-Vehicle	7.85	11.45	-	-

Table 10
Proof of safety: one-sided confidence limit on non-inferiority (T02)

Comparison	RR	Upper RR
Low-Vehicle	3.13	6.08
Med-Vehicle	9.42	17.29
High-Vehicle	16.43	29.87

4.2 Statistical evaluation of *in vitro* micronucleus assay

In this section, the results of the *in vitro* experiment for D/L Menthol were presented. Being an *in vitro* method it ensures that the cells were exposed to well-defined concentrations of D/L Menthol. The lower one-sided confidence limits for relative risks for comparisons versus control without an order restriction from the Dunnet-type are given in Tables 11 to 13. Applications of the different concentrations with D/L Menthol in the absence and presence of metabolic activation (S-9) in both experiments resulted in frequencies which were similar to and not significantly ($p \leq 0.05$) different from those observed in concurrent vehicle controls

for all concentrations analyzed. The genotoxic activity of the compound was enhanced in the presence of the metabolic activation system. Clearly, culture grown in the presence of MMC and one level of Cyclophosphamide (CP) showed statistically significant positive responses, i.e. a several-fold increase in micronuclei frequency, as compared to the corresponding controls in all experiments hence validating the sensitivity of the system to the known mutagenic activity of MMC and CP under the experimental conditions used. The results presented here point towards that there was a non-genotoxic mechanism behind D/L Menthol.

Similar to *in vivo,* the CA test was also used to test the trend among the number of micronucleus for *in vitro*. This test is based on a totally ordered alternative hypothesis to assess the concentration-response relationship. For the three CA tests in the experiment with treatment conditions 24/S-9, 4/S-9, and 4/S+9, the p-values obtained were 0.8981, 0.1362 and 0.2125, respectively. The p-values were distinctly larger which favor the null hypothesis of no significant trend among the number of micronuclei over the alternative hypothesis that the number of micronucleus increases with increasing concentration scores.

In addition to looking at statistical significance of the trend, it is also imperative to assess the biological relevance of the results. For this reason, the Williams-type trend test as recommended by HG was performed which was constructed for both a total order alternative as well as specific comparisons versus control. Tables 14 to 16 give the relative risk estimates and their lower confidence limits for Williams-type contrasts. In this test, the presence of a significant trend in the number of micronuclei with increasing concentration can be concluded if at least one of the lower limit confidence intervals for the relative risks excludes the value of one (Herberich and Horthorn, 2012). Clearly, no trend can be inferred using the Williams-type contrast because all the 97.5% lower simultaneous confidence intervals included the value 1. Moreover, the distance of the lower limits to 1, ranges from 0.01 to 0.44, were small that toxicologically insignificant was very likely. This is, in comparison to CA test, the advantage of Williams-type test wherein the lower limit of the relative risk can be used to interpret the effect size in terms of biological relevance. Furthermore, this test provides more information regarding the dose-response shape than a simple p-value for a global test of trend (Herberich and Horthorn, 2012).

Table 11

D/L Menthol, 24 hour treatment in the absence of S-9

Comparisons	RR	Lower RR	Pvalue
Conc. 1 / DMSO	1.51	0.76	0.29
Conc. 2 / DMSO	0.84	0.38	0.95
Conc. 3 / DMSO	1.48	0.74	0.32
Conc. 4 / DMSO	1.54	0.77	0.27
Conc. 5 / DMSO	0.99	0.46	1.87
Conc. 6 / DMSO	0.57	0.23	1.00
MMC1 / DMSO	3.51	1.91	0.00
MMC2 / DMSO	5.66	3.16	0.00

Table 12

D/L Menthol, 4 hour treatment in the absence of S-9

Comparison	RR	LowerRR	Pvalue
Conc. 1 / DMSO	0.99	0.51	0.83
Conc. 3 / DMSO	1.36	0.74	0.36
Conc. 4 / DMSO	1.41	0.77	0.30
Conc. 5 / DMSO	1.40	0.77	0.30
Conc. 6 / DMSO	1.16	0.62	0.63
MMC1 / DMSO	4.99	3.02	0.00
MMC2 / DMSO	5.50	3.35	0.00

