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Letter to the Editor

Comments to the paper by Nunes et al. (2007), Analysis of genotoxic potentiality of stevioside by comet assay, *Food and Chemical Toxicology* 45 (2007) 662–666

Nunes et al. (2007) orally administered 1 concentration (4 mg/ml) of stevioside (88.6% purity) to Wistar rats. DNA-damage was evaluated by the comet assay. They reported lesions in peripheral blood, liver, brain and spleen cells, the most pronounced effects being in the liver. Some comments have to be made to this paper. First of all, the structure shown in their Fig. 1b is not correct and it is not that of steviol, but that of *ent*-kaurenate. The authors used stevioside with a purity of only 88.6% and they administered only 1 concentration, i.e. the dose-dependence was not tested at all. In their Tables 1 and 2 the SD's are very large, sometimes much larger than the mean itself! There was no positive control included in the experiment. As the authors performed tests over a period of 6 weeks, they should have included an internal standard to check the electrophoresis parameters over that long period. They did not refer to the excellent work by Sekihashi et al. (2002) and Sasaki et al. (2000) who also tested stevioside and a large number of other compounds under strictly standardised conditions including dose dependency, a positive and negative control, and who did not find DNA-damage by steviol glycosides nor by steviol. Moreover, Sekihashi et al. (2002) also tested stomach and colon cells, and this is very relevant as steviol glycosides are not absorbed (Koyama et al., 2003; Geuns et al., 2003). The authors refer to a metabolism study of IV injected ^{131}I-stevioside. This metabolism might totally differ from that after oral uptake, and the ^{131}I might give a totally different metabolism. The metabolism of oral stevioside has been thoroughly studied by Simonetti et al. (2004) and Geuns et al. (2004, 2006, 2007) and it was shown that there is no accumulation or metabolism of steviol in the human body, except steviol glucuronide synthesis that is excreted in the urine. The scores of the control blood cells (their Fig. 2) vary from 0.6 ± 1.34 to up to 27 ± 13.3 at 6 weeks and increase and decrease at different time points (observe the values of the SD too). The authors suggest stress as the possible cause. However, this seems unbelievable and a lack of standardisation by use of an internal standard, and a lack of control of the quality of the feed, that might contain mutagenic compounds, seem more likely. Finally, the p-value discussed on p. 664, left column line 11 ($p < 0.001$) is different from that in Table 1 ($p < 0.01$).

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Response

Stevioside biological effects have been widely studied by different research groups, mainly because of the increasing consumption of stevioside around the world. Some authors have suggested that this compound has no mutagenic properties (Pezzuto et al., 1985; Suttajit et al., 1993; Matsui et al., 1996; Klongpanichpak et al., 1997), while many others have suggested that stevioside could be metabolized to steviol by liver and intestinal microflora (Wingard et al., 1980; Nakayama et al., 1986; Cardoso et al., 1996; Hutapea et al., 1997; Koyama et al., 2003). In this chemical structure, authors suggest that steviol could play a role in mutagenic events (Pezzuto et al., 1985; Matsui et al., 1996; Terai et al., 2002), while others did not point to this fact (Suttajit et al., 1993; Klongpanichpak et al., 1997). The controversial data concerning the mutagenicity can be explained thoroughly, for instance, due to different experimental approaches, type of cells, organisms assayed and stimuli used. Data comparison, according to Dr. Geuns, including that by Sekihashi et al. (2002) and Nunes et al. (2007), becomes very difficult. Sekihashi et al. (2002) used mice as experimental organisms and obtained their results after an acute (3 and 24 h) steviol oral administration. On the other hand, Nunes et al. (2007) used rats as experimental model and data were obtained after 45 days of oral aqueous stevioside solution subchronic intake. These differences imply that both studies are not replication of data and could themselves explain the controversial results.

The purity of stevioside used by Nunes et al. (88.62%) was higher than 85%, which was tried by Matsui et al. (1996) and Ishidate et al. (1984). Matsui and coworkers (1996) studied stevioside and steviol mutagenic properties through seven tests. They concluded that stevioside was not mutagenic, but steviol produced positive responses in three assays. Based on this, a lack of control of the feed quality, possibly containing mutagenic compounds, as suggested by Dr. Geuns, is unreliable and the DNA damage in peripheral total blood, liver, brain and spleen cells from rats should be due either to stevioside or its derivative steviol (Nunes et al., 2007).

Cardoso and coworkers (1996) studied the biological distribution and excretion of ^{131}I-stevioside. Although the injected ^{131}I-stevioside metabolism could be different from oral uptake; a high radioactivity level was found in liver, 1 min after injection. After 10 min, 44.69% of the injected dose was found in the tissue and the authors suggested that stevioside could be metabolized in the liver, since it was detected in the bile (Cardoso et al., 1996). Concerning the absence of steviol absorption, as suggested by Geuns et al. (2003), there are still some discrepancies in the literature (see for example: Nakayama et al., 1986; Koyama et al., 2003) and this issue must be better investigated.

In Nunes et al. (2007), if some comet counts of the control blood cells presented high standard deviation (SD) values, statistical analysis supported the significant differences between control and treated groups. Obviously, stress could be a reliable parameter to explain this, once both control and treated animals showed increased number of lesions. In addition, stress as source of DNA damage has been reported elsewhere through REM sleep deprivation in rats (Fonseca et al., 2004).

Incidentally, in a paper published in the "Journal of Agricultural and Food Chemistry" (Geuns et al., 2006), data presented on Table 1 (page 2795) were not discussed along the paper. In this table, the lack of significant differences among marker values before and after tissue damage could be just due to an insufficient sample size. Authors carried out experiments with nine volunteers, although in material and methods section they had cited ten. Furthermore, there are huge variations of the standard deviations. For example, mean value $\pm$ SEM from lactate dehydrogenase, before stevioside administration, is 184 ± 41.6. So, the SD would be approximately 125 U/L to the same mean value. The variation within a group is higher than that one among all groups. Because of this, the statistical test employed did not detect a significant difference. In statistical analysis, an important question appears, in case strong variation occurs among data associated to a $p > 0.05$: is the sample size large enough to guarantee the lack of significance between the differences? This issue should be discussed in the paper.

Finally, agreeing with Dr. Geuns, Figure 1b (page 663) from the paper by Nunes et al. (2007) shows a steviol precursor, because it lacks an OH radical in 13-carbon. Moreover, the correct p-value is $p < 0.001$ (page 664 – left

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column, line 11), instead of $p < 0.01$ from Table 1. We are quite sorry about it, but errors may happen. For example, it occurred in Dr. Geuns' letter, in which he cited the title of his own paper as "Stevioside Metabolism by Human Volunteers" in place of "Metabolism of Stevioside by Healthy Subjects" as present in the journal "Experimental Biology and Medicine" web site.

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