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1 ***In vitro* bioassay investigations of the endocrine disrupting potential of steviol glycosides**
2 **and their metabolite steviol, components of the natural sweetener *Stevia***

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22

23 **Abstract**

24 The food industry is moving towards the use of natural sweeteners such as those produced by
25 *Stevia rebaudiana* due to the number of health and safety concerns surrounding artificial
26 sweeteners. Despite the fact that these sweeteners are natural; they cannot be assumed safe.
27 Steviol glycosides have a steroidal structure and therefore may have the potential to act as an
28 endocrine disruptor in the body. Reporter gene assays (RGAs), H295R steroidogenesis assay
29 and Ca^{2+} fluorimetry based assays using human sperm cells have been used to assess the
30 endocrine disrupting potential of two steviol glycosides: stevioside and rebaudioside A, and
31 their metabolite steviol. A decrease in transcriptional activity of the progesterone receptor was
32 seen following treatment with 25,000 ng/ml steviol in the presence of progesterone (157
33 ng/ml) resulting in a 31% decrease in progesterone response ($p = <0.01$). At the level of
34 steroidogenesis, the metabolite steviol (500-25,000 ng/ml) increased progesterone production
35 significantly by 2.3 fold when exposed to 10,000 ng/ml ($p = <0.05$) and 5 fold when exposed
36 to 25,000 ng/ml ($p = <0.001$). Additionally, steviol was found to induce an agonistic response
37 on CatSper, a progesterone receptor of sperm, causing a rapid influx of Ca^{2+} . The response
38 was fully inhibited using a specific CatSper inhibitor. These findings highlight the potential
39 for steviol to act as a potential endocrine disruptor.

40 **Key terms:** Endocrine disruptors, *in vitro* bioassays, steviol, reproductive disorders, CatSper;
41 *Stevia*

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46 **1. Introduction**

47 Steviol glycosides are high intensity sweeteners that can be isolated and purified from the
48 leaves of *Stevia rebaudiana* (Bertoni). These glycosides of the diterpene derivative steviol
49 consist mainly of stevioside and rebaudioside A. These chemicals have sweetening potentials
50 200-300 times that of sucrose, are thermally stable and account for approximately 5-10% of
51 dry leaf weight (Soejarto, *et al.*, 1982).

52 The increasing prevalence of diabetes and obesity worldwide, in conjunction with a growing
53 concern over the safety of chemical sweeteners such as aspartame has led to a growing
54 demand for natural non-calorie sweeteners such as steviol glycosides which are presumed
55 safe to eat and are of acceptable taste. With increasing health consciousness, the use of
56 steviol glycosides as a food additive is now encouraged to slow down the world-wide sugar
57 consumption and therefore contribute towards combating the increasing diabetes and obesity
58 rates (Brahmachari, *et al.*, 2011). Steviol glycosides are now authorised for use in a range of
59 commonly consumed food products (The European Commission, 2011), resulting in steviol
60 equivalents within a range of 20,000 up to 3,300,000 ng/ml abundantly present in foods
61 (Table 1).

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69 *Table 1: Examples of authorised uses of steviol glycosides (E-Number 960, expressed as*
70 *steviol equivalents) in food.*

Food category	Maximum level steviol equivalents (ng/ml or ng/g)	Restrictions/Exceptions
Cocoa and Chocolate products; as covered by Directive 2000/36/EC	270,000	Only energy-reduced or with no added sugars
Chewing gum	3,300,000	Only with no added sugar
Flavoured Drinks	80,000	Only energy reduced or with no added sugar
Potato – cereal, flour or starch-based snacks	20,000	
Jam, Jellies and marmalades and sweetened chestnut puree; as defined by Directive 2001/113/EC	200,000	Only energy-reduced jams, jellies and marmalades
Fruit and vegetables (preparations excluding compote)	200,000	Only energy-reduced

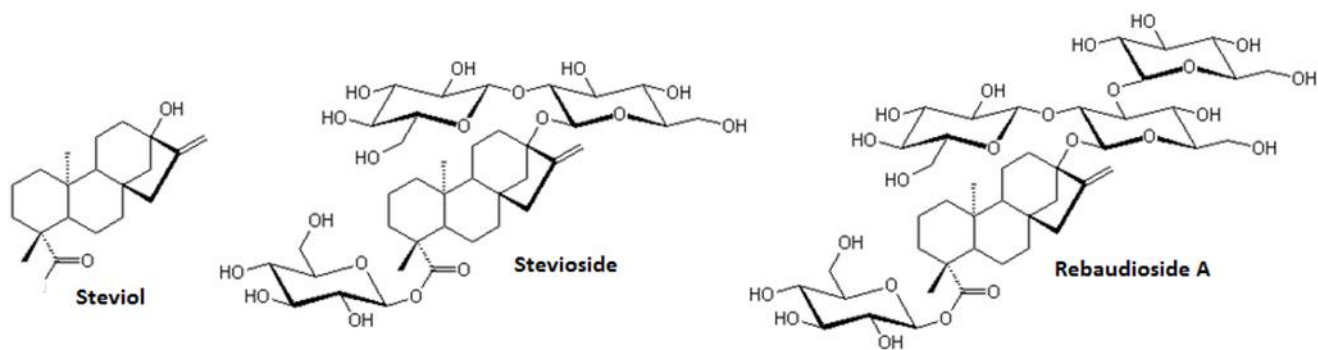
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72 The safety of steviol glycosides for the proposed use as food additives has been evaluated by
73 the Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of Food
74 and Agriculture Organization (FAO) of the United Nations, and the World Health
75 Organisation (WHO). JECFA have established an ADI (Acceptable Daily Intake) for steviol
76 glycosides (expressed as steviol equivalents) of 4 mg/kg bw/day (FAO, 2010). This ADI
77 takes into account a no-observed-adverse-effect-level (NOAEL) and applies a 100-fold safety
78 uncertainty factor extrapolated from a 2-year carcinogenicity study on rats consuming 2.5%
79 stevioside in the diet, equating to 967 mg stevioside/kg bw/day or 388 mg steviol

80 equivalents/kg bw/day (Xili *et al.*, 1992). However, conservative estimates of steviol
81 glycoside exposure, both in adults and in children, suggest that the ADI would in some cases
82 be exceeded at the maximum proposed use levels (The European Commission, 2011).
83 Consumption studies demonstrate that intact steviol glycosides are poorly absorbed by
84 humans after oral exposure (Pawar, *et al.*, 2013). However, they may be hydrolysed to the
85 metabolite steviol by intestinal microflora in the colon, where the majority is absorbed across
86 the gut wall and the rest is excreted in the faeces. To aid excretion from the body, absorbed
87 steviol is rapidly transformed to steviol glucuronide in the liver via conjugation to an acyl-
88 glucuronide (Brusick, 2008). Consequently, while steviol glycosides and steviol are not
89 detected in the blood or urine of human subjects, steviol glucuronide has been reported in
90 urine and steviol in faeces (Wheeler, *et al.*, 2008).

