Assessment of Toxicity of Myristicin and 1’-Hydroxymyristicin in HepG2 Cell Line

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Running title:In vitro Myristicin and 1’-Hydroxymyristicin toxicity

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Authors' contribution:

Each author had a real and concrete contribution and all the co- authors have been approved the article's publication.

L. Marabini, C.L. Galli, M. Marinovich develop the project and designed the experiments.

L. Marabini conducted all the bioinformatics analyses

L. Neglia and E. Monguzzi carried out genotoxicity tests

Significance statement

The literature data concerning to this alkenylbenzene compound are limited and inconsistent. This study has provided additional informations regarding the myristicin genotoxicity and its metabolism. New data are needed to deal with human risk assessment more properly.

Keywords Alkenylbenzenes; Myristicin; genotoxicity; comet assay; micronucleus; in vitro toxicity; apoptosis

ABSTRACT

Background and Objective: Myristicin belongs to a class of potentially toxic chemicals (alkoxysubstitutedallylbenzenes) and despite the structural analogy with safrole, data on this compound are very controversial and unclear. In this work it was assessed the cytotoxic and genotoxic potential of myristicin and 1'-hydroxy-myristicin after 24 h of exposure in HepG2 cells.

Materials and Methods: The compounds were tested up to 600 µM concentration, for 24 hours. The genotoxicity was assessed with alkaline and neutral comet assay and micronucleus assay. The data were analyzed by One –Way ANOVA.

Results: It is to be emphasized thatonly the synthetic phase 1 metabolite (1'-hydroxymyristicin) showed a genotoxic effect starting from the concentration of 150 µM both in comet and micronucleus tests. However, it is important to point out that the same concentration cause a statistically significant ($p<0.001$) apoptotic process.

Conclusion: The consumption of a traditional diet determine very low levels of exposure to the parent myristicin. This fact implies as the primary metabolic pathway the O-demethylation (5-allyl-2,3-dihidroxyanisole) and not Phase I metabolism, which leads to the conclusion that this substance could not present a significant risk to humans.

70
71
72 **INTRODUCTION**
73
74 Myristicin is an alkoxy-substituted allylbenzene (alkenylbenzene) present in a variety of
75 botanical species such as fennel, parsley, carrot, parsnip, basil, anise, dill, celery and in some
76 spices consumed by humans, such as nutmeg, macis, cinnamon, and clove. Myristicin is also
77 found in some food additive oils or in traditional medicine[1,2].
78 In the last year's, the consumption of botanical and botanical ingredients has increased, due to
79 the fact that they are used as plant food supplements with the aim of enhancing health. Usually,
80 plant food supplements and phytochemicals are considered safe because of their natural origins[3].
81 This assumption is not correct because it is known that some herbal preparations can contain
82 individual potential harmful chemicals, among which are alkylbenzenes, in
83 particular,allylalkoxybenzenes, with toxic and well known genotoxic properties[4-6]. Special
84 attention has been given to estragole, methyleugenole and safrole since, although, at very high
85 levels of exposure, they were found to be genotoxic and carcinogenic in animals[7,8]. The
86 genotoxic potential of myristicin is still questioned even if there are overall evidence that it
87 produces DNA adducts albeit in smaller quantities and less persistent than estragole, safrole and
88 methyleugenol[9,10]. The compound shows neither mutagenic activity in *Salmonella typhimurium*
89 TA100 and TA98 at up to cytotoxic doses with and without metabolic activation nor UDS
90 (Unscheduled DNA Synthesis) in hepatocytes from male Fisher 344[11,12]. The discrepancy
91 emerged from *in vivo* and *in-vitro* experiments, could likely be a different ability of DNA
92 damage repair activity in the models considered. Myristicin induces apoptotic death in human
93 neuroblastoma SK-N-SH cells accompanied by an accumulation of cytochrome c and by
94 activation of caspase 3[13]. More evidence of apoptosis induction were observed in a study in
95 hamster ovary CHO cells[2] and, more recently, a study revealed that myristicin induces apoptosis

