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Final version
Corrigendum October 2022

Scientific Committee on Consumer Safety

SCCS

OPINION

on Genistein and Daidzein

The SCCS adopted this document
by written procedure on 16 September 2022

-CORRIGENDUM adopted by written procedure on 11 October 2022-

ACKNOWLEDGMENTS

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This Opinion has been subject to a commenting period of eight weeks after its initial publication (from 13 January to 14 March 2022). Comments received during this period were considered by the SCCS. For this Opinion, the main changes were in the section 3.3.6. mutagenicity and genotoxicity, as well as relevant parts in the discussion, conclusion and reference sections.
A corrigendum has been adopted by written procedure on 11 October 2022 to correct the bioavailability oral route for daidzein to 25% in the MoS calculation and the SCCS conclusion (section 3.4: leave-on products with 0.02% Daidzein) + related text in section 3.5. under *Toxicokinetics.*

1. ABSTRACT

The SCCS concludes the following:

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of phytoestrogens,*

   (a) does the SCCS consider genistein safe when used in cosmetic products up to a maximum concentration of 0.007%?

   (b) does the SCCS consider daidzein safe when used in cosmetic products up to a maximum concentration of 0.02%?

   From the safety assessment based on the available relevant data on the aglycone form of genistein and daidzein, and in consideration of the potential endocrine disrupting properties of phytoestrogens, the SCCS considers that:

   (a) the use of genistein (CAS No 446-72-0, EC No 207-174-9) in cosmetic products up to a maximum concentration of 0.007% is safe.

   (b) the use of daidzein (CAS No 486-66-8, EC No 207-635-4) in cosmetic products up to a maximum concentration of 0.02% is safe.

2. *Alternatively, according to the SCCS what is the maximum concentration of genistein and daidzein that is considered safe for individual and combined use in cosmetic products?*

/

3. *Does the SCCS have any further scientific concerns with regard to the use of genistein and daidzein or other related phytoestrogens in cosmetic products?*

/

Keywords: SCCS, scientific opinion, genistein (CAS No 446-72-0, EC No 207-174-9), daidzein (CAS No 486-66-8, EC No 207-635-4), Regulation 1223/2009

Opinion to be cited as: SCCS (Scientific Committee on Consumer Safety), Scientific opinion on genistein and daidzein, preliminary version of 12 January 2022, final version of 16 September 2022, corrigendum of 11 October 2022, SCCS/1641/22

About the Scientific Committees

Two independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
These Committees are: the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and they are made up of scientists appointed in their personal capacity.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide Opinions on questions concerning health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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MANDATE FROM THE EUROPEAN COMMISSION

Background on substances with endocrine disrupting properties

On 7 November 2018, the Commission adopted the review[1] of Regulation (EC) No 1223/2009 on cosmetic products ('Cosmetics Regulation') regarding substances with endocrine disrupting (ED) properties. The review concluded that the Cosmetics Regulation provides the adequate tools to regulate the use of cosmetic substances that present a potential risk for human health, including when displaying ED properties.

The Cosmetics Regulation does not have specific provisions on EDs. However, it provides a regulatory framework with a view to ensuring a high level of protection of human health. Environmental concerns that substances used in cosmetic products may raise are considered through the application of Regulation (EC) No 1907/2006 ('REACH Regulation').

In the review, the Commission commits to establishing a priority list of potential EDs not already covered by bans or restrictions in the Cosmetics Regulation for their subsequent safety assessment. A priority list of 28 potential EDs in cosmetics was consolidated in early 2019 based on input provided through a stakeholder consultation. The Commission carried out a public call for data[2] in 2019 on 14[3] of the 28 substances (to be treated with higher priority-Group A substances) in preparation of the safety assessment of these substances. Both genistein and daidzein were included in the above-mentioned 14 substances for which the call for data took place.

Background on genistein and daidzein

Phytoestrogens are plant-derived compounds with structural similarity to 17β-estradiol, a steroid hormone produced primarily by the ovaries during the reproductive lifespan and is responsible for the development of female secondary sexual characteristics and maintenance of the female reproductive tract. Among the phenolic compounds classified as phytoestrogens are isoflavones, which are typically found in legumes, including soybeans, chickpeas, fava beans, as well as in fruits and nuts (pistachios, peanuts, etc.). Soy and its by-products are mostly used in food and dietary supplements.

Among the most common isoflavones are genistein (CAS No 446-72-0, EC No 207-174-9) and daidzein (CAS No 486-66-8, EC No 207-635-4) that correspond to the chemical names 'Genisteol 4',5,7-Trihydroxyisoflavone' and 'Daidzeol 7,4'- Dihydroxyisoflavone', respectively. Both substances are included in the European database for information on cosmetic substances and ingredients (CosIng) with the reported function of 'skin conditioning', however additional functions have been reported, including 'antioxidant', 'skin protecting', etc. Currently, genistein and daidzein are not regulated under the Cosmetic Regulation (EC) No. 1223/2009.

During the call for data, stakeholders submitted scientific evidence to demonstrate the safety of genistein and daidzein in cosmetic products. The Commission requests the SCCS

[1] https://ec.europa.eu/transparency/regdoc/rep/1/2018/EN/COM-2018-739-F1-EN-MAIN-PART-1.PDF
[2] https://ec.europa.eu/growth/content/call-data-ingredients-potential-endocrine-disrupting-properties-used-cosmetic products_en
[3] Benzophenone-3, kojic acid, 4-methylbenzylidene camphor, propylparaben, triclosan, Homosalate, octocrylene, triclocarban, butylated hydroxytoluene (BHT), benzophenone, homosalate, benzyl salicylate, genistein and daidzein

to carry out a safety assessment on genistein and daidzein in view of the information provided.

Terms of reference

(1) In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of phytoestrogens,

(a) does the SCCS consider genistein safe when used in cosmetic products up to a maximum concentration of 0.007%?

(b) does the SCCS consider daidzein safe when used in cosmetic products up to a maximum concentration of 0.02%?

(2) Alternatively, according to the SCCS what is the maximum concentration of genistein and daidzein that is considered safe for individual and combined use in cosmetic products?

(3) Does the SCCS have any further scientific concerns with regard to the use of genistein and daidzein or other related phytoestrogens in cosmetic products?

2. OPINION

This Opinion relates to the assessment of safety of two specific isoflavones – genistein (CAS No 446-72-0, EC No 207-174-9) and daidzein (CAS No 486-66-8, EC No 207-635-4), when used in cosmetic products. The Opinion has also taken into consideration other sources of exposure to the two isoflavones from the use of certain phytoestrogen preparations in cosmetics and food/food supplements.

In developing the Opinion, the SCCS has considered the data and information received following the Commission's Call for Data on genistein and daidzein. These included a submission by an individual submitter, and a joint submission by Mulon Conseil on behalf of three submitters (hereinafter referred to as Mulon Conseil Submission). Information was also received from two Member State authorities (UK, IT). In addition, the SCCS also considered the information from published scientific literature that was deemed relevant to the safety assessment of genistein and daidzein.

In this regard, it is worth pointing out that many of the published studies relate to the test material being either an isoflavone containing food (e.g. soy or soy products), an isoflavone extract, or total genistein or daidzein (i.e. in both aglycone and glycoside forms). Since this Opinion relates specifically to genistein (CAS No 446-72-0, EC No 207-174-9) and daidzein (CAS No 486-66-8, EC No 207-635-4), only those studies that had used the aglycone form of the two substances were considered of relevance by the SCCS for safety assessment.

3.1 CHEMICAL AND PHYSICAL SPECIFICATIONS

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Genistein
Daidzein

3.1.1.2 Chemical names

Genistein:

IUPAC Name: 5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one (PubChem)
5,7-dihydroxy-3-(4-hydroxyphenyl)-4-benzopyrone
5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (Merck index, 11th edition, 4395)
4',5,7-trihydroxyisoflavone
Prunetol (Merck index)
Genisteol (Merck index)

Daidzein:

IUPAC Name: 4',7-Dihydroxyisoflavone (Individual company submission)
7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one (PubChem)
Preferred IUPAC name: 7-Hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
7-hydroxy-3-(4-hydroxyphenyl)-4-benzopyrone (ECHA)
Daidzeol (PubChem)

3.1.1.3 Trade names and abbreviations

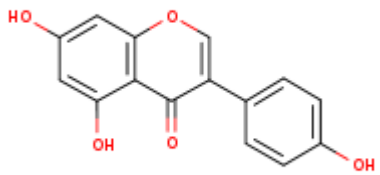
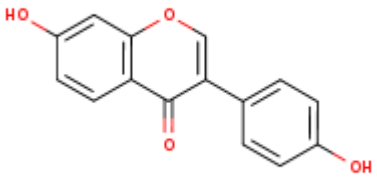
Glycine Soja extract; Soy isoflavones 90%

3.1.1.4 CAS / EC number

Genistein:
CAS Number: 446-72-0
EC Number: 207-174-9

Daidzein:
CAS Number: 486-66-8
EC Number: 207-635-4

3.1.1.5 Structural formula

	
Genistein	Daidzein

3.1.1.6 Empirical formula

Genistein: $C_{15}H_{10}O_5$
Daidzein: $C_{15}H_{10}O_4$

3.1.2 Physical form

Solid (PubChem)

3.1.3 Molecular weight

Genistein: 270.24 g/mol
Daidzein: 254.24 g/mol
[PubChem]

3.1.4 Purity, composition and substance codes

SCCS comment
Full reports of the chemical characterisation of genistein and daidzein in terms of purity and identity in representative batches must be provided, and the validity of the analytical methodologies used must be documented.