Table 13

D/L Menthol, 4 hour treatment in the presence of S+9

Comparisons	RR	LowerRR	Pvalue
Conc. 2 / DMSO	1.27	0.68	0.47
Conc. 3 / DMSO	1.36	0.73	0.36
Conc. 5 / DMSO	1.27	0.68	0.47
Conc. 6 / DMSO	1.29	0.69	0.44
CP1 / DMSO	1.55	0.85	0.17
CP2 / DMSO	3.19	1.87	0.00

Table 14

Williams-type contrasts for relative risks on the number of MN (4h/-S9)

Comparison	RR	Lower RR
C1: Vehicle vs. Concentration 6	1.16	0.67
C2: Vehicle vs. Concentrations 6 and 5	1.27	0.80
C3: Vehicle vs. Concentrations 6, 5 and 4	1.32	0.84
C4: Vehicle vs. Concentrations 6, 5, 4 and 3	1.33	0.86
C5: Vehicle vs. Concentrations 6, 5, 4, 3 and 1	1.25	0.81

Table 15
Williams-type contrasts for relative risks on the number of MN (24h/-S9)

Comparison	RR	LowerRR
C1: Vehicle vs. Concentration 6	0.56	0.26
C2: Vehicle vs. Concentrations 6 and 5	0.75	0.41
C3: Vehicle vs. Concentrations 6, 5 and 4	0.96	0.55
C4: Vehicle vs. Concentrations 6, 5, 4 and 3	1.06	0.63
C5: Vehicle vs. Concentrations 6, 5, 4, 3 and 2	1.01	0.61
C5: Vehicle vs. Concentrations 6, 5, 4, 3, 2 and 1	1.09	0.66

Table 16
Williams-type contrasts for relative risks on the number of MN (4h/+S9)

Comparison	RR	LowerRR
C1: Vehicle vs. Concentration 6	1.29	0.75
C2: Vehicle vs. Concentrations 6 and 5	1.28	0.80
C3: Vehicle vs. Concentrations 6, 5 and 3	1.31	0.83
C4: Vehicle vs. Concentrations 6, 5, 3 and 2	1.30	0.83

Proof of Safety vs. Proof of Hazard

In this section, the data were evaluated using proof of safety and proof of hazard. From the previous findings it can not be ruled out that D/L Menthol was a non-genotoxic compound that should be assessed using a threshold concept. For this reason the proof of safety assuming a three-fold threshold of tolerability was performed. For the proof of hazard, using Dunnett's approach the nonsignificant p-values obtained in all the comparisons of concentrations versus control indicate that D/L Menthol concentrations were harmful (see Tables 11-13). As discussed earlier, the use of a nonsignificant p-value in the simple proof of hazard, however, is inappropriate. Consequently, the results of proof of safety were presented.

Tables 17-19 show the relative risk estimates and their upper confidence limit for Williams-type contrasts for the Poisson model. Though the given p-values speak nonsignificant of the test, notice also that the one-sided upper confidence limits were roughly close to the chosen three-fold threshold leading to the conclusion that some of the concentrations might be harmful. The upper limit under the treatment conditions 24h-S9 of Concentrations 1, 3 and 4 exceeded the tolerable threshold which might indicate harmful effect. However, there distant to 3 seems to be ignorable. The final determination regarding the harmlessness or harmfulness is a complicated process where toxicologists collaborate with biostatisticians (Horthorn and Hasler, 2008).