91 Human studies into “safe” levels of steviol glycoside consumption have shown that daily
92 doses of rebaudioside A up to 1,000,000 ng/person/day were well-tolerated by individuals
93 with type-2 diabetes mellitus or normal glucose metabolism (Maki *et al.*, 2008). This dose
94 equates to approximately 16,600 ng/kg bw/day for a 60 kg person and corresponds to
95 approximately 330,000 ng steviol equivalents/person/day or to 5,500 ng steviol
96 equivalents/kg bw/day (Maki *et al.*, 2008).

97 Steviol glycosides and steviol possess a steroidal structure (*Fig.1*) and therefore may have the
98 potential to act as an endocrine disrupting chemical (EDC).



99 *Figure 1: Chemical structure of steviol, stevioside and rebaudioside A*

100 The WHO defines an EDC as “an exogenous substance or mixture that alters function(s) of
101 the endocrine system and consequently causes adverse health effects in an intact organism, or
102 its progeny, or (sub)populations” (WHO, 2002). It is now known that EDCs can act via
103 multiple mechanisms within the cell and body. These mechanisms may include the
104 mimicking or blocking of transcriptional activation elicited by naturally present hormones via
105 binding to hormone receptors or interference with hormone production, secretion and control
106 systems in the steroidogenesis pathway (Tabb and Blumberg, 2005).

107 A limited number of studies have been presented to date on the effects of steviol glycosides
108 on the endocrine system. Some studies conversely report no adverse effects on the
109 reproduction, organs, sperm or foetal development of male or female rats (EFSA, 2010). The
110 majority of these studies have used *Stevia rebaudiana* extracts as opposed to the pure steviol
111 glycosides.

112 *In vitro* bioassays are extremely useful tools for the detection and mechanistic study of EDCs
113 (Connolly *et al.*, 2011). The current study aims to investigate the endocrine disrupting
114 activity of the natural sweeteners stevioside and rebaudioside A and their metabolite steviol.
115 Mammalian reporter gene assays (RGAs) incorporating natural steroid receptors have been
116 used to assess effects on nuclear receptor transcriptional activity (Willemsen *et al.*, 2004),
117 while the H295R human adrenal carcinoma cell line which has all the important enzymes and
118 genes needed for steroidogenesis has been used as a model to study effects on hormone
119 production (Hecker and Giesy, 2008). Ca^{2+} fluorimetry based assays using human sperm
120 cells, have also been used to assess the effects of EDCs on the Ca^{2+} channel of sperm
121 (CatSper), which has progesterone as its natural ligand (Schiffer *et al.*, 2014; Tavares *et.al.*,
122 2013). CatSper regulates several important sperm functions and is absolutely required for
123 male fertility (Smith *et.al.*, 2013).

124 To the best of our knowledge this is the first reported study investigating the endocrine
125 disrupting potential of the natural sweeteners stevioside and rebaudioside A and their
126 metabolite steviol at the level of nuclear receptor transcriptional activity using oestrogen,
127 androgen, progestagen and glucocorticoid RGAs, on steroidogenesis using the H295R
128 steroidogenesis model and on the non-genomic progesterone receptor of sperm, CatSper.

129 **2. Methods**

130 ***2.1 Chemicals and reagents***

131 Cell culture reagents were supplied by Life Technologies (Paisley, UK). The standards 17 β -
132 oestradiol, testosterone, hydrocortisone, progesterone, steviol, stevioside, rebaudioside A and
133 forskolin were obtained from Sigma-Aldrich (Poole, Dorset, UK). Dimethyl sulfoxide
134 (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were also supplied by Sigma-
135 Aldrich. Lysis reagents and luciferase assay system was purchased from Promega
136 (Southampton, UK). Fluorescent Ca²⁺ indicator Fluo-4 was provided by Invitrogen (USA).
137 MDL12330A was supplied by Tocris (USA). Human serum albumin was purchased from
138 Irvine Scientific (USA).

139 ***2.2 Cell culture***

140 Four reporter gene assay (RGA) cell lines, the MMV-Luc (oestrogen responsive), TARM-
141 Luc (androgen and progestagen responsive), TM-Luc (progestagen responsive) and TGRM-
142 Luc (glucocorticoid and progestagen responsive) were previously developed by transforming
143 human mammary gland cell lines with the luciferase gene under the control of a steroid
144 hormone inducible promoter (Willemsen *et al.*, 2004). The H295R human adrenocortical
145 carcinoma cell line, used in the steroidogenesis model, was obtained from the American Type
146 Culture Collection (ATCC CRL-2128, Manassas, VA, USA).

147 All cell lines were routinely grown in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark)
148 at 37°C with 5% CO₂ and 95% humidity. The RGA cell lines were cultured in cell culture
149 medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum
150 and 1% penicillin streptomycin. For culturing the MMV-Luc cell line, DMEM without
151 phenol red was used due to the weak oestrogenicity of phenol red. The H295R cell line was
152 cultured in cell culture medium containing DMEM with Ham's F-12 nutrient mixture (1:1)
153 supplemented with 1% ITS + Premix and 2.5% NuSerum (BD Biosciences, Bedford, MA,
154 US).

155 **2.3 Cell viability assays**

156 **2.3.1 MTT assay**

157 The MTT assay was performed to monitor the toxic effects of test compounds in the RGA
158 cell lines. Briefly, clear flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) were seeded
159 with 4 x 10⁵ cells/ml of the appropriate cell line. After 24 h stevioside and steviol (500, 1,000,
160 5,000, 10,000 and 25,000 ng/ml) and rebaudioside A (5,000, 10,000, 25,000, 50,000 and
161 100,000 ng/ml) were added to the cells at a final DMSO concentration of 0.1%. Test
162 compounds were then incubated for a further 48 h. Viable cells convert the soluble yellow
163 MTT into insoluble purple formazan by the action of mitochondrial succinate dehydrogenase.
164 Following incubation, supernatant was discarded and 50 µl of MTT solution/well (5mg/ml
165 stock in PBS diluted in 1:2.5 in assay media) was added and cells were incubated for a further
166 3 h. The supernatant was removed and 200 µl of DMSO was added to each well and
167 incubated for a further 10 min with agitation at 37°C to dissolve the formazan crystals.
168 Optical density was measured using a Sunrise spectrophotometer at 570 nm with a reference
169 filter at 630 nm (TECAN, Switzerland). Samples were analysed in triplicate wells and in
170 three independent experiments. Viability was calculated as a percentage absorbance of the
171 sample when compared with the absorbance of the DMSO solvent control.

