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Risk assessment of consumption of methylchavicol and tarragon: The genotoxic potential *in vivo* and *in vitro*

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ABSTRACT

Methylchavicol (or estragole), a natural flavouring substance present in tarragon, was confirmed as a genotoxic chemical in the *in vitro* UDS test in cultured rat hepatocytes and in the *in vivo* UDS test in hepatocytes of exposed rats. Deep-frozen tarragon was clearly less genotoxic than methylchavicol at equivalent dose levels, and desiccated tarragon was negative. Both forms of tarragon tested *in vitro* have the ability to decrease significantly the genotoxicity of methylchavicol added to the culture medium at concentrations $\leq$10 μM for deep-frozen and $\leq$55 μM for desiccated tarragon. The decrease may be attributed to antimutagenic properties of tarragon leaves and/or to adsorption of methylchavicol, which would decrease its bioavailability. Desiccated tarragon powder was not genotoxic in the *in vivo* UDS test when administered up to the maximum dose of 6.25 g/kg bw (18.75 mg/kg bw of methylchavicol). *In vivo*, desiccated tarragon did not show antimutagenic properties, because it did not decrease the genotoxicity of methylchavicol added at high concentrations.

Considering the low exposure level at the maximum daily tarragon consumption, the rapid detoxification and excretion in humans and the no-genotoxic-effect-level of methylchavicol by the oral route when given to rats as tarragon leaves, a high margin of exposure exists. We can conclude that tarragon consumption presents no genotoxic risk to humans.

1. Introduction

Many chemically pure compounds present in food products, including flavouring substances, have been submitted to toxicity assays. Some of them were found to be mutagens *in vitro* and/or *in vivo* systems and some were even shown to be carcinogens in rodents. One can wonder whether the toxicological properties of the suspected substance present in food are the same when administered as a pure substance or when consumed as plant food.

Methylchavicol or estragole (also named 1-allyl-4-methoxybenzene, p-methoxyallyl-benzene, 1-methoxy-4-(2-propenyl)benzene, iso anethole or p-allyl anisole; CAS 140-67-0), is an alkylbenzene derivative structurally related to eugenol and anethole, which are neither mutagenic nor carcinogenic, and to methyleugenol and safrole, both of which are mutagenic and carcinogenic. Many aromatic plants, such as basil, chervil, tarragon and others contain methylchavicol. However, tarragon (*Artemisia Dracunculus*) is one of the aromatic plants that contains the highest percentage of methylchavicol, with a relatively small variability according to species, crop-time and place of origin. The aim of the present study is to assess the genotoxic potential of methylchavicol, either alone or as a component of tarragon leaves. There is general concern about the carcinogenicity and the mutagenicity of purified or synthesized methylchavicol. Indeed, methylchavicol has been found to be a mouse and rat hepatocarcinogen in long-term studies [1,2]. For these reasons, we performed a risk assessment of methylchavicol using *in vitro* and *in vivo* regulatory genotoxicity methods and desiccated and deep-frozen tarragon leaves as used in cooking.

In bacteria, methylchavicol is overall non-mutagenic in *Salmonella typhimurium* and *Escherichia coli* WP2 uvrA [4] and in the *Rec* assay in *Bacillus subtilis* [3], although Swanson et al. [5] demonstrated a mutagenic activity of estragole in strain TA1535 of *S. typhimurium* without metabolic activation. However three of the methylchavicol metabolites are mutagenic: 1′-hydroxyestragole is mutagenic in *S. typhymurium* TA100 with metabolic activation

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and 2′3′-oxide-1′-hydroxyestragole and 2′3′-oxide-1′-estragole are mutagenic in *S. typhymurium* TA100 and TA1535 with and without metabolic activation. Furthermore, ethylchavicol demonstrated a weak mutagenic activity in *S. typhymurium* TA1535 with metabolic activation in the presence of phospho-adenosyl-phosphosulphate (PAPS) [6]; this study tends to demonstrate the role of the sulfo-conjugate metabolite in the mutagenic activity.

In the *in vitro* chromosome aberration test, methylchavicol was shown to have no effect in V79 cells when tested in a concentration range from 10-6 to 10-3 M, with and without metabolic activation [7].

In the *in vitro* unscheduled DNA synthesis (UDS) test in rat hepatocytes, methylchavicol was shown to be genotoxic in the range 10^{-5}–10^{-3} M [7–9]; the genotoxicity of 1′-hydroxyestragole was more potent than that of methylchavicol [9].