3.1.5 Impurities / accompanying contaminants

SCCS comment
Full reports on the impurity profile of genistein and daidzein in representative batches must be provided, and the validity of the analytical methodologies used must be documented. The identity and concentration of any impurities of concern that may be present must also be provided.

3.1.6 Solubility

Genistein:

Solubility in water (nmol/ml)
12.94 ± 0.53 (pH 6 buffer)
659.70 ± 10.96 (pH 10.8 buffer)
243.14 ± 2.04 (soybean oil) [Huang *et al.*, 2008]

0.015 ± 0.005 mg/ml (in water) [Minghetti *et al.*, 2006]
12.3 ± 0.7 mg/ml (in PEG 400) [Minghetti *et al.*, 2006]

Daidzein:

Solubility in pH 6 buffer: 18.76 ± 0.33 nmol/ml
Solubility in pH 10.8 buffer: 1875 ± 292.01 nmol/ml
Solubility in soybean oil: 10.88 ± 2.73 nmol/ml [Minghetti *et al.*, 2006

0.013 ± 0.01 mg/ml (in water) [Minghetti *et al.*, 2006]
11.6 ± 0.1 mg/ml (in PEG 400) [Minghetti *et al.*, 2006]

Solubility values in other solvents are also reported by Wu *et al.* (2010) and Nan *et al.* (2014).

SCCS comment
The aqueous solubility of the isoflavone aglycones is generally very low and is pH dependent due to the acidic nature of the phenolic groups. The solubility values in aqueous media reported by Minghetti *et al.* (2006) and Huang *et al.* (2008) are equivalent to <0.1 g/L, which indicates that both substances are practically insoluble in water.

3.1.7 Partition coefficient (Log P_{ow})

Genistein:
2.84 (PubChem)

Daidzein:
2.55 (ChemID)
3.16 (Calculated by Vega Software) [Minghetti *et al.*, 2006]

3.1.8 Additional physical and chemical specifications

	Genistein	**Daidzein**
organoleptic properties (colour, odour, taste if relevant)	Pale yellow powder (ChemSpider) Rectangular or six-sided rods from 60% alcohol. Dendritic needles from ether (PubChem)	Fine off-white solid (ChemSpider) Pale yellow prisms from diluted alcohol (Merck index, 11th Edition, 2868)
melting point	303 °C (Minghetti *et al.*, 2006); 301.5 °C (PubChem), 297-298 °C (slight decomposition) (Merck index)	320 °C (Minghetti *et al.*, 2006); 323 °C (PubChem) Decomposes at 315-323 °C (Merck index)
boiling point	555.5 °C (ChemSpider)	512.00 to 513.00°C at 760.00 mm Hg (estimated) (PubChem)
flash point	217.1 °C (ChemSpider)	201 °C (ChemSpider)
vapour pressure	$5.2x10^{-12}$ mm Hg at 25 °C (estimated) (PubChem)	/
Density	217.1 g/mL (ChemSpider)	/
Viscosity	/	/
pKa	pKa1 = 7.63, pKa2 = 9.67; pKa3 = 10.80 (estimated) (PubChem) pKa1 = 7.25 ± 0.84, pKa2 = 9.53 ± 0.15 at 298.2 K (Nan 2014)	7.2 (Individual company submission) pKa1 = 7.51 ± 0.07, pKa2 = 9.47 ± 0.14 at 298.2 K (Nan 2014)

pH	/	/
refractive index	/	/
UV/visible light absorption spectrum	λmax: 262,5 nm (ε 138) (Merck index)	λmax: 250 nm (log ε 4.44) (Merck index), λmax: 252 nm (ChemSpider)

3.1.9 Homogeneity and Stability

SCCS comment
Data on the stability of genistein and daidzein under the experimental conditions of the reported studies, and under the conditions of use, must be provided along with information on any hydrolysis products.

3.2. EXPOSURE ASSESSMENT & TOXICOKINETICS

3.2.1 Function and uses

Genistein and daidzein are isoflavones used in the formulation of leave-on cosmetic products. Both isoflavones belong to polyphenolic category of compounds. The majority of the isoflavone cosmetic ingredients are derived from soybean plants (Glycine max L.). In plants, both genistein and daidzein are largely present in glucoside forms.

Their primarily function is reported as antioxidants, skin protectants, skin conditioning agents, and hair-conditioning agents. Other functions are also reported.

Cosmetic activities of isoflavone extracts are mainly:

- Collagen synthesis stimulation,
- Enhancer of hyaluronic acid production,
- free radical formation limitation,
- Anti-inflammatory activity,
- Antioxidant activity.

Therefore, cosmetic products with isoflavones extracts, including genistein and daidzein, are mainly used in antiaging products.

Ref.: [Mulon Conseil Submission]

SCCS comment
In CosIng database, the functions for both genistein and daidzein are listed as 'skin conditioning - Miscellaneous'.

3.2.2 Dermal / percutaneous absorption

Dermal/percutaneous absorption *in vitro*

The evaluation of *ex vivo* human skin permeation of daidzein and genistein was performed by Minghetti (2006). The skin permeation studies were conducted by using modified Franz diffusion cell and human epidermis as a membrane. Different vehicles including enhancers were tested: water, PEG400, diethylene glycol monoethyl ether (Transcutol), caprylocaproyl macrogol-8 glycerides (Labrasol), propylene glycol, oleic acid. The solubility of daidzein, genistein and dry soybean extract were tested in the solvents. Within the conditions of the study, PEG400 was the most effective vehicle tested.

The phytoestrogen tested was a dry soy extract (24% in PEG400) containing exclusively aglycone of genistein and daidzein (11.7% and 12.3% respectively).

Results:

TABLE 2
Permeation parameters of GEN and DAI from the dry soy extract saturated vehicles

	GEN			DAI		
Vehicle	Permeated amount after 24 hr (μg/cm^2 ± s.d.)	Flux (μg/cm^2/hr)	Permeability coefficient (K_p) (cm^2/hr)	Permeated amount after 24 hr (μg/cm^2 ± s.d.)	Flux (μg/cm^2/hr)	Permeability coefficient (K_p) (cm^2/hr)
Polyethylene glycol 400	31.7 ± 8.7	1.3 ± 0.4	1.5×10^{-4}	23.9 ± 11.9	1.0 ± 0.5	1.2×10^{-4}
Labrasol	5.6 ± 0.4	–**	–	–*	–	–
Transcutol	5.8 ± 0.2	–**	–	–*	–	–
Propylene glycol	12.3 ± 3.2	0.5 ± 0.1	1.2×10^{-5}	9.8 ± 3.9	0.4 ± 0.2	1.5×10^{-5}
Oleic acid	–*	–	–	–*	–	–
Water	–*	–	–	–*	–	–

*Not detectable, **lower than 0.1 μg/cm^2/hr.

When pure daidzein, dissolved in PEG 400, was investigated, higher fluxes were obtained compared to the soy extract (see the Table a below).

Table a

TABLE 4
Permeation parameters of pure GEN and DAI from PEG400 (mean ± s.d.)

	Retained amount after 24 hr (μg/cm^2)	Permeated amount after 24 hr (μg/cm^2)	Flux (μg/cm^2/hr)	Permeability coefficient (K_p) (cm^2/hr)
DAI	80.7 ± 3.9	37.3 ± 16.8	1.7 ± 0.8	1.5×10^{-4}
GEN	36.0 ± 0.4	155.7 ± 18.2	7.1 ± 0.8	5.8×10^{-4}

Ref.: Minghetti *et al.*, 2006; Mulon Conseil Submission

SCCS comment
The study has been described from open literature, which does not provide adequate experimental details. The original study report was not provided for evaluation; however, from the publication it is evident that the dermal/percutaneous absorption experiments were not performed according to the SCCS basic criteria for dermal absorption studies (SCCS Notes of Guidance, 2021), because skin samples from only one donor were used.
Dermal/percutaneous absorption *in vivo*

In 2001, the transdermal absorption of the isoflavones, daidzein and genistein, applied on the forearm skin in olive oil was studied *in vivo* on one subject (a premenopausal women). The ability of the soy isoflavones to penetrate the skin structure was evaluated by monitoring plasma and urine levels. The pure isoflavones were applied to the skin as a suspension in extra virgin olive oil (10 mg genistein or daidzein in 1 g oil). In this study, both the capacity of genistein and daidzein to permeate the skin barrier and to reach the systemic circulation was implicitly assumed. The concentrations of the isoflavones and their metabolites were monitored in plasma and urine by GC-MS methods. It was found that the concentration of genistein in plasma was 3-fold higher than the plasma concentration of daidzein. In contrast, daidzein excretion was 2-3-fold higher than that of genistein in urine.