172

2.3.2 AlamarBlue® assay

173 The viability of the H295R cells was determined using the AlamarBlue® assay. After the
174 removal of the media for hormone analysis, 1 ml of 1:10 (v:v) AlamarBlue® in cell culture
175 medium was added to each well and incubated for 6 h. A 100 µl volume was then removed
176 from each well and added into clear flat-bottomed 96-well microtitre plates (Nunc, Roskilde,
177 Denmark). Using a Sunrise spectrophotometer (TECAN, Switzerland) the absorbance was
178 measured at 570 nm and 600 nm. Viability was calculated as the % absorbance of the sample
179 in comparison with the absorbance of the solvent control (0.1%, v:v DMSO in media).

180

2.4 Reporter gene assays (RGAs)

181 The RGA procedure has previously been described by Frizzell *et al.*, (2011). Briefly, cells
182 were seeded at a concentration of 4×10^5 cells/ml, 100 µl/well in specialised white walled,
183 clear and flat bottomed 96-well plates (Greiner Bio-One, Frickenhausen, Germany). After
184 24 h, stevioside, steviol (500, 1,000, 5,000, 10,000 and 25,000 ng/ml), rebaudioside A (5,000,
185 10,000, 25,000, 50,000 and 100,000 ng/ml) and the steroid hormone standards were added to
186 the cells at a final DMSO concentration of 0.1%. The positive controls used with their
187 respective cell lines were: 1.36 ng/ml 17β-oestradiol (MMV-Luc), 14.5 ng/ml testosterone
188 (TARM-Luc), 157 ng/ml progesterone (TM-Luc) and 181 ng/ml hydrocortisone (TGRM-
189 Luc). A solvent control (0.1%, v:v DMSO in media) was also included for each cell line.
190 Antagonist tests were carried out by incubating the test compounds with the relevant positive
191 control for the cell line being tested. The cells were incubated for 48 h. The supernatant was
192 discarded and the cells washed once with PBS prior to lysis with 20 µl cell lysis buffer
193 (Promega, Southampton, UK). Finally, 100 µl luciferase substrate (Promega, Southampton,
194 UK) was injected into each well and luciferase activity measured using a Mithras Multimode
195 Reader (Berthold, Osterode, Germany). RGAs were performed in triplicate for each

196 experimental point and repeated in at least two independent exposures. The response of the
197 cell lines to the various compounds was measured and compared with the solvent and
198 positive controls.

199 ***2.5 Steroidogenesis assay***

200 As steviol glycosides are metabolised to steviol *in vivo*, steviol was tested at five different
201 concentrations (500, 1,000, 5,000, 10,000 and 25,000 ng/ml). The highest soluble
202 concentration achieved in DMSO was 25,000 ng/ml. The H295R assay was performed as
203 described previously (Gracia *et al.*, 2007). Briefly, the cells were seeded at a concentration of
204 3×10^5 cells/ml, 1 ml per well, in 24-well plates (BD Biosciences, Bedford, MA, US). The
205 cells were allowed to attach for 24 h before removing the media and replacing with fresh
206 media containing the test compounds dissolved in DMSO at a final concentration of 0.1%
207 (v:v). Forskolin was used as a positive control at a concentration of 10uM. A solvent control
208 (0.1%, v:v DMSO in media) was also included. Subsequently, the media was collected from
209 the wells following 48 h incubation and stored at -20°C until hormone quantification was
210 carried out. A 48 hour incubation time allows the concentrations of these hormones to reach a
211 plateau-phase under these conditions. The AlamarBlue® cell viability assay was carried out
212 on the remaining cells in each well. Each experimental point was performed in triplicate in at
213 least two independent exposures.

214 ***2.6 Hormone detection and quantification***

215 Frozen media was thawed prior to analysis. Oestradiol, testosterone and progesterone levels
216 in the media were quantified by enzyme-linked immunosorbent assays (ELISAs)
217 (Immunodiagnosics, Marburg, Germany). These highly specific kits are based on the
218 principle of competitive binding and are intended for the quantitative *in vitro* diagnostic
219 measurement of oestradiol (0–2000 pg/ml), testosterone (0–16 ng/ml) and progesterone (0–40

220 ng/ml) in serum and plasma, with sensitivities of 9.714 pg/ml, 0.083 ng/ml and 0.045 ng/ml
221 respectively. ELISA kits were carried out according to manufacturer's instructions with the
222 exception of the standard curves which were prepared in the same culture medium used for
223 the H295R assay. Prior to media analysis, it was confirmed that steviol (500-25,000 ng/ml)
224 did not cross-react with the progesterone ELISA antibody (data not included). The intra-assay
225 coefficient of variation was less than 10%. Standard curves were included on each ELISA
226 plate. The mean absorbance obtained from each standard was plotted against its concentration
227 using dose-response curves generated with Graph Pad Prism software.

228 ***2.7 Sperm preparation and measurement of changes in intracellular Ca²⁺*** 229 ***concentration***

230 Samples of human semen were obtained from healthy volunteers with their prior consent.
231 Sperm were prepared as described (Schiffer *et.al.*, 2014). Briefly, sperm were purified by a
232 "swim-up" procedure in human tubular fluid (HTF⁺) containing (in mM): 97.8 NaCl, 4.69
233 KCl, 0.2 MgSO₄, 0.37 KH₂PO₄, 2.04 CaCl₂, 0.33 Na-pyruvate, 21.4 lactic acid, 2.78 glucose,
234 21 HEPES, and 4 NaHCO₃ adjusted between pH 7.3-7.4 with NaOH. After washing, human
235 serum albumin (3 mg/ml) was added to HTF⁺. Sperm were incubated for at least 1 h in HTF⁺
236 with 3 mg/ml serum albumin at 37 °C in a 10% CO₂ atmosphere.