96 in human leukemia K562 cells[14], besides changes in the mitochondrial membrane potential, the
97 release of cytochrome c, activation of caspase-3 and cleavage of PARP and DNA fragmentation.
98 Furthermore, the same study showed that myristicin down-regulated genes involved in DNA
99 damage response pathways, such as genes for the nucleotide excision repair, the double strand
100 break repair, the DNA damage signaling and stress response[14].
101 Alkenylbenzenes can undergo different metabolic pathways (Fig. 1). It is reported in the
102 literature that a notable increase in the formation of the 1'-hydroxy metabolites occurs after an
103 increase in dose of the parent compound, which is accompanied by a shift in metabolic
104 pathways[15,16]. This metabolite can then become the substrate of sulfotransferase enzymes, going
105 through a reaction of esterification. The esterified metabolite can dissociate and give the reactive
106 carbocation, able to link nitrogenous bases forming adducts with DNA. In human hepatic cells
107 (HepG2), it is evident that myristicin yields DNA adducts quantitatively equivalent to that of
108 safrole[17]The aim of this work is to study the *in vitro* cyto- and genotoxicity of myristicin and 1'-
109 OH myristicin (Fig 1), using a metabolically active model of human hepatoma cell line (HepG2).
110 We believe that more information is needed to obtain sufficient data for a correct risk
111 assessment.
112
113

114 ## MATERIALS AND METHODS

115 **Chemicals and reagents**

116 RPMI-1640 medium, pyruvic acid, L-glutamine, penicillin-streptomycin solution, 3-(4-5-
117 dimethylthiazol-2-1l)-2,5-diphenyl tetrazolium bromide (MTT), Neutral Red solution (0,33%),
118 dimethyl sulfoxide (DMSO), trypsin-EDTA solution 1X, low-melting point agarose (LMA),
119 agarose for routine use, propidium iodide (1mg/ml in water), sodium chloride (NaCl), tris
120 (hydroxymethyl)aminomethane, sodium hydroxide (NaOH), potassium chloride (KCl), Triton X-
121 100, hydrochloric acid (HCl), sodium-citrate, citric acid and sucrose were obtained from Sigma-

122 Aldrich, Italy. Fetal bovine serum (FBS), Sytox green and 6 μm fluorescent beads were purchased
123 from Invitrogen.-Life technologies (Italy)
124 Myristicin was purchased from Sigma-Aldrich (Milan, Italy) while 1'-hydroxymyristicin was
125 synthesized and provided from Division of Toxicology, Wageningen University (Wageningen, The
126 Netherlands).
127 Myristicin and 1'-hydroxymyristicin both were dissolved in DMSO and solutions obtained were
128 dissolved 1:1000 in RPMI-1640 medium (Fig.1).
129

130 **Cell cultures**

131 HepG2 cells, a human hepatocellular carcinoma cell line, were purchased from
132 Istitutozooprofilattico (Brescia, Italy). Cells were maintained in RPMI-1640 medium added with
133 10% of heat inactivated FBS, 0.01% of pyruvic acid, 0.03% of L-glutamine and 1% penicillin-
134 streptomycin solution and placed at 37°C, under humidified air supplemented with 5% CO_2.
135 Confluent monolayers were exposed to myristicin (600μM) or 1'-hydroxymyristicinconcentrations
136 (from 50 to 600 μM) in RPMI-1640 medium for 24 hours at 37°C.
137

138 **Cytotoxicity assessment**

139 **MTT assay**

140 This assay was conducted according to Schiller et al.[18], HepG2 cells were grown in a 96-well plate,
141 myristicin or 1'-hydroxymyristicin were added and then removed after 24 hours. MTT dye (final
142 concentration 0.5 mg/ml) was added to each well. After removal of MTT solution, cells were lysed
143 with 150 μl of DMSO in order to dissolve the formazan crystals. The plate was read at 550 nm with
144 the spectrophotometer (Multilabel counter Victor Wallace 1420, Perkin-Elmer, Italy) and
145 absorbance was determined. Samples with a cell viability less than 50% were subsequently
146 excluded from genotoxicity analysis.
147