The excretion rate of the studied phytoestrogens in urine and their concentration in plasma was significantly decreased after repeated transdermal application.
The urinary recovery of administered daidzein and genistein after the first application was 15.9% and 7.7% respectively and this dropped to 1.6% and 0.7% after the second application, one month later.

Ref.: Vänttinen and Moravcova, 2001; Mulon Conseil Submission

SCCS comment
This study is from the open literature and the original study report was not provided for the SCCS evaluation. From the study description, it is evident that the study was not performed in line with the SCCS basic criteria for dermal absorption studies (SCCS Notes of Guidance SCCS/1628/21), as the skin samples from only one donor were used. However, it indicated that both genistein and daidzein become systemically available after topical application *in vivo* in humans.

Combined *in vitro* and *in vivo* study

In vitro skin absorption of daidzein was studied, compared with genistein, and completed with *in vivo* skin absorption tests conducted on nude mice for 8-weeks with soy isoflavones and aglycone mixture. The *in vivo* uptake of genistein was greater than that of daidzein, which was similar to the results of *in vitro* skin absorption. The study indicated that stratum corneum (SC) is the major contributor to the barrier function against the skin absorption of daidzein. Daidzein permeation showed greater enhancement when the skin was pre-treated with two enhancers, α-terpineol and oleic acid. Terpenes are known to act at the lipid polar heads of ceramides, while fatty acids act at the lipidic tail portion of intercellular lipid bilayers. This suggests that the lipid bilayers are the main barrier blocking the transit of daidzein in both the neutral and ionic forms. Both routes of the polar head and non-polar tail of the lipids contribute to daidzein's absorption. It is assumed that genistein may produce higher perturbation of the lipid bilayer.

Table 3
Permeation data of genistein and daidzein from pH 10.8 aqueous solutions across various skin types

Compound	Skin type	Flux (nmol/cm²/h)	K_p ($\times 10^{-2}$ cm/h)[a]	Enhancement ratio (ER)[b]
Genistein	Intact skin	8.22 ± 1.27	1.25 ± 1.10	–
	Delipid skin	20.30 ± 0.95	3.08 ± 0.14	2.46
	α-Terpineol treatment[c]	10.38 ± 1.06	1.57 ± 0.16	1.26
	Oleic acid treatment[c]	10.96 ± 1.22	1.66 ± 0.18	1.33
Daidzein	Intact skin	5.45 ± 1.03	0.29 ± 0.06	–
	Delipid skin	36.87 ± 1.75	1.97 ± 0.09	6.79
	α-Terpineol treatment[c]	24.22 ± 3.19	1.29 ± 0.17	4.45
	Oleic acid treatment[c]	20.75 ± 3.70	1.11 ± 0.20	3.83

Each value represents the mean ± S.D. ($n = 4$).
[a] K_p (cm/h), permeability coefficient = flux (nmol/cm²/h)/solubility (nmol/ml).
[b] Enhancement ratio (ER), K_p across treated skin/K_p across intact skin.
[c] The concentration of α-terpineol or oleic acid in a 25% ethanol/water was 3% (v/v).

Ref.: Huang *et al.*, 2008; Mulon Conseil Submission

SCCS comment
This study is from the open literature and the original study report was not provided for the SCCS evaluation. From the study description, it is evident that the study was not performed in line with the SCCS basic criteria for dermal absorption studies (SCCS Notes of Guidance, 2021). However, it indicated that daidzein and genistein become systemically available after topical *in vitro* and *in vivo* application to skin from nude mice.

SCCS overall comments on dermal/percutaneous absorption
The dermal/percutaneous absorption of daidzein and genistein has been reported in studies from the open literature. Original study reports were not available for evaluation, but the study descriptions indicated that they were not performed according to the SCCS basic criteria for dermal absorption studies (SCCS Notes of Guidance, 2021). The studies, however, indicated that both daidzein and genistein become systemically available after topical application to human and nude mouse skin (*in vivo* and *in vitro*). A flux of 37.3 ± 16.8 µg/cm² over 24 h through human skin was reported for pure daidzein in PEG400, whereas a higher flux rate of 155.7 ± 18.2 µg/cm² was reported for pure genistein in PEG400.

In the absence of studies on dermal/percutaneous absorption that are in accordance with the SCCS requirements, the SCCS will use a default value of 50% for dermal absorption of both daidzein and genistein in safety assessments.

3.2.3 Other studies on toxicokinetics

The toxicokinetics of soy isoflavones, including genistein and daidzein, has been intensively reviewed elsewhere (e.g., EFSA, 2015: VKM, 2017; Mulon Conseil Submission). Toxicokinetic and metabolism data in humans and test animals indicate that isoflavones are well absorbed and distributed in the form of glucuronide conjugates, with only a small proportion as aglycones. Orally ingested isoflavones are enzymatically metabolised in the gut and the liver, and by microorganisms in the intestine. Following consumption of soy or purified isoflavone preparations, isoflavones in plasma and urine of humans show a biphasic appearance, with peaks at 1–2 h (through small intestinal passive absorption), and again at 4–8 h of the uptake due to hydrolysis by gut bacteria (King and Bursill 1998; Fanti *et al.*, 1999; Franke *et al.*, 1999; Setchell *et al.*, 2003; Zubik and Meydani, 2003).

2.2.3.1. Genistein

Metabolism and Bioavailability

The aglycone resulting from hydrolysis of the glucoside form of genistein (genistin) can be absorbed via passive diffusion (Decroos *et al.*, 2005), and varies considerably between individuals (30–96%). Therefore, the oral dose of isoflavones has been considered as a poor indicator of the actual internal exposure (van der Velpen *et al.*, 2014; EFSA, 2015). The microbial conversion of genistein in soy-fed gnotobiotic rats resulted preferentially in the formation of dihydrogenistein, indicating that further conversion to 5-hydroxy-equol was slowed down, which may explain why it has not been detected *in vivo* (Matthies *et al.,* 2012).

In humans, the phase II conjugation products (glucuronides and sulphates) have been reported as the major metabolites of genistein resulting from liver metabolism and microbial transformation, which account for >95% of the total genistein concentration found in plasma, with hydroxylated derivatives, formed by cytochrome P450 enzymes, being minor metabolites (Rozman *et al.*, 2006; EFSA, 2015). This also means that less unconjugated isoflavone is present in the serum in humans compared to that in rodents. Although large interindividual variations have been observed in human clinical pharmacokinetic studies, the plasma level of total genistein is in micro molar range, while genistein (aglycon) is in hundred nano molar range *in vivo*. Genistein (aglycone) has also been found to be less than 1% in human urine. This is an important observation because the conjugated form has relatively little biological activity (Yuan *et al.*, 2012; Islam *et al.*, 2015).

In a study in female BALB/c mice, the absolute bioavailability of genistein amounted to 9–14 % after intravenous (i.v.) and oral administration of the pure substance at doses of 1.2 mg/kg genistein (Andrade *et al.*, 2010). In another study, Coldham *et al.* (2002) determined absorption after oral and intravenous dosing with 4 mg/kg bw ^{14}C-genistein in rats. The total absorption of radioactivity from the gut (parent compound and metabolites) was 56% in males and 111% in females, whereas the absolute oral bioavailability of the parent compound (genistein) was 7% in male rats and 15% in female rats.

Yang *et al.* (2010) investigated the systemic availability in male FVB mice (8–10 weeks old). In addition to genistein, genistein-7-glucuronide, genistein-4'-glucuronide, genistein-7-sulphate, and genistein-4'-sulphate were identified, with average maximum plasma concentration (C_{max}) values of 0.71 µM, 0.98 µM, 0.53 µM, 0.25 µM and 0.65 µM, respectively, after oral dosing at 20 mg/kg genistein.

A review by Yang *et al.* (2012) indicated that the oral bioavailability of genistein in rodents was in the range of 7 to 62% in rat, and 12 to 89% in mice.

Busby *et al.* (2002) investigated several administrations of genistein in healthy volunteers. C_{max} for genistein aglucone, as percentage of the total genistein, varied between 0.4 % and 3.9. A study by Setchell *et al.* (2011) provided relevant information by comparing the proportion of unconjugated genistein in the plasma of rats and humans. The percentage of unconjugated genistein was 4% in rats and 0.26% in female humans. The authors suggested that this information could be used to derive a chemical specific adjustment factor to enable interspecies extrapolation.

Shelnutt *et al.* (2000) conducted a study in 12 volunteers (six males and six females) who ingested a soy beverage prepared to provide a dose of 1 mg/kg genistein; 10% of the dose of genistein was recovered in the 24-hour urine.

Several studies have indicated that the mean proportion of unconjugated genistein in the plasma of adults ranges between 0.8–1.7%, after the *intake of various soy foods or soy extract (Gu et al., 2006; Hosoda et al., 2011; Setchell et al.*, 2011; Soukup *et al.*, 2014). A review of human studies by Yang *et al.* (2012) has indicated that orally-ingested genistein is absorbed rapidly due to the small molecular weight and lipophilic nature. The elimination half-life ($t_{1/2}$) for genistein (aglycone form) is reported to be in the range of 3.2–4.0 hours in men following the intake of soy food or soy isoflavone formulation. A study by Setchell *et al.* (2003), using an oral dose of 0.8 mg ^{13}C-labelled genistein/kg bw in premenopausal women, determined that t1/2 values of total genistein were 7.41 ± 0.39 hours. However, according to the authors, the urinary isoflavone concentrations correlated poorly with maximal serum concentrations, indicating the limitations of urine measurements as a predictor of systemic bioavailability.