Table 17

Proof of safety: one-sided CI on non-inferiority (4h/-S9)

Comparison	Estimate	Upper RR	Pvalue
Conc. 1 / DMSO	0.99	1.94	0.81
Conc. 3 / DMSO	1.37	2.56	0.99
Conc. 4 / DMSO	1.42	2.65	0.99
Conc. 5 / DMSO	1.42	2.62	0.99
Conc. 6 / DMSO	1.16	2.21	0.94
MMC1 / DMSO	4.87	8.10	1.00
MMC2 / DMSO	5.34	8.80	1.00

Table 18

Proof of safety: one-sided CI on non-inferiority (24h/-S9)

Comparison	Estimate	Upper RR	Pvalue
Conc. 1 / DMSO	1.52	3.11	1.00
Conc. 2 / DMSO	0.84	1.90	0.69
Conc. 3 / DMSO	1.49	3.06	1.00
Conc. 4 / DMSO	1.54	3.17	1.00
Conc. 5 / DMSO	0.99	2.16	0.85
Conc. 6 / DMSO	0.56	1.41	0.26
MMC1 / DMSO	3.50	6.50	1.00
MMC2 / DMSO	5.57	10.96	1.00

Table 19

Proof of safety: one-sided CI on non-inferiority (4h/+S9)

Comparison	Estimate	Upper RR	Pvalue
Conc. 2 / DMSO	1.28	2.43	0.97
Conc. 3 / DMSO	1.37	2.58	0.99
Conc. 5 / DMSO	1.28	2.44	0.97
Conc. 6 / DMSO	1.30	2.46	0.98
CP1 / DMSO	1.58	2.91	1.00
CP2 / DMSO	3.20	5.40	1.00

5 Discussion, Conclusion and Recommendation

The ultimate goal of this project was to elucidate the induced chromosomal damage potential of T01 and T02 using *in vivo* and D/L Menthol using *in vitro* micronucleus assay. Using *in vivo*, as discussed in the previous section, the two different statistical approaches, i.e. Poisson and quasi-Poisson models, led to the same conclusion – negative results for T01 at different sampling time and a positive result for T02. The crucial part in the analysis is the consequence of failing to account for overdispersion, as in the case of fitting the Poisson model, which may lead to incorrect inferences. The presence of extra variability may be due to several factors, such as individual biological variation in response to the compounds under study, errors in weighing and pipetting of the chemicals, the period between injecting the chemical and killing the mice and so on. To describe the influence of ignoring the between-animal variability, the traditional ANOVA-like approach was performed and compared with the analysis which takes into account the extra-variability. Evidently, the comparison of incidences of micronucleated polychromatic erythrocytes (MNPCE) using a quasi-Poisson model was shown to be more appropriate than a similar procedure using a Poisson model. The latter statistical approach becomes more liberal with increasing overdispersion, giving confidence limits for significance that are too narrow.

In the case of *in vitro* micronucleus assay, though the culture was the randomized experimental unit it would be more appropriate if the between-cultures variability was taken into account. However, because of the commonly used two cultures only, the estimation of a related dispersion parameter for proportion/count is rather unstable. This means if more cultures are available, model with extra-variation like quasi-Poisson model is recommended. For this reason, HG propose an approach for the pooled-over culture data, i.e. pooling all the number of micronuclei over a culture, using a Poisson model. Findings revealed that D/L Menthol did not induce a clear dose-dependent increase in the micronuclei occurrence. Several studies have been undertaken to assess the potential of menthol to induce chromosomal damage. Ishide *et al.* (1984) examined the induction of chromosomal aberrations in Chinese hamster fibroblast cultures treated with menthol, with or without metabolic activation. At the maximum concentration of 0.02 mg menthol/ml in culture for 48 h, no induction of chromosomal aberrations was evident. Similar conclusion was obtained in a study conducted by Anderson and Jensen (1984), i.e. no induction of genotoxicity by menthol using concentrations of menthol from 6.4 to 800 mg/plate. Murthy *et al*. (1991)

provided, for the first time, the data on the effect of menthol in human chromosomes. The findings revealed that menthol did not induce chromosomal aberrations and sister chromatid exchange assays in human chromosomes.