148 **Neutral Red assay**

149 The neutral Red assay was based on Rodrigues method[19]. HepG2 cells were grown in 96-well
150 plates and subsequently treated. Then plates were washed with PBS and 200 µl of the neutral red
151 solution, 25 µg/ml in the culture medium, were added to each well after a centrifugation at 5000
152 rpm for 5 minutes. Neutral red (0.33%) was dissolved in culture medium the day before the test and
153 left at 37°C during the night. The day of the test, plates with neutral red solution was incubated at
154 37°C for three hours and then cells were rinsed with PBS and lysed with a solution containing
155 acetic acid, ethanol, and water (1:50:49), in order to let neutral red going out from lysosomes. After
156 30 minutes of agitation, plates were read at 550 nm with the spectrophotometer (Multilabel counter
157 Victor Wallac1420, Perkin-Elmer Italy) and absorbance was determined. The absorbance measured
158 correlates with the number of living cells for each well, considering that each sample is referred to
159 the negative control to which is attributed a 100% cell viability. Samples with a cell viability less
160 than 50% were excluded from genotoxicity analysis.

161

162 **Apoptosis evaluation (Annexin V assay)**

163 This assay measures a numberof cells that are going toward an apoptotic process, differentiating in
164 early and late stages of this mechanism. Annexin V is a human protein Ca^{2+}- dependent that for this
165 assay is labeled with a fluorophore. Annexin V has an high affinity for phosphatidylserine (PS), a
166 phospholipid that normally stays on the cytoplasmic surface of cell membrane and that during
167 apoptosis is translocated on the outer side of the membrane, becoming able to be linked by annexin
168 V. The test was performed with Alexa Fluor 488 Annexin V/Dead cell Apoptosis Kit (Invitrogen).
169 Cells were seeded 24 hours before treatment in 60 mm plates at a density of $6.5x10^5$ cells/ml. After
170 treatment, cells were collected with trypsin and centrifuged for 5 min at 2000 rpm. Cells are then
171 suspended in 1 ml of PBS+5% FBS and counted with trypan blue. A volume of 10^6 cells/ml is
172 calculated for each sample and cells are subsequently combined with 100 µl annexin binding buffer
173 0.5x. Annexin binding buffer 0.5x was obtained with Na citrate (0.1%) Then 5 µl of Annexin V

174 were added to each sample and finally also 1 µl of working solution (propidium iodide dissolved
175 1:10 in ABB 0.5x) was added to each sample. Samples were left at RT in the dark for 15 min. In the
176 end, 400 µl of ABB 1:10 were added and samples were read in flow cytometry at a wave length of
177 excitation of 496 nm with an emission of 519 nm. Results are expressed as apoptotic cells
178 percentage for each sample.
179
180 **Genotoxicity evaluation**
181 **Alkaline Comet Assay**
182 Experiments were carried out according to Singh et al [20] HepG2 cells were plated in 60 mm culture
183 dishes and after 24 hours they were exposed to studied compounds. Then the cells were collected
184 with trypsin and centrifuged at 2000 rpm for 5 minutes. Pellet was suspended in 1 ml of culture
185 medium with a 20 G syringe needle. A total of $2*10^4$ cells/ml were suspended in 200 µl of 0.5%
186 low-melting- point agarose (LMA) in PBS and then transferred onto pre-coated microscope slides
187 with 1% agarose for routine use in PBS and covered with a coverglass. Slides were stored at 4°C for
188 10 minutes, then coverglasswas removed and the second layer of LMA was added to each slide.
189 After 10 minutes at 4°C, slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA,
190 10 mMTris, 250 mMNaOH, 10% DMSO, 1% Triton X-100, pH 10) at 4°C for 1 hour. Slides were
191 then rinsed with neutralization solution (0.4 M Tris, pH 7.5) and placed in a horizontal gel
192 electrophoresis tank (PBI) filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM Na-
193 EDTA, pH > 13) and left this way for 35 min, in order to let DNA unwinding. Then electrophoresis
194 run was done at 300 mA for 45 min, followed by 5 min of neutralization with neutralization
195 solution and finally, slides were fixed with ethanol at – 20°C for 5 min. Slides were left to dry at
196 room temperature and then nuclei were stained with propidium iodide (20 µg/ml in water) and
197 analyzed using fluorescence microscope (Axioplan 2, Zeiss; Milan, Italy) at 25- fold magnification.
198 For each sample, at least 100 randomly selected nucleoids were examined. Images of nucleoids
199 were analyzed with TriTek Comet Score Imaging software 1.5 and tail length, tail moment and % of