237 Changes in intracellular Ca²⁺ concentration in human sperm were measured in 384 multi-well
238 plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30 °C.
239 Sperm were loaded with the fluorescent Ca²⁺ indicator Fluo-4 (10 μM) for 45 min at 37 °C.
240 After incubation, excess dye was removed by centrifugation (700 x g, 10 min, RT). The
241 sperm pellet was resuspended in HTF⁺ to 5 × 10⁶ sperm/ml. Aliquots of 50 μl were loaded to
242 the wells of the multi-well plate. Fluorescence was excited at 480 nm and emission was
243 recorded at 520 nm with bottom optics. Fluorescence was recorded before and after injection

244 of 25 μ l (1:3 dilution) of negative control (buffer), positive control (progesterone, 5 μ M) and
245 steviol manually with an electronic multichannel pipette. Steviol was dissolved in DMSO to
246 10 mM and further diluted in HTF⁺. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (%),
247 indicating the percentage change in fluorescence (ΔF) with respect to the mean basal
248 fluorescence (F_0) before addition of steviol, positive control and negative control. For the
249 inhibition experiment, sperm were incubated with MDL12330A for 5 min prior to addition of
250 steviol.

251 ***2.8 Statistical Analysis***

252 All values shown are expressed as mean \pm standard deviation (SD) of at least two
253 independent exposures for the compounds tested (for two independent exposures n=2 and for
254 three independent exposures n=3). Data from the cell viability, reporter gene, steroidogenesis
255 and Ca²⁺ fluorimetry assays were analysed using Microsoft Excel and Graphpad PRISM 5
256 software (San Diego, CA). A one way analysis of variance (ANOVA) and Dunnett's Multiple
257 Comparison Test was used to determine significant differences between treatments and the
258 corresponding controls. The mean concentrations were tested for significant difference at the
259 95% confidence level, a *P*-value of <0.05 was considered as significant (*P*≤0.05 *, *P*≤0.01 **
260 and *P*≤0.001 ***).

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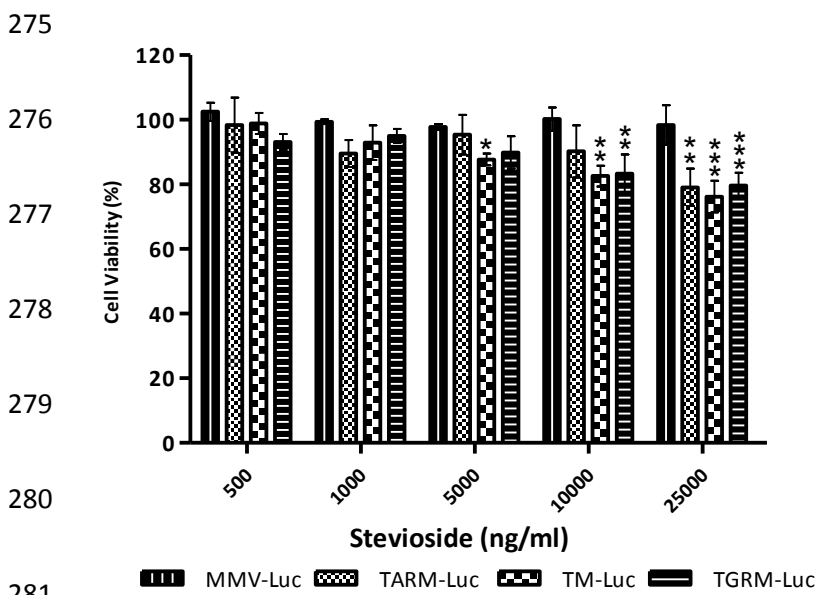
262 **3 Results**

263 ***3.1 Cell viability and cytotoxicity***

264 Stevioside (500-25,000 ng/ml), steviol (500-25,000 ng/ml) and rebaudioside A (5000-
265 100,000 ng/ml) were assessed for cytotoxicity by the MTT assay in the MMV-Luc (oestrogen
266 responsive), TM-Luc (progestagen responsive), TARM-Luc (androgen and progestagen
267 responsive) and TGRM-Luc (glucocorticoid and progestagen responsive) cell lines (*Fig.2*).

268 Cytotoxicity was observed at the higher concentrations of stevioside in the TARM-Luc
 269 (25,000 ng/ml $p \leq 0.001$), TM-Luc (5,000, 10,000, and 25,000 ng/ml, $P \leq 0.001$) and TGRM-
 270 Luc (10,000 and 25,000 ng/ml, $P \leq 0.001$) RGA cell lines.

271 Steviol (500-25,000 ng/ml) was assessed for cytotoxicity in the H295R cell line by the
 272 AlamarBlue® assay. Cytotoxicity was not observed at any of the concentrations tested (data
 273 not shown). The viability of the cells at the sample concentrations did not differ significantly
 274 from the DMSO solvent control ($P \geq 0.05$).

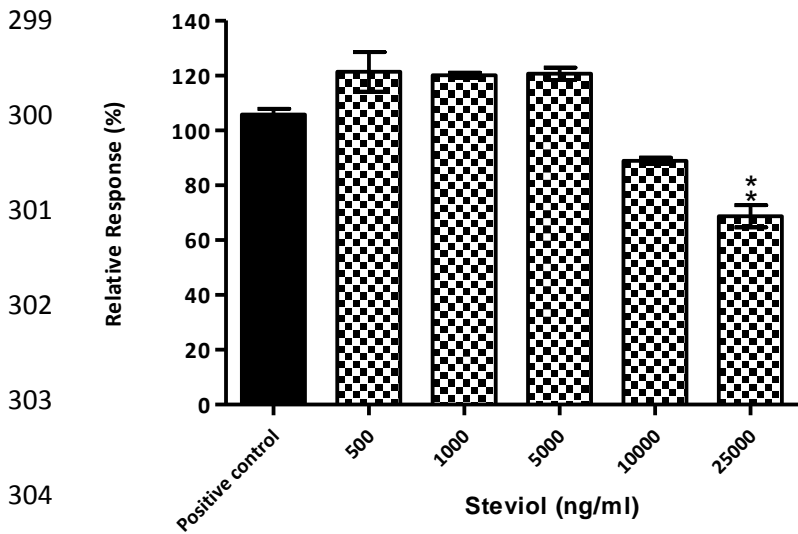


282 *Figure 2: Viability of the RGA cell lines following exposure to (a) 500-25,000 ng/ml*
 283 *stevioside for 48 h compared to the solvent control, as determined in the MTT assay. The*
 284 *MMV-Luc cell line is specific for the detection of oestrogens, TARM-Luc for androgens and*
 285 *progestagens, TM-Luc for progestagens and TGRM-Luc for glucocorticoids and*
 286 *progestagens. Values are means \pm SD for three independent exposures in triplicate (n = 3). P*
 287 *= <0.05 (*) P \leq 0.01 (**) P = <0.001 (***)*