In terms of claiming a possible genotoxicity of the given compounds, using proof of safety vs. proof of hazard, HG conclude that the former is more appropriate than the latter. In this study a three-fold threshold of tolerability for the non-inferiority test was assumed. However, it is worthwhile to note that this chosen threshold indicates the difficulty of claiming harmlessness with sample size of 5. HG discuss that for a primary claim of being not genotoxic, more animals than five are needed. In toxicological studies the sample size is often determined on the basis of regulatory guidelines. Moreover, aside from using non-inferiority test for proof of safety an alternative approach such as the step-up estimation of the maximal safe dose can also be performed (HG, 2009).

In terms of the interpretation of the results in *in vivo* and *in vitro,* there are several criteria for determining a positive response, one of which is a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations. If none of the criteria are satisfied then the test substance is considered to be nonmutagenic in this system. Sofuni et al (1990) considered the dose response to be (strong) positive if it had two significant doses out of three dose groups and decided it to be weakly positive if it had only one significant dose and there was a significant trend. However, both biological and statistical significance should be considered together in an evaluation.

Taking together, as it was mentioned above, findings revealed no compelling evidence of chromosomal damage as measured by micronuclei formation for compounds T01 and D/L Menthol except T02. However, it is highly recommended to confer with toxicologist for the biological relevance of the findings.

References

Altman, D.G. & Bland, J.M. (1995). Absence of evidence is not evidence of absence. *British Medical J.,* **311(7003)**, 485.

Anderson, P. H. & Jensen, J. J. (1984). Mutagenic investigation of peppermint oil in the Salmonella/mammalian microsome test. *Mutation Research,* **138**, 17-20.

Armitage, P. (1955). Test for linear trends in proportions and frequencies. *Biometrics ,* **11(3),** 375-386.

Bretz, F. & Hothorn, L.A. (2002). Detecting dose-response using contrasts: asymptotic power and sample size determination for Poisson data. *Stat Med*., **21(22),** 3325-3335.

Cochran, W.G. (1954). Some methods for strengthening the common χ^2 tests. *Biometrics*. **10**, 417-451.

Hayashi, M., Yoshimura, T. and Ishidate, Jr. M. (1989). A procedure for data analysis of the rodent micronucleus test involving historical control. *Environmental and Molecular Mutagenesis*, **13**, 347-356.

Herberich, E. & Hothorn, L. (2012). Statistical evaluation of mortality in long-term carcinogenicity bioassays using a Williams-type procedure. *Regulatory Toxicology and Pharmacology*, **64**, 26-34.

Hauschke, D., Kieser, M. & Hothorn, L. A. (1999). Proof of safety in toxicology based on the ratio of two means for normally distributed data. *Biometrical Journal*, **41(3),** 295-304.

Hothorn, L.A. & Gerhard, D. (2008). Statistical evaluation of the *in vitro* micronucleus assay. Reports of the Institute of Biostatistics No. 09/2008.

Hothorn, L.A. & Gerhard, D. (2009). Statistical evaluation of the *in vivo* micronucleus assay. *Arch Toxicol*, **83**, 625-634

Hothorn, L.A. & Hasler, M. (2008). Proof of hazard and proof of safety in toxicological studies using simultaneous confidence intervals for differences and ratios to control. *Journal of Biopharmaceutical Statistics*, **18**, 915-933.

Ishidate M., Jr, Sofuni T., Yoshikawa, K., Hayashi, M., Nohmi, T., Sawada, M. and Matsuoka, A. (1984) Primary mutagenicity screening of food additives currently used in Japan . *Food and Chemical Toxicology*, **22**, 623-636.

Kim, B.S., Cho, M., & Kim, H.J. (2000). Statistical analysis of *in vivo* rodent micronucleus assay. *Mutation Research*, **469**, 233-241.

Kirkland, D. J. (2000). Statistical Evaluation of Mutagenicity Test Data. Cambridge University Press.

Krishna, G. & Hayashi, M. (2000). In vivo rodent micronucleus assay: protocol, conduct and data interpretation. *Mutation Research*, **455(1-2)**, 155-166.

Lee, J., Han, G., Fulp, W. & Giuliano, A. (2012). Analysis of overdispersed count data: application to the Human Papillomavirus Infection in Men (HIM) Study. *Epidemiol Infect,*1087–1094.