In the *in vivo* UDS test, methylchavicol was genotoxic in rat hepatocytes after oral administration, after a 4-h expression time at 0.5, 1 and 2 g/kg bw given orally and after 12-h expression time at 2 g/kg bw [7]. Methylchavicol induced formation of 30 pmoles DNA adducts/mg DNA in B6C3F1 mouse liver at 0.25, 0.5, 1.0 and 3.0 μM/animal when administered intraperitoneally at 1, 8, 15 and 22 days after birth [10] whereas 1′-hydroxyestragole induced two major and two minor DNA adducts at C-8 and N-7 of guanine in mice treated by the same route [11].

In order to compare the genotoxic effect of methylchavicol when administered either pure or contained in tarragon leaves, both forms were tested at equivalent doses *in vitro* and *in vivo* in the unscheduled DNA synthesis test in rats. Furthermore, tarragon was tested either alone or enriched with pure methylchavicol in order to investigate its antimutagenic potential. The unscheduled DNA synthesis assay in the liver was chosen it was demonstrated to be the most sensitive test both *in vitro* and *in vivo* [7] and because the liver is the target organ in rodents in long-term carcinogenicity studies [1,2]. In order to complete the battery of genotoxic tests, an *in vivo* bone-marrow micronucleus test was also performed.

2. Materials and methods

All the experiments were performed according to Good Laboratory Practice OECD guidelines.

2.1. Chemicals

Methylchavicol (CAS 140-67-0) was purchased at Sigma–Aldrich (France; batch # 18807AB, purity 99%). Tarragon was used in crunch deep-frozen or powder of desiccated forms. Deep-frozen tarragon (batch #X01 601 containing 0.21% methylchavicol) and desiccated tarragon (batch #40P00453 containing 0.40% methylchavicol) were obtained from Nutracos (London, UK). William's E (WE-C) medium was purchased from Gibco (France), collagenase Ia was from Sigma–Aldrich and dimethyl sulfoxide from Merck (France). Dimethylhydrazine was obtained from Fluka (France), cyclophosphamide from Baxter (France), 2-acetamidofluorene from Sigma (France) and corn oil from Cooper (France).

2.2. Animals and treatments

EOPS Fischer male rats were used for the *in vitro and in vivo* UDS assays and male and female Sprague–Dawley rats for the *in vivo* micronucleus assay. The animals were obtained from Charles River France, Saint-Germain-sur-l'Arbresle, France.

2.3. In vitro UDS assay

The study was performed according to OECD guideline 482 [12].

Animals were deeply anaesthetized with pentobarbital. The liver was then perfused using a buffered collagenase solution (Hepes buffer containing 4 mM $CaCl_2$ and 0.025% collagenase Ia) at 20 mL/min for 5 min at 37 °C. The cytotoxicity was determined on a small sample of each isolated cell suspension by use of the trypan-blue vital dye-exclusion technique (5 volume of cell suspension + 1 volume of a 0.4% trypan-blue solution). The proportion of viable cells was determined for each cell suspension using a Malassez cell. The percentage of viable cells always exceeded or was close to 70%. The cell suspension was diluted to provide approximately 1.5×10^5 viable cells/mL and 3 mL of cell suspension were transferred to each well of 6-well plates. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 for approximately 90 min until attachment of the cells, then 10 μCi/mL [^{3}H]thymidine and solvent or positive control (2-acetamidofluorene, 6.25 μM), or test preparation in solution were added. Cell toxicity at the end of the treatment period was measured by use of the trypan-blue assay. Slides from each treatment and from positive and solvent controls were submitted to autoradiography. The cell nuclei and the cytoplasm were stained with Harris hemalun. Grain counting was performed by use of an image-analysis system (Visilog, Noesis, France).

Nuclear and cytoplasmic grain counts were obtained from at least 50 cells per slide, 3 slides per group (test preparation treatments, solvent controls, positive controls). Nuclear (NC) and cytoplasmic (CC) grain counts were recorded, and the net nuclear grains (NNG) per cell determined (NNG = NC − CC). The percent of cells in repair (NNG ≥5) was also determined in each group.