200 DNA in the tail were measured. Moreover, nucleoids were classified into five different categories
201 according to area, shape, and intensity of fluorescence of their tail.(A: normal nucleoid;B, C, D:
202 damaged nucleoids; E: ghosts).
203
204 **Neutral Comet Assay (NRA)**
205 Slides with alayer of lysed cells and LMA were placed in the horizontal electrophoresis tank with a
206 buffer (pH 8.3) containing 90 mM Tris, 2 mM EDTA, 90 mM boric acid and left this way for 15
207 min before starting the electrophoretic run at 80 mA for 25 min. Nucleoids were stained with
208 propidium iodide (20 µg/ml in water) and analyzed using fluorescence microscope (Axioplan 2) at
209 25- fold magnification[20].
210 For each sample, at least 100 randomly selected nucleoids were examined. Images of nucleoids
211 were analyzed with TriTek Comet Score Imaging software 1.5 and tail length, tail moment and % of
212 DNA in the tail were measured.
213
214 **Micronucleus assay**
215 Experiments were done according to Bryce et al.[21] making an analysis of micronuclei in flow
216 cytometry, associated also with a measure of cell viability through fluorescent microspheres
217 (beads). Cell viability measure made through fluorescent beads is considered more accurate than
218 measure obtained with normally used cytotoxicity assays, which can overestimate a number of
219 living cells. The day before treatment, cells were seeded at a density of 6.5 $x10^{5}$ cells/ml. After
220 treatment, a period of 24 hours followed in which cells were left in the medium at 37°C, in order to
221 give time to have cell division. The day of the experiment, cells were collected and centrifuged for
222 5 min at 2000 rpm, then each sample was suspended in 1 ml of PBS + 2% FBS and counted by
223 trypan blue method, in order to obtain a quantity of $5*10^{5}$ cells/ml for each sample. Calculated
224 volume was suspended in PBS + 2% FBS in order to reach a total volume of 1 ml for each sample.
225 After 5 minutes of centrifugation, 300 µl of propidium iodide (2 µg/ml) were added to each tube

226 and samples were left in the dark at real temperature for 10 min. Samples were centrifuged and
227 pellets were suspended in 1 ml of PBS + 2% FBS, after another centrifugation of 2000 rpm for 5
228 min, pellets were left in the dark at RT for 30 minutes with just 50 µl of supernatant covering them.
229 Then 500 µl of Lysis 1 solution (0.584 mg/ml NaCl, 1.13 mg/ml Na-citrate, 0.3 µl/ml
230 IGEPAL©CA630, 0.5 mg/ml RNAse, 0.4 µM Sytox Green) were added to each sample. After 1
231 hour at RT in the dark, 500 µl of Lysis 2 solution (85.6 g/ml sucrose, 16.4 mg/ml citric acid, 0.4
232 µM Sytox Green, 2 drops/ml beads) were added to each sample. After at least 30 min in the dark at
233 RT, samples were transferred to FACS tubes and stored at 4°C until flow cytometry analysis. MN
234 number was determined through the acquisition of at least 20,000 gated nuclei for each sample and
235 it is expressed as fold increase respect negative control. Fold increase ≥ 3 was considered a positive
236 result for this test. Nuclei/beads ratio was determined for each sample and referred to that of
237 negative control, in order to have an evaluation of relative cell survival.
238
239 **Statistical Analysis**
240 Triplicate experiments were performed with independent samples. The results were analyzed using
241 ANOVA t-test to assess statistical significance, one-way or two-way ANOVA analysis followed by
242 post-hoc Dunnett Results were considered statistically significant at $P < 0.05$. Analysis was carried
243 out using the software package 6.0 GraphPad Prism version (Graph Pad Prism Software Inc. La
244 Jolla USA). Statistical differences were considered at the $p<0.05$, $p<0.01$ or $p<0.001$ level vs. the
245 control group as indicated in the figures and captions. In the following, the results are expressed as
246 means ± standard deviation.
247
248
249
250 **RESULTS**
251 **MTT AND NRA**