Margolin, B.H. & Risko, R.J. (1988). The statistical analysis of in vivo genotoxicity data: Case studies of the rat hepatocyte UDS and mouse bC (eds): "Evaluation of Short-Term Test for Carcinogens. Report of the International Proram on Chemical Safety's Collaborative Study on In Vivo Assays." Cambridge: Cambridge University Press vol. 1 pp. 129-142.

Murthy, P.B.K., Ahmed, M.M., Miller, B., Pujadas, E. & Gocke, E. (1991). Lack of genotoxicity of menthol in chromosome aberration and sister chromatid exchange assays using human lymphocytes *in vitro*. *Toxic in Vitro*, **5**, 337-34.

OECD 487 (2009). OECD guideline for the testing of chemicals draft proposal for a new guideline: *in vitro* mammalian cell micronucleus test (Mnvit).

Paul, S. & Saha, K.K. (2007). The generalized linear model and extensions: a review and some biological and environmental applications. *Environmetrics,* **18(4)**, 421-443.

Parodi, S. & Bottarelli, E. (2006). Poisson regression model in epidemiology - an introduction. Medic. Vet., 25-44.

Ramon, L., Walter, S. & Rudolf, F. (2002). SAS for linear models, fourth edition. North Carolina: SAS Institute Inc.

Shahrim, Z., Baharuddin, P., Yahya, N., Muhammad, H., Bakar, R. & Ismail, Z. (2006). The *in vivo* rodent micronucleus assay of Kacip Fatimah (*Labisia pumila*) extract. *Tropical Biomedicine,* **23(2)**, 214-219.

Sofuni, T., Matsuoka, A., Sawada, M., Ishidate Jr., M., Zeiger, E. & Shelby, M.D. (1990). A comparison of chromosome aberration induction by 25 compounds tested by two Chinese hamster cells (CHO and CHL) systems in culture. *Mutation Research*, **241**,175-213.

Appendix

Table A

D/L Menthol, 24 hour treatment in the absence of S-9

Treatment	Replicate	Total MNBN Cells Scored	Total Cells Scored	Proportion of MNBN Cells Scored	Significance
DMS	A	10	1073	0.009	
DMS	B	12	1080	0.011	
	Total	22	2153	0.010	-
Conc. 1	A	15	1101	0.014	
Conc. 1	B	13	1094	0.012	
	Total	28	2195	0.013	NS
Conc. 2	A	7	1066	0.007	
Conc. 2	B	8	1071	0.007	
	Total	15	2137	0.007	NS
Conc. 3	A	13	1080	0.012	
Conc. 3	B	14	1082	0.013	
	Total	27	2162	0.012	NS
Conc. 4	A	15	1077	0.014	
Conc. 4	B	13	1081	0.012	
	Total	28	2158	0.013	NS
Conc. 5	A	10	1088	0.009	
Conc. 5	B	8	1091	0.007	
	Total	18	2179	0.008	NS
Conc. 6	A	6	1100	0.005	
Conc. 6	B	4	1033	0.004	NS
	Total	10	2133	0.005	
Conc. 7	A	0	0		
Conc. 7	B	0	0		
	Total	0	0		
MMC1	A	31	1106	0.028	
MMC1	B	35	1101	0.032	
	Total	66	2207	0.030	
MMC2	A	56	1145	0.049	
MMC2	B	55	1149	0.048	
	Total	111	2294	0.048	