According to EFSA, the maximum concentration of genistein aglycone varied between 0.4% and 3.9% expressed as a percentage of the total isoflavone concentration. EFSA stated that the absolute bioavailability of genistein must be low in humans.

Distribution

A study by Coldham and Sauer (2000) on the distribution of genistein in rats after oral administration of ^{14}C-labelled genistein found radioactivity in every organ. After 2 and 7 hours, the highest levels of radioactivity (>1,000 ng genistein-equivalent/g tissue) were in the gastrointestinal tract and in the excretory organs, liver and kidney. Intermediary concentrations (<1,000 to 250 ng genistein-equivalent/g tissue) were found in the reproductive organs - testis/ovary, uterus, prostate and vagina - and low radioactivity concentrations (<100 ng genistein-equivalent/g tissue) in brain, fat, thymus, spleen, skeletal muscle and bone.

Chang *et al.* (2000) observed high proportions (up to 90%) of genistein aglycone in several tissues, in particular the reproductive tissues, after oral administration in rats. This observation was confirmed by Zhou *et al.* (2008) who also observed the highest genistein concentrations in the gastrointestinal tract and in the excretory organs, liver and kidney after oral administration in rats. The concentrations in the reproductive organs were equal to the concentrations in the skeletal muscle and in fat.

Genistein was identified in breast, prostate and amniotic fluid (EFSA, 2015; Geer *et al.*, 2015).

The UK Committee on Toxicity (2003) noted that genistein binds weakly to sex hormone-binding globulin and concluded that phytoestrogens are unlikely to prevent binding of estrogen or androgens at genistein levels found in blood (<5 µM [<1351 µg/L]).

2.2.3.2 Daidzein [adapted from EFSA, 2015]:

Janning *et al.* (2000) investigated the toxicokinetics of daidzein in female DA/Han rats. Animals were ovariectomized 2 weeks before substance administration. Daidzein was administered to groups of rats either at 10 mg/kg bw i.v. (n=16) or orally (gavage) at 10 and 100 mg/kg bw (n=12, respectively). For investigation of biliary excretion, bile from bile-cannulated rats was investigated up to 7 h after i.p. administration of 10 mg/kg bw daidzein. Plasma-concentration-time curves after oral administration pointed to rapid absorption. The oral bioavailability was 9.7% and 2.2% for the doses of 10 and 100 mg/kg bw, respectively. At all of the time points studied, amounts of daidzein conjugates exceeded those of free daidzein. Despite notable differences in the absolute amounts of daidzein in tissue samples of individual rats, levels were usually 3- 5-fold higher than in

plasma. Low amounts were also detected in uteri, whereas concentrations in skeletal muscle or the brain were below the analytical detection limit. Results from bile-cannulated rats showed that daidzein is efficiently excreted with bile, mainly in conjugated form. Daidzein metabolites, excreted over a period of 8 h with bile amounted to approximately 30 % of the dose.

According to Bayer *et al.* (2001), the extensive liver metabolism led to a low oral bioavailability. Their study measured excretion of daidzein in male and female Fischer F344 rats after administration of daidzein (100 mg/kg bw, dissolved in corn oil) by gavage. For both sexes, 86% of the dose was excreted as unchanged daidzein in the faeces within 36 hours after administration, and 8–9% of the dose was excreted in the urine within 24 hours after administration (Bayer *et al.*, 2001).

In female BALB/c mice, absolute oral bioavailability for daidzein amounted to 29–34% (Andrade *et al.*, 2010). Studies investigating distribution of daidzein in humans or animals have not been reported. However, daidzein was measured in human prostate tissue after 2 weeks of intervention with an isoflavone-containing supplement (Rannikko *et al.*, 2006). Furthermore, daidzein was also measured in breast tissue after soymilk intake (Bolca *et al.*, 2010).

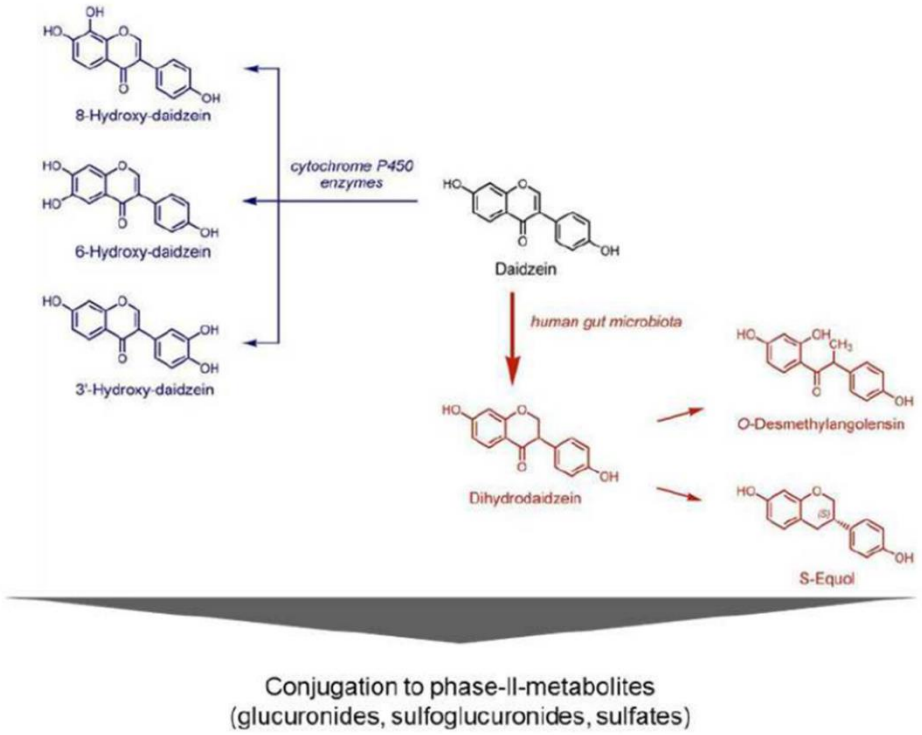
From the study of Busby *et al.* (2002), who investigated several oral formulations containing daidzein in healthy volunteers, C_{max} for daidzein aglycone, as percentage of the total daidzein, varied between 1.4% and 4.2%.

Rüfer *et al.* (2008) investigated the pharmacokinetics of pure daidzein in the aglycone and glycosidic form after oral administration in humans. Only results for the aglycone form is reported here: 7 men aged 22-30 years ingested 1 mg daidzein equivalent/kg bw. Blood samples were drawn before isoflavone administration and 1, 2, 3, 4.5, 6, 8, 10, 12, 24, and 48 h after the dose administration. Urine was collected before and 0–6, 6–12, and 12–24 h after the intake of the isoflavones. The concentrations of daidzein and its major metabolites in plasma and urine were measured with isotope dilution capillary gas chromatography–mass spectrometry. The following pharmacokinetic parameters were calculated for daidzein: AUC_{inf}: 8.3 ± 2.7 µmol x h/l; C_{max}: 0.43 ± 0.12 µmol/l; t_{max}: 8.3 ± 0.7 h and t1/2: 10.9 ± 1.9. Compared to the glycosidic form, lower AUC and C_{max} values were obtained for daidzein in the aglycone form. The fractional absorption for daidzein (% of dose based on recovery in urine) was 11.6% of the ingested amount. However, urinary isoflavone concentrations correlated poorly with maximal serum concentrations, indicating the limitations of urine measurements as a predictor of systemic bioavailability (Setchell *et al.*, 2011).

The study by Setchell *et al.* (2011) provided relevant information by comparing the proportion of unconjugated daidzein in plasma in rat and humans. The percentage of unconjugated daidzein was 8.1% in rats and 0.98% in female humans. At least some data on the metabolism of daidzein is available for humans, monkeys, rats and mice. Monkeys, rats, and mice are described as 100% equol producers, meaning that the microbiotas of these animals are uniformly able to transform daidzein to a considerable extent to S-equol (Gu *et al.*, 2006). In contrast, the microbial metabolism of daidzein in humans is characterised by large interindividual variability, and only a part of the population is able to produce S-equol. As a consequence of this heterogeneity, microbial metabolites other than equol, e.g., dihydrodaidzein or O-demethylangolensin, can be present in human plasma at higher concentrations than S-equol.