252 Concentrations of myristicin and 1'-hydroxymyristicin suitable to conduct reliable genotoxicity
253 studies were established on the basis of concentrations that did not cause a reduction of more than
254 50% cell viability Cells exposed to myristicin (range 50 – 600 μM) for 24 h did not show a
255 significant cell viability reduction, both with MTT and NRA up to 600 μM (data not shown).
256 Differently, cells exposed to 1'-hydroxymyristicin, at the same range of concentrations, showed a
257 dramatic viability reduction ($p<0.001$) starting from 150 μM in MTT test and from 50 μM
258 concentration in NRA (Fig.2). The MTT and NRA dose–response were very similar.

259

260 **ALKALINE COMET ASSAY (pH> 13)**

261 **Myristicin**

262 Cells were exposed for 24 h to 450 and 600 μM myristicin concentration (Fig.3). None of the
263 parameters showed a significant difference in respect to control.

264

265 **1'-Hydroxymyristicin**

266 Cells were exposed for 24 h to 50-450 μM concentrations of 1'-hydroxymyristicin (Fig. 4a, b, c).
267 Tail moment and nucleoids classification showed a significant difference between cells exposed to
268 1'-hydroxymyristicin 450 μM and non- treated cells. A significant increase was measured in the
269 percentage of nucleoids category (A, BCD damaged and E) from 150 μM and above (Fig.4a,b,c).

270

271 **NEUTRAL COMET ASSAY (pH 8)**

272 Using the neutral version of comet assay that identifies double strand damage, a significant dose-
273 response increase of DNA damage has been observed only in cells exposed to 1'-
274 hydroxymyristicin, 150 and 450 μM (Fig. 5 a,b). In this test, the parameters normally utilized are

275 tail length and tail moment. These results supported the increase of nucleoids E (see alkaline comet
276 assay results) and the reduction of viability already highlighted.

277

278 **MICRONUCLEUS ASSAY**

279 The increase of Micronucleus frequency, detecting the presence of damaged chromosomes in cells
280 after division, confirms the extent of DNA damage observed in the comet assay (alkaline and
281 neutral test). As shown in Fig.6, cells exposed only to 150 and 450 µM 1'-hydroxymyristicin
282 showed a marked increase in the number of micronuclei largely exceeding the threshold level (n=3)
283 for this test. The decrease of the effect at 450 µM 1'-hydroxymyristicinis likely due to the
284 cytotoxicity (see also Fig.2).
285 No genotoxic response was elicited by myristicin (600 µM).

286

287 **ANNEXIN V ASSAY**

288 The cytotoxicity and the type of DNA damage have led us to investigate a possible apoptotic effect
289 associated with 1' hydroxymyristicin treatment.
290 A significant increase ($p<0.01$, and $p<0.001$) in apoptotic cell numbers (both in early Fig. 7 A and
291 late apoptotic stage Fig. 7 B and therefore not only as phosphatidylserine (PS) expression on the
292 outer leaflet membrane but also as a triggered apoptotic process), was actually observed in cells
293 exposed to concentration of 150 and 450 µM1'-hydroxymyristicin (Fig7 A and B).These evidence
294 support the results previously obtained with alkaline comet assay (Nucleoids E) and also with MTT
295 and NRA.