288 **3.2 Reporter gene assay**

289 An agonist response was not observed for stevioside, steviol or rebaudioside A in the MMV-
 290 Luc, TM-Luc, TARM-Luc or TGRM-Luc cell lines (data not shown). Additionally, no
 291 antagonist response was seen for stevioside and rebaudioside A in the MMV-Luc, TM-Luc,
 292 TARM-Luc or TGRM-Luc cell lines (data not shown). Steviol appeared to exhibit an
 293 antagonist response in the progesterone responsive TM-Luc cell line, with a 28.1% reduction
 294 in progesterone response at the highest concentration of 25,000 ng/ml ($P \leq 0.05$) (Fig.3). At
 295 this concentration of steviol, no reduction in cell viability was observed in the MTT assay.
 296 Although there was a trend in reduction of glucocorticoid transcriptional activity at 25,000
 297 ng/ml steviol, this reduction was not significant ($P \geq 0.05$).

298



304

305 *Figure 3: Antagonist effects in the TM-Luc cell line cell lines for 500-25,000 ng/ml steviol.*
 306 *Responses measured are compared to the relative positive control (157 ng/ml progesterone).*
 307 *Response is expressed as the percentage response \pm SD for three independent exposures in*
 308 *triplicate (n = 3). $P = \leq 0.01$ (**)*

309 **3.3 Hormone production results**

310 Steviol did not induce significant changes in the production of oestradiol and testosterone
311 (data not shown). However, progesterone production increased significantly by 2.3 fold when
312 exposed to 10,000 ng/ml ($P = <0.05$) and 5 fold when exposed to 25,000 ng/ml ($P = <0.001$)
313 (Fig.4).

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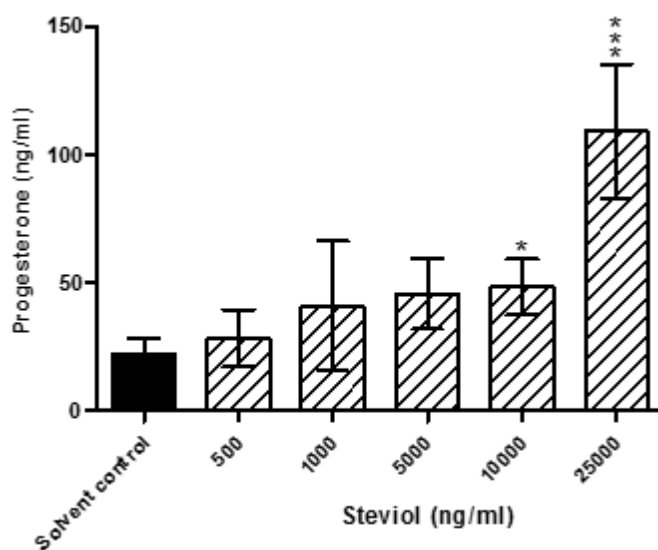
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323 *Figure 4: Progesterone production by H295R cells following exposure to 500-25,000 ng/ml*
324 *steviol for 48 h. Values are means \pm SD for three independent exposures in triplicate ($n = 3$).*
325 *$P = <0.05$ (*) $P \leq 0.001$ (***)*

326 **3.4 Effects on progesterone receptor of sperm, CatSper**

327 Steviol was found to induce Ca^{2+} signals in human sperm cells ($n=5$) (Fig. 5a). The induced
328 response was found to be inhibited $98.65 \pm 1.91\%$ by the specific CatSper inhibitor
329 MDL12330A ($n=3$) (Fig. 5b). Analysis of the dose-response relation yielded an EC_{50} of
330 $10.82 \pm 1.06 \mu M / 3,446.17 \pm 337,61 \text{ ng/ml}$ ($n=4$) (Fig. 5c-d). The EC_{02} was also calculated

331 which corresponds to the lowest effective dose of steviol for inducing effects on CatSper,
332 $0.676 \pm 0.623 \mu\text{M} / 215.3 \pm 198.4 \text{ ng/ml}$ (n=4).