The *in vitro* UDS study was performed at concentrations chosen on the basis of the content of methylchavicol in tarragon, i.e. 0.21% in deep-frozen tarragon, and 0.40% in desiccated tarragon. In the first assay, the maximum concentration studied was 4076 μg deep-frozen tarragon/mL corresponding to 55 μM methylchavicol-equivalent in final concentration. In the second assay, the maximum concentration studied was 4076 μg desiccated tarragon/mL corresponding to 109 μM methylchavicol-equivalent in final concentration.

Concurrent with treatments with tarragon alone, treatments with tarragon powder (containing 0.55 μM methylchavicol-equivalent) enriched in pure methylchavicol (up to 55, 10, 5.5 and 1 μM methylchavicol-equivalent in the first assay or to 109, 55, 10, 5.5 and 1 μM methylchavicol-equivalent in the second assay) were also performed as well as treatments with pure methylchavicol alone at 55, 10, 5.5 and 1 μM.

A third assay was conducted with various proportions of desiccated tarragon + pure methylchavicol, always corresponding to the final concentration of 10 μM methylchavicol-equivalent.

2.4. In vivo UDS assay

The study was performed according to OECD guideline 486 [13]. The method was the same as the *in vitro* UDS assay except that groups of three male Fischer rats per sampling time were treated by gavage with a solution (methylchavicol) or a suspension (tarragon leaves) in corn oil. Two expression times of 2–4 h or 12–16 h after treatment were chosen. The doses administered were 2–0.8–0.25 g/kg bw for pure methylchavicol alone, 6.25–2 g/kg bw desiccated tarragon, 6.25 g/kg bw of desiccated tarragon enriched with methylchavicol up to 2 g/kg bw and 6.25 g/kg bw desiccated tarragon enriched with methylchavicol up to 0.8 g/kg bw. The negative control group received only the vehicle (corn oil) 2–4 h or 12–14 h before sampling of the hepatocytes. The positive control group received either dimethylhydrazine (10 mg/kg bw, orally) for the 2–4-h expression time or 2-acetamidofluorene (25 mg/kg bw, orally) for the 12–16-h expression time.

2.5. In vivo rat bone-marrow micronucleus assay

The study was performed according to OECD guideline 474 [14]. Groups of 5 male and 5 female rats were used. Treatment comprised two successive administrations at 24-h intervals by gavage. Methylchavicol was given as a solution in corn oil at the maximum tolerated dose of 1000 mg/kg bw/day × 2 and at two lower doses (500 and 250 mg/kg bw/day × 2). The negative control group received only the vehicle (corn oil) 24 and 48 h before sampling. The positive control group received cyclophosphamide (25 mg/kg bw, single treatment) by the intraperitoneal route, 24 h before sampling. At the sampling time, the animals were sacrificed by CO_2 asphyxia; the femurs were removed, and the bone marrow was extracted with foetal calf serum. The cell pellets obtained by centrifugation were spread on slides. The smears were stained by use of a technique derived from the May-Gruenwald Giemsa method.

On coded slides the number of polychromatic erythrocytes having one or more micronuclei was determined for at least 2000 polychromatic erythrocytes per animal.

The polychromatic/normochromatic erythrocyte ratio was determined by analyzing at least 1000 erythrocytes per animal.

2.6. Criteria for genotoxic activity

In the *in vitro* UDS assay a test preparation is considered positive in the system at any dose tested when the group mean NNG value is greater than 0 NNG and 20% or more of cells are in repair (NNG value ≥5) or if an increase is observed in both NNG and the percentage of cells in repair compared with the concurrent control group. Historical controls of the laboratory were −4.18 ± 1.80 NNG and 4.46 ± 2.99% cells in repair for negative controls and 19.32 ± 6.54 NNG and 90.89 ± 8.89% cells in repair for positive controls.

In the *in vivo* UDS assay a test preparation is considered positive if at any dose and at either time point: the test preparation yields group mean NNG values greater than 0 NNG or 20% or more of cells are responding (NNG values ≥5) or an increase is observed in both NNG and the percentage of cells in repair compared with the concurrent control group. Historical controls of the laboratory were −1.60 ± 1.48 NNG and 2.02 ± 0.54% cells in repair for negative controls and 13.23 ± 1.94 NNG and 90.21 ± 3.43% cells in repair for positive controls at 2–4 h expression time and −1.88 ± 1.15 NNG and 1.19 ± 1.15% cells in repair for negative controls and