NS: not significant at 5% level of significance using Dunnet-type procedure

Table B
D/L Menthol, 4 hour treatment in the absence of S-9

Treatment	Replicate	Total MNBN Cells Scored	Total Cells Scored	Proportion of MNBN Cells Scored	Significance
DMS	A	12	1061	0.011	
DMS	B	11	1065	0.010	
		23	2126	0.011	-
Conc. 1	A	11	1071	0.010	
Conc. 1	B	12	1077	0.011	
		23	2148	0.011	NS
Conc. 2	A	0	0		
Conc. 2	B	0	0		
		0	0		
Conc. 3	A	12	1074	0.011	
Conc. 3	B	20	1094	0.018	
		32	2168	0.015	NS
Conc. 4	A	16	1074	0.015	
Conc. 4	B	17	1078	0.016	
		33	2152	0.015	NS
Conc. 5	A	18	1108	0.016	
Conc. 5	B	16	1123	0.014	
		34	2231	0.015	NS
Conc. 6	A	14	1119	0.013	
Conc. 6	B	14	1111	0.013	
	Total	28	2230	0.013	NS
Conc. 7	A	0	0		
Conc. 7	B	0	0		
	Total	0	0		
MMC	A	66	1212	0.054	
MMC	B	67	1209	0.055	
	Total	133	2421	0.055	
MMC	A	76	1279	0.059	
MMC	B	79	1278	0.062	
	Total	155	2557	0.061	

NS: not significant at 5% level of significance using Dunnet-type procedure

Table C

D/L Menthol, 4 hour treatment in the presence of S+9

Treatment	Replicate	Total MNBN Cells Scored	Total BN Cells Scored	Proportion of MNBN Cells Scored	Significance
Vehicle (DMS)	A	10	1091	0.009	
Vehicle (DMS)	B	12	1092	0.011	
		22	2183	0.010	-
Conc. 1	A	0	0		
Conc. 1	B	0	0		
		0	0		-
Conc. 2	A	13	1089	0.012	
Conc. 2	B	15	1090	0.014	
		28	2179	0.013	NS
Conc. 3	A	16	1092	0.015	
Conc. 3	B	14	1090	0.013	
		30	2182	0.014	NS
Conc. 4	A	0	0		
Conc. 4	B	0	0		
		0	0		-
Conc. 5	A	14	1084	0.013	
Conc. 5	B	14	1087	0.013	
		28	2171	0.013	NS
Conc. 6	A	14	1106	0.013	
Conc. 6	B	15	1115	0.013	
	Total	29	2221	0.013	NS
Conc. 7	A	0	0		
Conc. 7	B	0	0		
	Total	0	0		-
CP1	A	15	1095	0.014	
CP1	B	20	1122	0.018	
	Total	35	2217	0.016	NS
CP2	A	40	1158	0.035	
CP2	B	36	1170	0.031	
	Total	76	2328	0.033	

NS: not significant at 5% level of significance using Dunnet-type procedure

Auteursrechtelijke overeenkomst

Ik/wij verlenen het wereldwijde auteursrecht voor de ingediende eindverhandeling:
Statistical Evaluation of In Vivo and In Vitro Micronucleus Assays in Toxicology

Richting: **Master of Statistics-Biostatistics**
Jaar: **2013**

in alle mogelijke mediaformaten, - bestaande en in de toekomst te ontwikkelen - , aan de Universiteit Hasselt.

Niet tegenstaand deze toekenning van het auteursrecht aan de Universiteit Hasselt behoud ik als auteur het recht om de eindverhandeling, - in zijn geheel of gedeeltelijk -, vrij te reproduceren, (her)publiceren of distribueren zonder de toelating te moeten verkrijgen van de Universiteit Hasselt.

Ik bevestig dat de eindverhandeling mijn origineel werk is, en dat ik het recht heb om de rechten te verlenen die in deze overeenkomst worden beschreven. Ik verklaar tevens dat de eindverhandeling, naar mijn weten, het auteursrecht van anderen niet overtreedt.

Ik verklaar tevens dat ik voor het materiaal in de eindverhandeling dat beschermd wordt door het auteursrecht, de nodige toelatingen heb verkregen zodat ik deze ook aan de Universiteit Hasselt kan overdragen en dat dit duidelijk in de tekst en inhoud van de eindverhandeling werd genotificeerd.

Universiteit Hasselt zal mij als auteur(s) van de eindverhandeling identificeren en zal geen wijzigingen aanbrengen aan de eindverhandeling, uitgezonderd deze toegelaten door deze overeenkomst.

Voor akkoord,

Olivo, John Closter

Datum: **11/09/2013**