Conjugated isoflavones may be deconjugated, as a recent study using U2OS and T47D cells showed, but the degree of deconjugation observed was low (0.2–1.6 %) (Islam *et al.*, 2015). Using human breast tissue S9 fraction, the average total deconjugation after 24 hours was 2.4% and 2.0%, respectively, whereas the rat breast tissue S9 fraction was about 30 times more potent in deconjugating the 7-O-glucuronides (Islam *et al.*, 2015). This is in line with the finding that only low concentrations of unconjugated daidzein (< 50 pmol/g breast tissue) was found in breast tissue homogenate from healthy women after the intake of soy milk or a soy supplement (Bolca *et al.*, 2010). The authors estimated overall total glucuronidation of 98% in breast tissue, although not all phase II metabolites were determined. Oxidative phase I metabolites of daidzein, mainly 6-hydroxy-, 8-hydroxy and 3'-hydroxy-daidzein are found in humans, rats and mice (Breinholt *et al.*, 2000; Kulling *et al.*, 2001; Rüfer *et al.*, 2008). Although the extent of their formation is described as low, all these minor metabolites bear a catechol structure and might be easily oxidised to form reactive o-quinones. Moreover, o-quinones are described as reactive metabolites towards nucleophiles. No quantitative data were available on phase I metabolites that would allow a comparison between species. The main phase II metabolites of daidzein is the 7-glucuronide-4'-sulphate, whereas in female rats and mice the monoglucuronides are the predominant conjugates (Hosoda *et al.*, 2011; Soukup *et al.*, 2014). The metabolism of daidzein is depicted in Figure 1.

Figure 1: Metabolism of daidzein



Most ingested daidzein is excreted as phase II conjugates and as phase II conjugates of microbial-derived metabolites in the urine (King e al., 1998). Faecal elimination has been found to be a minor route. Larkin *et al.* (2008), in their review, reported that total faecal excretion of isoflavones in humans accounts for less than 5%, and is predominantly in the unconjugated form, with less than 10% being conjugated. These same authors also state that the majority of urinary excretion of daidzein occurs within the first 24 hours after

ingestion. Xu *et al.* (1994) however report that total faecal excretion of isoflavones was only 1-2% of the ingested amount.

The urinary recovery rate was determined in several studies. Shelnutt *et al.* (2000) conducted a study in 12 volunteers (six males and six females) who ingested a soy beverage prepared to provide a dose of 0.6 mg/kg daidzein aglycone equivalents; 27.4% of the dose of daidzein (aglycone plus phase II conjugates) was recovered in the 24-hour urine. Based on urinary dihydrodaidzein, total recovery was 50.9% for daidzein. S-equol was not measured in this study and therefore not included in the excretion rates determined over time. The apparent terminal half-lives for daidzein glucuronide, the main metabolite found in urine, was 3.8 ± 0.4 hours, including the microbial metabolites in the calculation made for daidzein. In other studies, the percentages of a daidzein dose excreted in the urine were reported to be 46.9% (Lu *et al.*, 1995), 35.8% (Watanabe *et al.*, 1998), 62 % (King and Bursill, 1998) and 50% (range 18–95%) (Setchell *et al.*, 2003). At least part of the variability is certainly based on the fact that not all known microbial metabolites were included in each calculation and that enzymatic hydrolysis, which was used in all studies to quantify the resulting aglycones, might be not complete in every single case.

The half-life ($t_{1/2}$) reported in most human studies is the half-life of total daidzein. This means that the values were calculated on the basis of daidzein measurement after enzymatic hydrolysis of the different phase II conjugates which accounted for > 95% of the total daidzein concentration found in plasma. The values are summarised in the review of Yang *et al.* (2012). In one study, ^{13}C-labelled daidzein was used. The $t_{1/2}$ value of total daidzein was determined to be 7.18 ± 0.49 hours for a dose of 0.8 mg daidzein/kg bw (Setchell *et al.*, 2003b).

SCCS overall comments on toxicokinetics

Genistein is rapidly and nearly completely absorbed through the oral route *in vivo* due to small molecular weight and favourable lipophilic property. Toxicokinetic and metabolism studies in human and test animals have indicated that, like other isoflavones, genistein is absorbed and distributed mainly in the form of glucuronide conjugate, with only a small proportion as aglycone. After absorption, genistein (aglycone) is rapidly and extensively metabolised enzymatically (in the gut and liver), and by microorganisms (in the intestine), and the concentration of unconjugated genistein in plasma in humans is very low. This is an important consideration for safety assessment because the conjugated form has relatively little biological activity.

For daidzein, the available information on human toxicokinetics is limited. Most studies refer to the isoflavone or glycosidic form of daidzein, which is of limited relevance for the safety evaluation of daidzein in aglycone form used in cosmetic applications. Based on the data available on the toxicokinetics of daidzein, species differences between rodents and humans have been observed. In humans, it seems that daidzein is well absorbed, but it is not possible to derive a reliable percentage value for oral bioavailability from the current data, as the available information indicates that human isoflavone bioavailability depends upon the relative ability of gut microflora to degrade these compounds. After systemic absorption, daidzein undergoes extensive first-pass metabolism, which accounts for its low bioavailability in humans. Because of the insufficient data on oral bioavailability, and on the first-pass metabolism in the gut, the SCCS will use a value of 25% to account for oral bioavailability when MoS calculation is based on oral studies.

The available information indicates that ADME of isoflavones is also different for rodents and for humans. Therefore, oral bioavailability information from rodents cannot be

extrapolated to humans as such. In humans, studies in volunteers provide some information on the oral bioavailability, based on the percentage recovery in urine of the administrated dose. However, there are limitations in the derivation of oral bioavailability based on this approach because urinary isoflavone concentrations are poorly correlated with maximal serum concentrations, indicating the limitations of urine measurements as a predictor of systemic bioavailability.

For genistein, Setchell *et al.* (2011) and Yang *et al.* (2012) reported that the percentage recovery in urine of the administrated dose (genistein) varies between 7.7 and 30%. From a collective consideration of the available information, the SCCS will use a value of 25% for oral bioavailability of genistein.

For daidzein, King and Bursill (1998), Rüfer *et al.* (2008), Setchell *et al.* (2011), Shelnut *et al.*, (2000) the percentage recovery in urine of the administrated dose (Daidzein) varies between 11.6 and 62%.

Apart from the oral studies, data from studies using subcutaneous administration are also available for both genistein and daidzein (see section 3.3.5), which might allow identification of a point of departure for risk assessment. Due to the uncertainties associated with the oral absorption percentage of daidzein in humans, and with the rapid conjugation of the absorbed genistein (aglycone), the SCCS considers that the use of data from subcutaneous studies is also justified in these cases and provides more reliable information than the data from oral studies - for which assumptions of 25% oral bioavailability have been considered for genistein and for daidzein.

3.2.4 Calculation of SED

Leave-on products with 0.007% genistein

Amount of product applied daily:	g/day	=	7.82
Typical body weight of human:	kg	=	60
Amount of product applied daily:	mg/kg bw	=	130
Concentration of genistein:	%	=	0.007
Amount of genistein applied daily	mg/kg bw	=	0.0091
Absorption through the skin	%	=	50
Systemic exposure dose (SED)	mg/kg bw/day	=	0.0046

Leave-on products with 0.02% daidzein

Amount of product applied daily:	g/day	=	7.82
Typical body weight of human:	kg	=	60
Amount of product applied daily:	mg/kg bw	=	130
Concentration of daidzein:	%	=	0.02
Amount of daidzein applied daily	mg/kg bw	=	0.026
Absorption through the skin	%	=	50
Systemic exposure dose (SED)	mg/kg bw/day	=	0.013

3.2.5 Other source of exposure: food intake

Isoflavones are a class of naturally occurring substances, present in a number of plants, especially in soybeans, red clover and kudzu root. Isolated isoflavones used in dietary supplements are defined as extracts from soybeans containing a mixture of predominantly glycosylated genistein, daidzein and Glycitein and isolated forms of these soy-based compounds and extracts from red clover.

Background dietary isoflavone exposure was estimated by an EFSA panel using levels of isoflavones in soy and soy-based products reported in the literature, in combination with food consumption data from the EFSA Comprehensive European Food Consumption Database for the group of women older than 40 years of age. Exposure to isoflavones from food supplements in the population of peri- and post-menopausal women was taken from the range of doses used in the intervention studies in the target population included in the systematic review. In addition, information from the relevant industry associations was obtained on the labelled amount of isoflavones in the supplements and the recommended range of daily doses. This information was compared in the EFSA Opinion with published data reporting on the measured isoflavone content in the food supplements (EFSA, 2015). The intake of isoflavones in the general European population is relatively low, most studies showing intake estimates below 1 mg/day, with the exception of the UK, where intake levels are up to 3 mg/day. Higher intakes have been reported among men than among women. Among vegetarians and consumers of soy, intake estimates are much higher than those for the general population, up to 20 mg/day, but still lower than what can be expected in a supplement (EFSA, 2015).

On the basis of the published studies included in the EFSA assessment, and the actual measured content of isoflavones in a number of food supplements available on the market, it can be estimated that intake of isoflavones from food supplements is extremely variable, ranging approximately 0.1–100 mg/day for soy isoflavones, 30–160 mg/day for isoflavones from red clover and 20–50 mg/day for kudzu root isoflavones (all the values above are expressed as aglycones). These values are in line with information provided by the relevant food sector business operators, with respect to the recommended daily doses of isoflavones in products mainly targeted at menopausal women.