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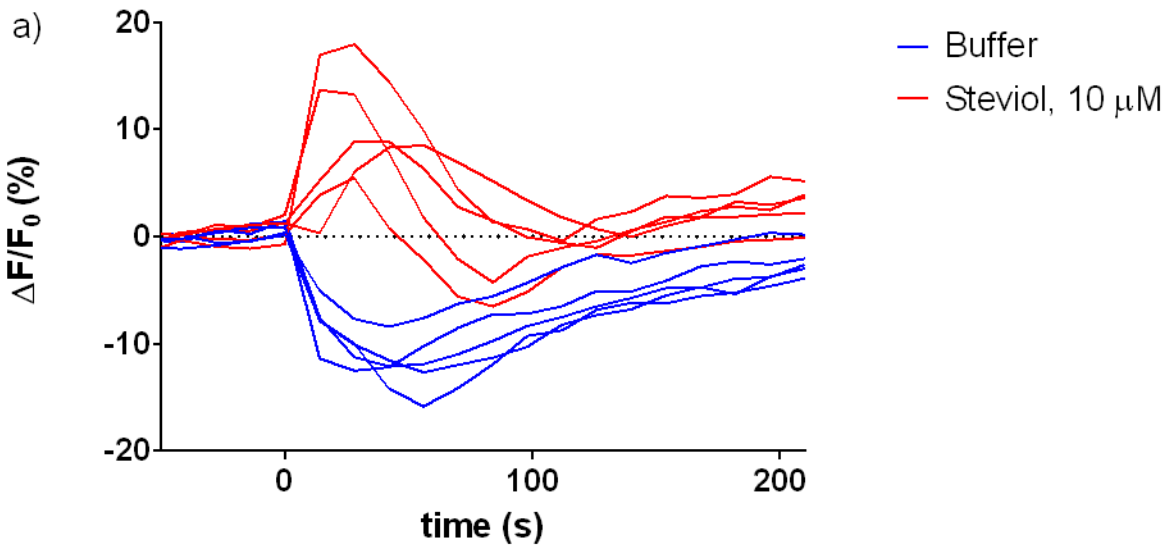
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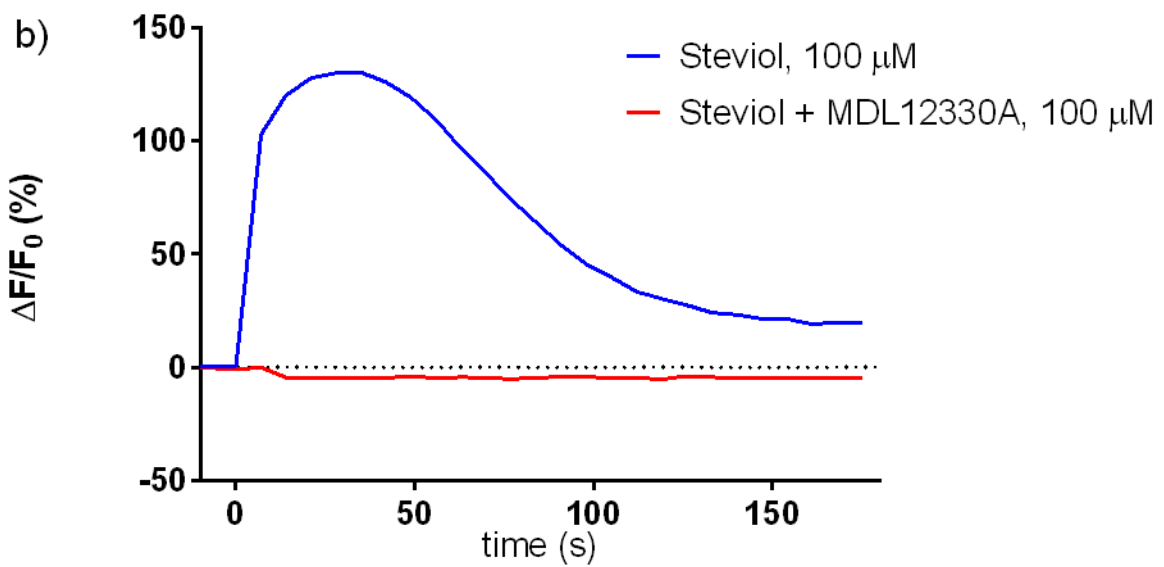
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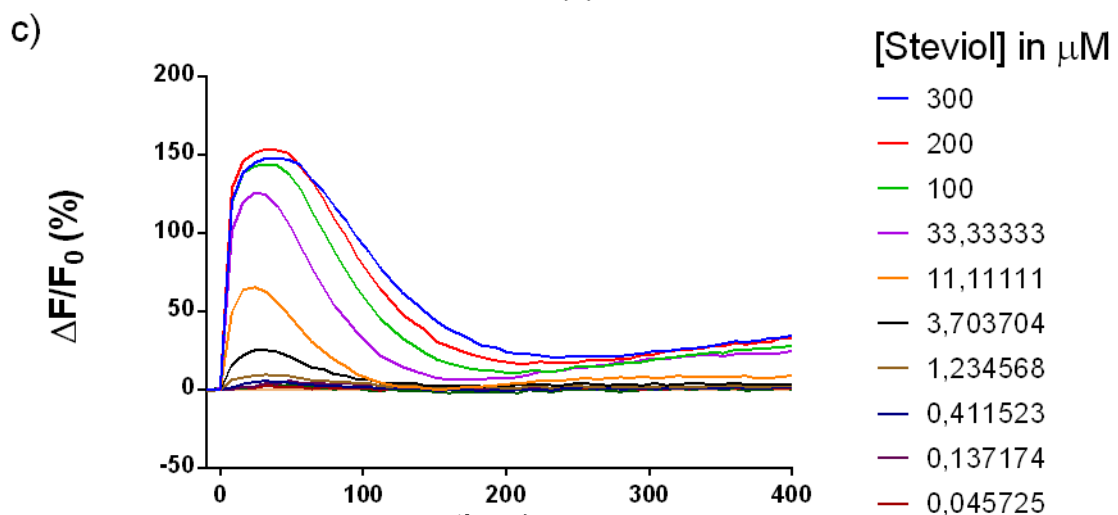


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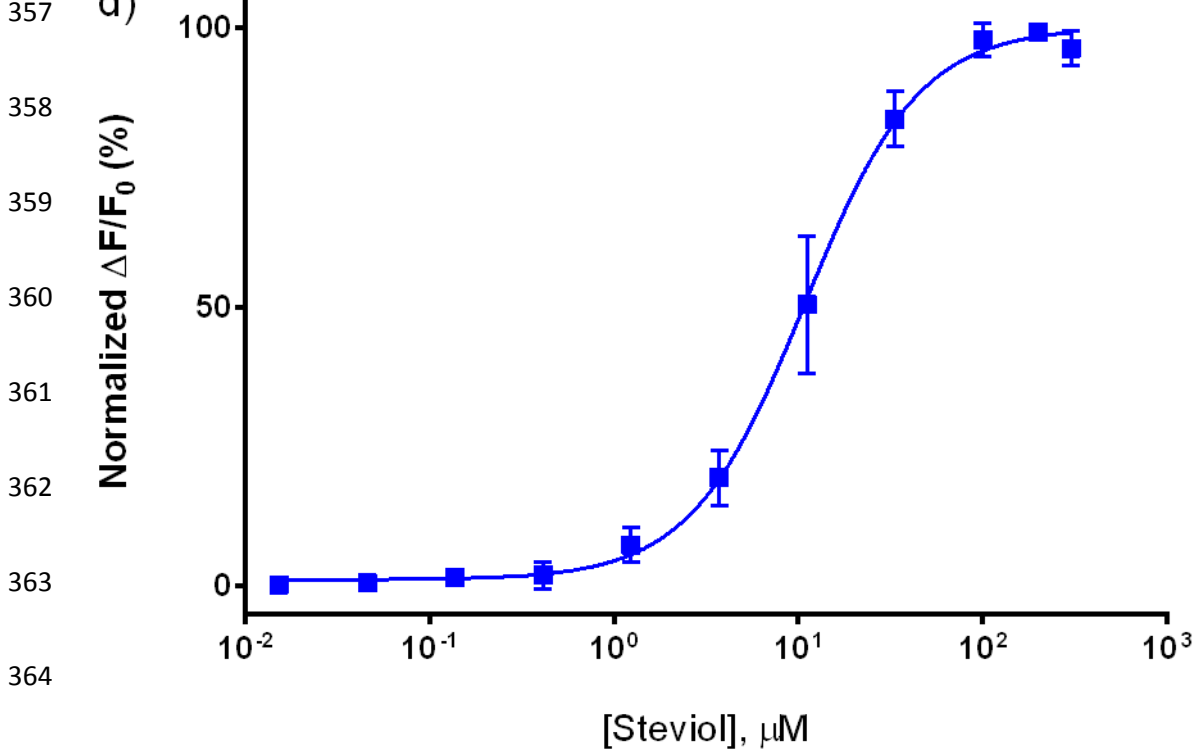
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366 *Figure 5 (a-d): (a) Ca^{2+} signals in human sperm cells induced by steviol, 10 μM , added at 0*