296

297

298

299 **DISCUSSION**

300

301 Toxicity of allylbenzenes, constituents of a variety of botanical-based food, is strongly dependent
302 on the presence of functional groups that may influence the chemical reactivity and, accordingly,
303 the biological activity of these natural constituents. The allylbenzene family is very diversified by
304 the presence or absence of alkylation products of their para-hydroxyl substituents, and/or position of
305 the double bond in the alkyl side chain. Besides this, also minor structural variations may elicit
306 differences in bioactivation/detoxification pathways that can affect the toxicological assessment.
307 This becomes relevant when considering the formation of reactive metabolites. In the scientific
308 literature, there are conflicting data on the toxicity of allyl alkoxy benzenes (myristicin, estragole,
309 methyl eugenol and safrole), propenylalkylbenzenes (anethole, isoeugenolmethylether),
310 allylhydroxybenzenes (chavicol and eugenol) and propenylhydroxybenzenes (isochavicol and
311 isoeugenol)[7].
312 While estragole, methyleugenol, safroleand anethole haveproved to be hepatotoxic, genotoxic and
313 carcinogenic, the genotoxic and possibly carcinogenic potential of myristicin at equivalent doses is
314 not to be expected .Dose-dependent formation of protein and DNA adducts in liver[22-29] was
315 observed with allyl alkoxy benzenes. Although DNA adducts in the liver of CD1 female mice were
316 isolated, the binding of myristicin to mouse-liver DNA was weaker than those of other compounds
317 such as safrole, estragole, and methyleugenol[10].
318 This study tries to clarifythe genotoxic potential of myristicin, oe of the constituents of nutmeg
319 powder, to which diverse populations are exposed through food and beverages. The human
320 hepatoma line (Hep G2) has retained the activities of various Phase I And Phase II enzymes which
321 play a crucial role in the activation /detoxication of genotoxic procarcinogens[30.] No carcinogenicity
322 studies of myristicin in animals were available in the literature. Miller et al.[25,26] performed
323 comprehensive sets of bioassays to characterize the hepatocarcinogenic potential of naturally
324 occurring and synthetic alkylbenzene derivatives including myristicin and its metabolites;

325 intraperitoneally treatment of male B6C3F1 mice 24 hours after birth and at days 8, 15, and 22 for a
326 total dose of 4.75 µmol/mouse did not show carcinogenic effects at 13 months.
327 The genotoxic potential of alkoxy-substituted allylbenzenes is likely due to the CYP-catalysed
328 formation of the 1'-hydroxy metabolite and subsequent activity of sulfotransferase 1A1
329 **(**SULT1A1), that catalyses the formation of the 1'-sull foxy conjugate. Myristicin*in vivo*, in the range
330 of human food intake, ,may metabolically be converted mainly by CYP1A1 and 2A6 to epoxy- or
331 hydroxy-derivatives that undergo glucuronidation and are readily excreted. At high doses in rodents,
332 O-demethylation becomes saturated and then takes place 1'-hydroxylation and epoxidation of the
333 allyl side-chain. This change in the balance of metabolic pathways, at high doses, leads to a
334 predominant formation of 1'-hydroxy-metabolites and the subsequent formation of 1'-sull foxy
335 metabolites by SULT 1A1 and SULT 1C2 that have been associated with the genotoxicity and
336 carcinogenicity [7,31].
337 The unstable sulfate ester forms a reactive electrophilic intermediate (carbonium ion or quinolinium
338 cation), which binds to proteins and DNA. Sulfate inhibition studies and i*n vivo–in vitro*
339 unscheduled DNA synthesis (UDS) assays of myristicin, elemicin, estragole, methyl eugenol and
340 the 1'-hydroxy metabolites of estragole and methyl eugenol[12,21,32] provide additional evidence that
341 the sulfate ester of the 1'-hydroxy metabolite is the ultimate toxic metabolite in animal. Data related
342 to the safety of estragole and safrole, structurally related to myristicin, indicate that at low dose
343 levels (below 1-10 mg kg b.w. in rodents and humans), allylalkoxybenzenes are rapidly cleared
344 from the body, with O-demethylation being the major metabolic route. Metabolic shifting from O-
345 demethylation to 1'hydroxylation results in increased formation of 1'hydroxymetabolite and
346 accordingly of the toxic reactive electrophilic 1'-sull foxy conjugate metabolite at higher dose level
347 (30 – 300 mg kg bw.) [31].
348 Our data showed no genotoxic potential of myristicin in comparison to other alkenylbenzenes with
349 similar structures (safrole, estragole)[33,34.] Besides, myristicin does not elicit any cytotoxicity
350 differently from its 1'hydroxymetabolite that elicited cytotoxic, genotoxic and apoptotic effects