According to the preparation processes and taking into account the physicochemical properties of isoflavones under aglycone or heteroside forms, food intake differs between Asian and the Western dietary practices: traditional Asian preparations mainly contain isoflavones under their aglycone form (i.e., genines), whereas modern western preparations (nonfermented) mainly contain isoflavones under their heteroside form (Wang *et al.*, 1994).

In the Nordic Council of Ministers report (2020), the authors have estimated the exposure of the Danish population to daidzein and genistein from diet. The major dietary contributors in these estimated intake scenarios of genistein, daidzein and glycitein are milk when replaced with soy milk and minced meat (dishes, beef patties, sauce Bolognese/ragu and meat balls) replaced by soy-based minced meat for women, girls and boys. Beans are significant contributors of genistein and daidzein for women while dried pulses are significant contributors of genistein and daidzein for girls. In general, soy sauce and cheese are among the food items that are minor dietary contributors of isoflavone intake among women, girls and boys.

Table 11. Total estimated daily soy, genistein, daidzein, glycitein and total isoflavone intake (mg/kg bw per day) among Danish women, girls and boys.

Target group	Body weight (kg)*	Estimated total soy consumption (mg/kg bw per day)	Estimated genistein exposure LOW** (mg/kg bw per day)	Estimated genistein exposure HIGH*** (mg/kg bw per day)	Estimated daidzein exposure LOW** (mg/kg bw per day)	Estimated daidzein exposure HIGH*** (mg/kg bw per day)	Estimated glycitein exposure LOW** (mg/kg bw per day)	Estimated glycitein exposure HIGH*** (mg/kg bw per day)	Estimated total isoflavone exposure LOW** (mg/kg bw per day)	Estimated total isoflavone exposure HIGH*** (mg/kg bw per day)
Total Women	71	385	0.04	0.06	0.02	0.05	0.003	0.003	0.05	0.1
Total Girls	28	1319	0.09	0.2	0.04	0.2	0.01	0.01	0.1	0.3
Total Boys	28	1508	0.1	0.2	0.05	0.2	0.01	0.01	0.2	0.4

Average body weights based on measured data from DANSDA. **LOW = lowest level reported in Forslund & Andersson (2017), ***HIGH = highest level reported in Forslund & Andersson (2017).

SCCS Conclusion of other sources of exposure

The available information suggests that certain categories of food and food supplements are the major sources of oral intake of genistein and daidzein along with other dietary isoflavones. The dietary intake values reported in different studies are however highly variable, but they indicate that average Asian diets contain much higher amounts of isoflavones than the average Western diets. Traditional Asian preparations mainly contain isoflavones in their aglycone form, whereas modern western preparations mainly contain isoflavones in the heteroside form.

3.3 TOXICOLOGICAL EVALUATION

3.3.1. Irritation and corrosivity

3.3.1.1 Skin irritation

According to one of the submitters, "No or negligible skin irritation was shown for the topically applied substances (stratum corneum disruption and skin erythema)" and reference was given to a publication from the open literature. The cited publication had assessed *in vivo* skin irritation in mice by means of transepidermal water loss (TEWL) and pH. The authors stated that for genistein, an increase in the delta for TEWL and pH was observed, whereas for daidzein, only a slight increment in the skin pH value was observed.

Ref.: Individual company submission, Huang *et al.*, 2008

SCCS comment

The study described is not in line with the SCCS requirements for skin irritation. Therefore, no conclusion on the skin irritation potential of genistein or daidzein could be drawn from this study. The information provided to ECHA under CLP notifications has indicated that daidzein causes serious eye irritation and skin irritation https://echa.europa.eu/fr/substance-information/-/substanceinfo/100.006.942.

Human studies

For the skin irritation endpoint, as manufacturer of a finish product, the submitter performed a Photopatch test (Potential skin irritation test after exposure to UVA/visible irradiation) and a Patch test on sensitive skin under occlusion.

Potential skin irritation on sensitive skin

The Patch test assesses the potential side effects (skin erythema and oedema reactions) that may occur after applying a cosmetic product to evaluate whether the topical product is safe for consumer use.
Twenty-five adult healthy volunteers of both sexes on sensitive skin, with no history of allergic skin reactions, and not under treatment with steroids, were included in the Patch test. The product was applied as it is by a Finn Chamber fixed to the skin of the arm. The samples were maintained *in situ* for 48 hours and the affected area has not been cleansed for the entire duration of the test. The evaluation of the reactions was carried out 15 min.,1, 24, hours after removal of the Finn Chambers.
According to the amended Draize scale (< 0.5 is the limit under which the product is classified as not irritating), the product was demonstrated as non-irritating for human skin. At the described conditions, the cosmetic product was well tolerated and assessed as non-irritating on sensitive skin.

Ref.: Individual company submission

SCCS comment
The provided information was not supported by data or original study reports. It appears that the test was performed with a finished product, for which the concentration of the ingredients genistein and/or daidzein has not been reported. Therefore, no conclusion on skin irritation could be drawn from this description.

3.3.1.2 Mucous membrane irritation / eye irritation

No data provided.

SCCS overall comments on skin/eye irritation
Adequate studies on skin and eye irritation have not been provided. However, the SCCS considers that the proposed low level of use (0.02% daidzein; 0.007% genistein) in a final cosmetic product is unlikely to cause skin/eye irritation.

3.3.2 Skin sensitisation

No data provided

SCCS comment
Adequate information on skin sensitisation of genistein and daidzein should be provided.

3.3.3 Acute toxicity

3.3.3.1 Genistein

Two acute toxicity studies were conducted in male and female Hanlbm Wistar rats fed a genistein-free diet and the Wistar Crl:(WI)BR rats fed standard animal diet. The rats were administered genistein (99.5–99.6% purity) in a single gavage dose of 2000 mg/kg bw and observed for 2 weeks. The rats were then killed and necropsied. Liver and kidney weights were measured in the Hanlmb rats.

All rats survived, and there were no gross effects at necropsy or changes in organ or body weights. In the Wistar Crl:(WI)BR rats, lethargy was noted in all males and one female on "day 1" and alopecia was observed on "days 14 and 15." The study authors concluded that genistein has low acute toxicity (McClain *et al.*, 2006).

3.3.3.2 Daidzein

LD50 (i.p., mouse): > 2000 mg/kg

Reference: https://pubchem.ncbi.nlm.nih.gov/compound/daidzein#section=Acute-Effects&fullscreen=true

From the SCCS literature search

An acute oral toxicity study (modified version of OECD TG 423) was described by Laddha *et al.* (2020). Animals were divided into four groups containing three animals in each. Group 1 was served as a normal control group that received 0.5% carboxymethyl cellulose (CMC) as a vehicle. Group 2, 3 and 4 received daidzein orally at doses of 300, 2000 and 5000 mg/kg bw, respectively. Animals were observed individually after dosing for any sign of changes in behaviour and physiological appearance during the first 30 min and then periodically during the first 24 h with special attention on the first 4 h. All the groups were kept under observation for 14 days. Animals tolerated the highest dose of 5000 mg/kg bw without significant changes of toxicological relevance.

SCCS comment
Original study data from the references mentioned above are not available.

The SCCS overall comment on acute toxicity
The available information indicates that the acute toxicity of both genistein and daidzein is low.

3.3.4 Repeated dose toxicity

3.3.4.1 Genistein

Repeated dose toxicological studies on genistein have been described in sections 3.3.4, section 3.3.10.1 (under level 5), and in the Annex-A Tables 9 and 9a.

3.3.4.2 Daidzein

3.3.4.2.1 Repeated dose (28 days) oral / dermal / inhalation toxicity

Guideline/Guidance:	OECD TG 407
Species/Strain:	Rat, Sprague-Dawley
Group size:	5/sex/dose
Test item:	daidzein
Purity:	/
Vehicle:	0.5% hydroxymethylcellulose (HMC)
Control:	Vehicle
Route of exposure:	Oral (no further details)
Duration:	28 days
Doses:	0, 25, 50 and 100 mg/kg bw/d
Study period:	not stated
GLP:	not stated

Groups of animals received oral doses of 0, 25, 50 and 100 mg/kg bw/d for 28 days. Animals were observed frequently for signs of toxicity and mortality. Body weight, food and water intake of all experimental animals were recorded weekly. At the end of 28 days, urine samples were collected using metabolic cages. Blood was withdrawn from retro-orbital plexus, collected in EDTA (disodium ethylenediaminetetraacetic acid) containing tubes and subjected to haematological analysis. Then, plasma was separated by centrifugation for the determination of biochemical and electrolyte analysis. All animals were humanly sacrificed after 28 days of treatment and were subjected to gross necropsy which included a detailed examination of the exterior surface of the body, all orifices, thoracic and abdominal cavities and their contents. Brain, heart, lung, liver, spleen, kidney, adrenals, stomach and gonads were removed and weighed immediately. Organ weight and body weight ratio were taken into consideration for the determination of relative organ weight. Various organs like brain, heart, lung, liver, spleen, kidney, adrenal gland, stomach and gonads were stored in 10% formalin solution for histopathological examination. All the tissues were embedded in paraffin and thin sections were prepared using a microtome. Hematoxylin and Eosin (H&E) stain were performed to check the histology.