367 *s (n=5) (b) Steviol-induced Ca^{2+} signal in the absence (blue) and presence (red) of the*

368 *CatSper inhibitor MDL12330A (c) Steviol-induced Ca^{2+} signals from one experiment,*

369 *included for estimation of steviol dose-response relationship in d). (d) Normalized dose-*

370 *response relationships of steviol, mean \pm SEM (n=4).*

371 **4 Discussion**

372 The endocrine disrupting effects of steviol glycosides (stevioside and rebaudioside A) and
373 their metabolite steviol have been investigated using mammalian *in vitro* bioassays that are
374 able to detect endocrine disruption at the level of nuclear receptor transcriptional activity and
375 steroidogenesis. Additionally, the effect of steviol on the progesterone receptor of sperm,
376 CatSper, was investigated.

377 Cell viability assays are useful controls to ensure that the effects seen in the mechanistic
378 studies are not a result of decreasing cell viability. Cytotoxicity was observed with the higher
379 concentrations of stevioside in the TARM-Luc (25,000 ng/ml), TM-Luc (5,000, 10,000, and
380 25,000 ng/ml) and TGRM-Luc (10,000 and 25,000 ng/ml) responsive RGA cell lines.
381 Interestingly the MMV-Luc cell line did not appear to be affected by concentrations up to
382 25,000 ng/ml. This observed difference may be due to the fact that the parent cell line of the
383 TARM-Luc, TM-Luc and TGRM-Luc cell lines is T47D while for MMV-Luc it is MCF-7. A
384 similar effect was seen in a previous study by Frizzell *et al.*, (2013), where the test
385 compound, alternariol, reduced cell viability in the three T47D cell lines but with no
386 reduction in viability in the MMV-Luc cells (Frizzell *et al.*, 2013). Studies investigating the *in*
387 *vitro* cytotoxicity of stevioside, steviol and rebaudioside A are scarce. However, Ukiya *et al.*,
388 (2013) investigated the potential cytotoxicity of steviol in breast (SK-BR-3), leukemia
389 (HL60), lung (A549) and stomach (AZ521) cancer cell lines by means of the MTT assay and
390 stated that steviol at a concentration of up to 31,845 ng/ml did not induce cytotoxic effects,
391 therefore in agreement with the present study. In spite of this, Paul et al (2012) showed a
392 significant decrease from the solvent control at 3,185 ng/ml stevioside with the effects (71%
393 of cells undergoing apoptosis) being more pronounced 72 h treatments. Additionally, results
394 from the trypan blue test in this study showed that there was some cytotoxic activity
395 occurring even at the lowest concentration tested 796 ng/ml. However, the differences in
396 findings could be due to the fact that in the trypan blue test, cell membrane integrity is

397 measured rather than mitochondrial membrane integrity as measured by the MTT assay. Also
398 in the MTT test, stevioside was exposed to the MCF-7 cells for a period of 72 h as opposed to
399 48 h in the current study.

400 Reporter gene assays provide specific and biologically relevant ways to screen substances for
401 their hormonal effects at the level of nuclear receptor transcriptional activity (Willemsen *et*
402 *al.*, 2004). The current study revealed no agonist response in any of the RGA cell lines for all
403 compounds tested, however an antagonist response was observed in the progesterone
404 responsive TM-Luc cell line whereby 25,000 ng/ml steviol induced a 29.1% decrease in the
405 progesterone receptor nuclear transcriptional activity. In agreement with these findings, a
406 study by Oliveira-Filho *et.al.*, (1989) reported that the number of binding sites for androgens
407 were not altered in rats chronically administered *Stevia rebaudiana* extract. However, effects
408 on the androgen receptor have previously been highlighted by Uehara *et.al.*, (1982) who
409 demonstrated that purified stevioside displaced 5 α -dihydrotestosterone specifically bound to
410 prostate androgen receptors *in vitro* (Uehara *et al.*, 1982). This finding was confirmed with
411 the synthetic androgen, methyltrienolone, a specific ligand of androgen receptors (Uehara *et*
412 *al.*, 1983). Differences in findings may be due to the varying forms of stevioside used in the
413 studies i.e. purified or plant extract and also the type of study carried out i.e. *in vitro* vs *in*
414 *vivo*.

415 Compounds can also be classed as EDCs through disrupting steroidogenesis; a complex
416 process regulated by enzymes. Harvey *et al.*, (2007) state that the adrenal gland was often
417 neglected in regulatory endocrine disruption screening and testing despite it being the most
418 common toxicological target organ in the endocrine system (Harvey *et al.*, 2007). In the
419 current study the H295R model was utilised to investigate the effects of steviol on
420 steroidogenesis. It was observed that steviol had no effect on oestradiol or testosterone
421 production; however, at 10,000 ng/ml and 25,000 ng/ml, significant increases in the

422 production of progesterone were observed. Progesterone is an intermediary of oestradiol and
423 testosterone but the increased progesterone levels observed did not result in a down-stream
424 increase of oestradiol or testosterone. However, the steroidogenesis pathway is an intricate
425 and complicated pathway and there may be other events taking place in the pathway that are
426 affecting the levels of testosterone and oestradiol. In addition, the H295R model has been
427 validated as an OECD *in vitro* screening assay to screen for endocrine disruptor action on
428 oestradiol and testosterone production. The validation process points to 48 hrs as an optimal
429 time point with the concentrations of these hormones seemingly reaching a plateau-phase
430 under these conditions and therefore most research to date has been carried out under these
431 validated conditions. However, further time points, gene and protein expression studies may
432 reveal additional effects on the levels of oestradiol and testosterone where the intermediary
433 progesterone is increased.