351 from a concentration of 150 µM. The appearance of the apoptotic effect must be considered because
352 it can mask the genotoxic effect. It is also evident that 600 µM myristicin in our experimental
353 conditions does not generate a sufficient 1'-hydroxymyristicin quantity to give the final toxicant
354 product.

355 **CONCLUSION**

356 .

357 Our data confirm no genotoxic potential of myristicin. The fact that only the hydroxyl metabolite is
358 genotoxic in a narrow range of doses underlines that the mechanism of carbocation formation (1'-
359 sull foxy conjugate toxic metabolite production) is necessary for genotoxic activity.
360 This is an important point to consider in the risk assessment of dietary exposure to myristicin,
361 which is mainly carried through consumption of the spices nutmeg and mace and of non alcoholic
362 beverages. The average exposure for myristicin may be as high as to 162 µg/day (3/684 µg/day
363 lower and upper limits equal to 0.05 and 11.4 µg/kg b.w. /day, respectively for an adult of the
364 average weight of 60 kg) in Europe. The results lead to the conclusion that myristicin presents no
365 significant risk to humans through consumption of a traditional diet because the very low levels of
366 exposure . The low doses cause essentially the primary involvement of the O-demethylation
367 leading to a safer metabolic path.
368

369 **Conflict of interest**
370 The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1 Proposed metabolic pathways of the alkenylbenzenemyristicin[15]

Fig. 2 Cytotoxicity evaluation, through MTT and Neutral red assay in HepG2 cells exposed to 1'-hydroxymyristicin for 24 h. Data are elaborated through One Way Anova (Dunnett's*post hoc* test) analysis.

Fig.3 Evaluation of genotoxic damage by alkaline comet test (pH >13) in HepG2 cells exposed to myristicin (450-600 μM) for 24 h. C-,vehicle (DMSO 0.1%); C+ positive control (mitomycin 0.1 μg/ml). Data are elaborated through One Way Anova (Dunnett's *post hoc* test) analysis.
A. Tail moment (μm). **B. %** BCD nucleoids **C. %** E nucleoids .

Fig. 4 Evaluation of genotoxic damage by alkaline comet test (pH >13) in HepG2 cells exposed to 1'-hydroxymyristicin (50-450 μM) for 24 h. C-,vehicle (DMSO 0.1%); C+ positive control (mitomycin 0.1 μg/ml). Data are evaluated through One Way Anova (Dunnett's *post hoc* test) analysis.
A. Tail moment (μm). **B. %** BCD nucleoids **C. %** E nucleoids

Fig.5 Neutral Comet test (pH 8) evaluation in HepG2 cells exposed to 1'-hydroxymyristicin (50-450 μM) for 24 h. C-,vehicle(DMSO 0.1%). Data are evaluated trough One Way Anova (Dunnett's *post hoc* test) analysis. **A.** Tail length (μm) **B.** Tail moment (μm)

Fig.6 Evaluation of genotoxic damage through micronucleus test in HepG2 cells exposed to myristicin and 1'-hydroxymyristicin. Detection of micronuclei is made in flow cytometry. C+ positive control (mitomycin 0.1 μg/ml). Micronuclei values are expressed as fold increase respect negative control (value of 1 on Y axis). A fold increase ≥3 gives an indication of positive results.

Fig.7 Evaluation of apoptotic cells by Annexin V test in HepG2 cells exposed to 1'-hydroxymyristicin for 24 h. C-, vehicle (DMSO 0.1%). C+, positive control (staurosporine 8.56 μM). Data are elaborated through One Way Anova (Dunnett's *post hoc* test) analysis.
A. Percentage of early apoptotic cells
B. Percentage of late apoptotic events