Results

Body weight, food and water intake of daidzein-treated animals was not different from controls. No statistically significant differences were observed in hematology and biochemical parameters of daidzein treated animals when compared to control animals. Electrolyte levels like sodium, potassium and phosphorus were also found to be in the normal range in the daidzein treatment group when compared with the control group. No significant alteration was observed in the urine volume of daidzein-administered animals when compared to controls. Also, kidney function was not impaired. No significant difference was observed in organ weight to body weight ratio of daidzein treated groups when compared with normal control animals. During the microscopical examination, no significant alteration was observed in tissue architecture of daidzein treated animals.
Organs like brain, heart, lung, liver, spleen, kidney, adrenal gland, stomach, testes and ovaries showed normal histology after daidzein treatment.

Ref.: Laddha *et al.*, 2020

SCCS comment
This study is described from open literature and the original study report is not available to the SCCS. However, it indicates that the highest dose of 100 mg/kg bw/d can be considered as NOAEL after 28-day repeated oral administration of daidzein.

3.3.4.2.2 Sub-chronic (90 days) oral / dermal / inhalation toxicity

Guideline/Guidance:	/
Species/Strain:	crossbred [(Large White × Landrace) × Duroc] weaned pigs
Group size:	12/sex/dose
Test item:	daidzein
Purity:	98%
Control:	NRC (1998) standard commercial feed without any soy source
Route of exposure:	Oral, diet
Duration:	70 days
Doses:	0, 40, 200 and 400 mg daidzein/kg diet
Study period:	not stated, publication submitted in 2013
GLP:	not stated

The treatment phase was divided in phase 1 (days 1-35) and phase 2 (days 36-70). Blood was taken for hematology and determination of hormone levels on day 35 and 70. On day 35 and 70, selected pigs were slaughtered by a lethal injection of sodium pentobarbital to obtain samples of small intestinal segment, mesenteric lymph, muscle, and organs (liver, heart, kidney, and spleen). The middle sections (4 cm) of duodenum, jejunum, and ileum were isolated and kept in 10% formalin for microscopic assessment of mucosal morphology. About 5 cm^3 of a regular shape sample from each organ was kept in the same way for morphological assessment. About 10 g of semitendinosus and liver samples was collected from each pig and kept at −20 °C for residue analysis of isoflavones. Isoflavones were determined in liver and semitendinosus muscle.

Results
At 200 mg/kg, average daily weight gain in phase one was higher when compared to controls, whereas it was lower at 400 mg/kg in phase 2. There were no effects ($P > 0.05$) of daidzein treatments on the content of triiodothyronine, tetraiodothyronine, IGF-1, GH, or estradiol in the serum of pigs in both phase 1 and phase 2. However, at 200 and 400 mg/kg serum testosterone levels were reduced when compared to controls (statistically significant in females only). Hematology analysis revealed that HCT, MCV, and MCH in pigs was significantly increased at the dose of 400 mg/kg of daidzein ($P < 0.05$), while other hematological parameters were not changed after 35 days of treatment. 200 mg/kg of daidzein supplementation also increased HCT ($P < 0.05$) of pigs compared to controls. Histological evaluation showed no effect of dietary supplementation of daidzein on villus height of the small intestine. However, pigs fed 200 and 400 mg/kg of daidzein had smaller ($P < 0.05$) crypt depth and greater ($P < 0.05$) villus height-to-crypt depth ratio (VCR) in the duodenum compared with that in controls on day 35, however, intestinal morphology was not affected by daidzein in phase 2. According to the histology score (organ damage index), there were few observable effects of 40 and 200 mg/kg of dietary daidzein supplementation on the morphology of all the organs and tissues examined in both phases 1 and 2. However, 400 mg/kg of daidzein supplementation could cause mild damage ($P < 0.05$) to the spleens of weanling pigs by day 70. At 400 mg/kg of daidzein there was an increase the histology score of livers at the end of phase 2 without gender difference.
Ref.: Xiao *et al.*, 2015

SCCS comment
This study is described from open literature and the original study report is not available to the SCCS. However, a variety of toxicologically relevant parameters have been investigated in the study, and adverse effects are observed at 400 mg/daidzein/kg diet and a NOAEL of 200 mg daidzein/kg diet. Based on the weight range of weaned, growing pigs, a body weight range between 12 and 50 kg and food consumption between 4 and 8 kg (https://agritech.tnau.ac.in/animal_husbandry/ani_pig_feeding%20mgt.html) can be assumed, resulting in an intake level of 16 mg/kg bw/d.

3.3.4.2.3 Chronic (> 12 months) toxicity

Under the conditions of a 2-year feed study with continuous exposure to the test compound from conception through termination (F1C), there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm. There was some evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on increased incidences of mammary gland adenoma or adenocarcinoma (combined) and pituitary gland neoplasms. The incidence of benign mammary gland fibroadenoma in female rats was significantly decreased in the 500-ppm group. Under the conditions of this 2-year feed study with exposure to the test compound from conception through 20 weeks followed by control feed until termination (F1T140), there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm. There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on marginally increased incidences of pituitary gland neoplasms.

Under the conditions of this 2-year feed study where offspring of three prior generations of animals exposed to the test compound were exposed from conception through weaning (PND 21) followed by control feed until termination (F3T21), there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm. There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on increased incidences of mammary gland adenoma or adenocarcinoma (combined).

The findings of the multigenerational NTP study on genistein in Sprague-Dawley rats regarding reproductive and developmental toxicity have been discussed in detail in section 3.3.5. In brief, the exposure to 500 ppm of Genistein caused lower body weights and some alterations in the reproductive system of female rats. Exposure to genistein caused lower body weights in one generation of male rats and increases in mammary gland hyperplasia and renal tubule calcification. Except for lower body weights in pups, there was no evidence for a carryover of genistein effects into unexposed generations. Although genistein showed adverse effects with dietary exposures of 100 or 500 ppm, there were no clear adverse effects on the reproductive or developmental parameters up to 100 ppm of genistein (NTP report TR 539– 2008).

3.3.5 Reproductive toxicity

3.3.5.1 Genistein

Oral route

An NTP multigenerational study reported in detail by Rozman *et al.* (2006) used Sprague-Dawley rats fed with diet containing genistein at 0, 5, 100, 500 ppm (equivalent to males: 0, 0.3, 7, 35 mg/kg bw/day; females: 0, 0.4, 9, 44 mg/kg bw/day; and females during

lactation: 0.7, 15, and 78 mg/kg bw/day), and followed the effects over 5 generations (F5 litters). The scheme of dosing genistein is shown in Figure 2.

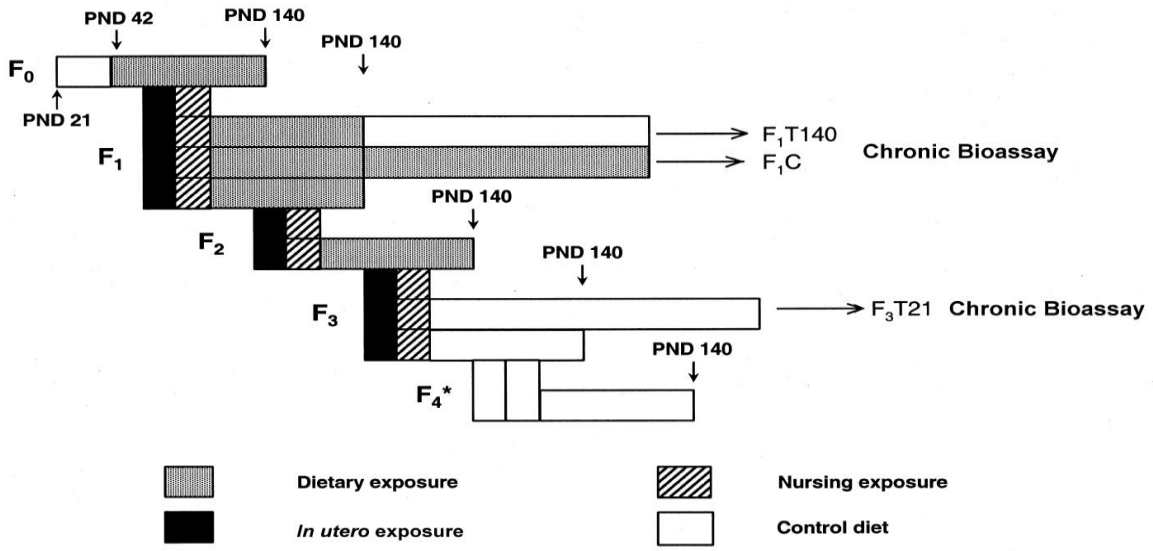


FIGURE 1
Dosing Schedule for the Multigenerational Reproductive Toxicology and Chronic Studies

Figure 2: Source: National Toxicology Program (NTP) (2008a). Multigenerational Reproductive Toxicology Study of Genistein (CAS No. 446-72-0)

The SCCS considered this as a pivotal study for this assessment because it provides the most comprehensive picture regarding the reproductive and developmental effects of genistein as it extends over five generations from a parental group of rats that were exposed to genistein through feed starting at the age of 6 weeks:

- The first and second generations of offspring were exposed to genistein during conception through their mothers, during weaning through their mothers' milk, and during their lifetimes through feed containing genistein.
- The third generation was exposed just during gestation and weaning, and
- The fourth and fifth generations were not exposed directly - to see if there were any carryover effects from the exposure of earlier generations.