434 Steviol was also found to induce an agonistic response on CatSper, the progesterone receptor
435 of sperm, with an EC_{50} of $10.82 \pm 1.06 \mu\text{M} / 3,446.17 \pm 337,61 \text{ ng/ml}$ and an EC_{02} of
436 $0.676 \pm 0.623 \mu\text{M} / 215.3 \pm 198.4 \text{ ng/ml}$. This response was fully inhibited ($98.65 \pm 1.91\%$)
437 using a specific CatSper inhibitor, indicating that the observed response is indeed mediated
438 through direct interaction between steviol and CatSper. This is of concern because CatSper
439 regulates several important sperm functions and is absolutely required for male fertility
440 (Smith *et.al.*, 2013). Other studies have similarly shown that structurally diverse chemicals
441 can act agonistically on CatSper (Schiffer *et.al.*, 2014; Tavares *et.al.*, 2013) and that these
442 chemicals can act additively in low doses to induce a Ca^{2+} response. This hints that even
443 low doses in the female reproductive tract could possibly affect human sperm cell function,
444 during their passage through the tract. To our knowledge, steviol has not been measured in
445 reproductive system fluids. However, due to the close anatomical relationship between the
446 distal colon/rectum with the reproductive tract in female and seminal vesicles/prostate in

447 males, it is possible that steviol absorbed in the distal colon/rectum, which bypasses the liver
448 and portal-circulation, can get into the reproductive fluids (Einer-Jensen and Hunter, 2005).

449 This study reports that steviol can modulate an increase in progesterone production and
450 antagonise the progesterone and agonise CatSper receptors. Progesterone plays a major role
451 in female reproductive health such as maintaining pregnancy, regulating the monthly
452 menstrual cycle and preparing the body for conception (Healy, 1990). Progesterone receptor
453 antagonists now have a use in clinical practice both as a contraceptive and in pregnancy
454 termination (Spitz, 2003). Planas and Kacute (1968) investigated the potential of aqueous
455 *Stevia* extract to act as a contraceptive in rats and found that the extract reduced fertility in
456 adult female rats of proven fertility and fertility continued to decrease for at least 50 to 60
457 days after intake was stopped (Planas and Kacute, 1968). A link between increased levels of
458 progesterone and weight gain has been reported (Galletti and Klopper, 1964; Lof *et al.*,
459 2009). Progesterone also has a significant role in insulin secretion. Insulin has an important
460 role in controlling blood sugar levels and therefore in patients with diabetes, insulin is either
461 at very low levels or low functioning. If insulin levels are altered, blood glucose regulation
462 will be adversely affected and subsequently, diabetes risk could increase (Diabetes UK,
463 2015). Straub *et al.*, (2001) reported that progesterone had the ability to inhibit glucose-
464 stimulated insulin secretion from isolated rat islets in a dose-dependent manner. However,
465 further in depth investigations of the relationship between progesterone levels in the body and
466 glucose-stimulated insulin secretion are required. The results of this study suggest that steviol
467 could potentially affect glucose and weight homeostasis indirectly through modulation of
468 steroid hormones. This is of heightened concern due to the fact that steviol glycosides are
469 non-calorific sweeteners aimed towards reducing the incidence of metabolic disease such as
470 diabetes and obesity.

471 A decrease in transcriptional activity of the progesterone receptor was observed following
472 exposure to 25,000 ng/ml steviol. In the steroidogenesis assay, a significant increase in
473 progesterone production was found from the lower concentration of 10,000 ng/ml steviol. A
474 report by EFSA (2011), estimated that exposure to steviol glycosides, expressed as steviol
475 equivalents, is around 5.6-6.8 mg/kg bw per day (5,600-6,800 ng/ml) for adult high level
476 consumers in the UK. Additionally, for European children, anticipated exposure of the high
477 level consumer is estimated to be 1.7-16.3 mg/kg bw per day (1,700-16,300 ng/ml).
478 Consequently, the levels studied within this investigation are in the range of dietary exposure
479 levels for children within the high level consumption range. Also, it is possible that as more
480 products containing the natural sweetener *Stevia* are developed and sold, exposure levels to
481 this compound will further increase (Goyal et al., 2009). *Stevia* will also be of particular
482 interest to certain groups of the population with metabolic conditions such as diabetes and
483 obesity and as a result these groups may have an even higher exposure to steviol glycosides.
484 Therefore, there is an urgent need to ascertain the current level of exposure to steviol
485 glycosides.

486 Metabolism studies in humans have reported very low blood levels of free steviol (JECFA,
487 2005). Steviol glucuronide has been reported to be primarily excreted in urine and steviol in
488 faeces (Wheeler et al., 2008). As previously outlined, absorbed steviol is rapidly transformed
489 to steviol glucuronide in the liver via conjugation to an acyl-glucuronide. Consequently, only
490 absorbed steviol which bypasses the liver and portal-circulation, e.g. absorbed in the distal
491 colon/rectum, will go unconjugated into general circulation and only until it passes the liver
492 (Brusick, 2008). Studies have reported that progesterone receptors are present in the epithelial
493 cells of the colon and progesterone has some effects directly on the gastrointestinal (GI) tract
494 (Guarino *et.al.*, 2011; Eliakim *et.al.*, 2000). Unconjugated steviol may be present at high
495 concentrations in the GI tract and may present the potential for steviol to inhibit progesterone

496 binding to the progesterone receptors. Thereby potentially enabling steviol to disturb
497 progesterone effects in the GI tract before being conjugated in the liver. It is increasingly
498 important that current metabolism studies are carried out to assess the metabolic pathways
499 and effects within the framework of the growing use of stevia glycosides and a widening
500 range of food products.

501 Despite the findings of this study, other research on steviol glycosides has concluded that
502 replacing table sugar or aspartame with these natural sweeteners has various health benefits.
503 Numerous plant glycosides have shown activity in the prevention of some dietary diseases
504 including cancer, obesity and diabetes (Bernal *et al.*, 2011). Steviol glycosides are non-
505 caloric and non-cariogenic sweeteners whose consumption may exert beneficial effects on
506 human health (Gardana *et al.*, 2010).

507 **5 Conclusion**

508 The metabolite of steviol glycosides, steviol, can antagonise the progesterone nuclear
509 receptor transcriptional activity and increase progesterone production. Additionally, steviol
510 was found to induce an agonistic response on Catsper, the progesterone receptor of sperm
511 cells. We have thus shown that steviol has the ability to affect progesterone signalling at three
512 different sites: 1) By lowering progesterone transcriptional activity 2) by increasing the
513 production of progesterone and 3) by acting as an agonist on Catsper, the progesterone
514 receptor of sperm. This study highlights the endocrine disrupting potential of natural
515 sweeteners such as those found in *Stevia rebaudiana* and suggests that emerging natural
516 sweeteners such as *Stevia* may not be safe alternatives to sugar and other synthetic
517 sweeteners. Further dietary exposure and metabolic studies are required to confirm their
518 safety.

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520

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524 **Conflicts of interest**

525 There are no conflicts of interest.

526 **References**

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