The primary measures examined during each generation were body weights, development of reproductive organs, and number of offspring per litter after each cycle of mating.

The doses were selected considering the serum concentrations of total genistein:

- adult rats fed the control and low dose (5 ppm) diets that are similar to human adults consuming a typical Western diet (<0.1 µmol/L);
- rats fed 100 ppm genistein diet that is similar to human adults consuming a typical Asian diet (0.1– 1.2 mmol/L, Adlercreutz *et al.* 1994) or soy nutritional supplements (0.5– 0.9 µmol/L, Doerge *et al.* 2000); and

- rats fed 500 ppm genistein diet that is similar to infants consuming soy formulas (2–7 µmol/L, Setchell *et al.* 1997).

The results showed that dietary exposure to:

- 500 ppm genistein (approx. 35 mg genistein/kg bw/day in males and 51 mg/kg bw/day in females) decreased body weights, accelerated vaginal opening, decreased anogenital distance, and altered estrous cyclicity in females continuously ingesting genistein. Significant decreases in postweaning body weights, and decreases in anogenital distance, were only observed in F1 males and not in the similarly exposed F2 generation. In animals exposed to 500 ppm, there was some evidence for reduced litter size in the F1 and F2 generations that were continuously exposed to the test chemical. Weaker effects on the incidences of male mammary gland hyperplasia were observed in 500 ppm males exposed only as adults or exposed only in utero and through lactation. No other impacts on fertility and no histopathologic lesions were observed in females. The male reproductive tract did not show significant alteration. Weaker effects on the incidences of male mammary gland hyperplasia were also observed in males exposed only as adults or exposed only in utero and through lactation.
- 100 ppm genistein (approx. 7.4 mg genistein/kg bw/day in males and 10.2 mg/kg bw/day in females), and also 500 ppm genistein, increased incidences of hyperplasia of the mammary gland and calcification of renal tubules in continuously exposed males, examined at 20 weeks of age. Other than decreased body weight gains in preweaning pups, there was no evidence for a carryover of genistein effects into the unexposed generations.

According to the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction (CERHR)'s Expert Panel Report on the Reproductive and Developmental Toxicity of Genistein (Rozman *et al.*, 2006), evidence is sufficient to conclude that genistein produces reproductive/developmental toxicity in the offspring (i.e., F1, F2, F3) of rats at 500 ppm (approximately 35 mg/kg bw/day in males and 44 mg/kg bw/ day in females) via oral administration as manifested by decreased anogenital distance and body weight in male and female pups, abnormal estrous cyclicity and decreased age and body weight at vaginal opening in female pups, and increased age at testicular descent in male pups. These effects do not manifest themselves in the F0 generation. The multigenerational design does not permit determination of whether the adverse effects were due to exposures during reproductive or developmental ages. The experimental animal data are assumed relevant to the assessment of human risk'.

There are several other detailed studies that confirm that the administration of genistein at doses ranging from 5 to 1250 ppm consistently show that only doses starting from 500 ppm cause adverse effects attributable to the predictable hormonal effect of genistein, which become severe at doses above 1250 ppm equivalent to 69.96 – 96.75 mg/kg b.w. As an example, the study conducted by McClain *et al.* (2006) according to GLP using genistein doses of 0, 5, 50, or 500 mg/kg bw/day for 52 weeks has reported the only significant organ weight effects considered by the authors to be treatment-related were at 500 mg/kg bw/day at 52 weeks. These included increased relative weights of adrenal and spleen (males and females), prostate (7%), testis (52%), ovary (394%), and uterus (275%) in the 500 mg/kg bw/day group. Increases in adrenal, spleen, and uterus weights were also observed following 26 weeks of treatment. Increased ovary weight was the only organ weight effect that persisted through the recovery period. At the 52-week necropsy,

uterine horn dilation was observed in 7 females of the 500 mg/kg bw/day group and watery cysts in ovaries were noted in 4, 3, and 12 females of the low-, mid- and high-dose group.

Histopathological changes in vagina and mammary gland were observed at 500 mg/kg bw/day. Extramedullary hemopoiesis was reported to occur in the spleen at all doses and was stated to be a compensatory response to decreased bone marrow resulting from bone thickening. Liver histopathology was observed in males and females at 500 mg/kg bw/day. Many of the histopathology observations at 52 weeks (i.e., effects in liver, bone, epididymides, prostate, ovaries, uterus, and vagina) were also observed at 26 weeks. Following the 8-week recovery period, osteopetrosis in females and epididymal vacuolation were the only persistent histopathological effects observed at the high dose. In male rats, prostate inflammation was observed at ≥50 mg/kg bw/day. In female rats, the study authors reported histopathology alterations in ovaries and uterus/cervix at ≥50 mg/kg bw/day and an increase in ovarian atrophy and prostate inflammation at 50 mg/kg bw/day.

Other findings included hyperplasia of the male mammary tissue at a LOAEL of 7 mg/kg bw/day and alveolar proliferation in female mammary tissue at LOAELs of 15 mg/kg bw/day (prenatal exposure) and 30 mg/kg bw/day (lactational/post-pubertal exposure). Other studies showed developmental effects including decreased litter size, decreased pregnancy rate, decreased mated dams delivering litters, disrupted estrous cycles, altered ovarian histopathology, prostate tissue changes, and accelerated vaginal opening at LOAELs ranging from 12.5–83 mg/kg bw/day.

The overall evidence, remarkably consistent, as listed in Annex-A Table A9s (from Rozman *et al.*, 2006) is sufficiently adequate to conclude that genistein produces developmental toxicity in rats following dietary exposure at a LOAEL of 7–9 mg/kg bw/day, which is equal to 100 ppm (BMDL10 of 20–26 mg/kg bw/day) in a five-generation study. Some of these effects were seen at similar doses in mice. The experimental animal data were assumed relevant to the assessment of human risk (Rozman *et al.*, 2006).

Subcutaneous route

Lewis *et al.* (2003) studied the effects of subcutaneous exposure to genistein in neonatal rats during the period from postnatal day (PND) 1 (the day of birth) to PND 21. Due to the lack of a significant exposure of the newborn pups via the mother's milk, the study used subcutaneous injection of genistein to the pups over the period PND 1–7, followed by daily gavage up to PND 21.

The study allocated mated female rats to three groups of 20 and allowed them to litter. Pups were dosed genistein subcutaneously 0.2 or 4 mg/kg (equivalent to 4 or 40 mg/kg by oral route, bioequivalent selected from the results of a previous study on comparative exposure by different routes) each day from PND 1 (the day of birth) to PND 6, and orally (4 or 40 mg/kg) each day from PND 7 to 21.

At PND 22, one male and one female pup from each litter were killed and serum samples taken for the analysis of testosterone, LH, and FSH in males, and estradiol, LH, FSH, and progesterone in females. There were no consistent treatment-related effects on hormone levels in males or female offspring at day 22. Blood progesterone concentration was reduced in mature females following 40 mg/kg genistein. Genistein at both doses had no effect on basal- or GnRH-stimulated LH secretion from the pituitary. Anogenital distance was no different in immature pups when assessed at day 2 postpartum and showed no biologically significant difference from control values at day 22. In female pups, the uterus weight at 40 mg/kg bw was two-fold higher than that of the control pups at day 22, but returned to control values in mature females at 12 weeks of age. The time of vaginal

opening was four days earlier in females in the high-dose group and in most developing female pups. At 40 mg/kg bw for the animals smeared from the time of vaginal opening, 13/20 showed permanent estrus (persistent cornification) with 19/20 of the animals smeared from day 43 showing this pattern. The time of preputial separation in males was not affected by administration of genistein. Testis weights were unaffected by either dose of genistein. The no observed adverse effect level (NOAEL) of genistein was considered to be between 4 and 40 mg/kg/day based on the hormonally induced functional changes at the higher dose due to the expected endocrine effects. From this study, the SCCS identified 4 mg/kg bw/day of genistein as the NOAEL for the subcutaneous route.

There were no effects in females dosed with 4 mg/kg genistein. Testis weights (analyzed by litter, with adjustment for body weight) were significantly reduced by DES but were unaffected by either dose of genistein.

3.3.5.2 Daidzein

Lamartiniere *et al.*, 2002

Guidelines/Guidances:	/
Test item:	daidzein
Purity:	98.5% (HPLC)
Impurity:	1.5 % Methanol
Vehicle:	feed (phytoestrogen-free AIN-76A diet)
Route of exposure:	oral (feed)
Duration of exposure:	2 weeks before breeding for the dams until sacrifice of the offspring when tumor diameters reached 2.5 cm, and maximum 230 days of age.
Doses:	0, 250 and 1000 mg daidzein/kg feed (corresponding to 19 and 66 mg/kg bw/d in females)
Species:	Rat
Strain:	Sprague-Dawley CD
Sex:	male and female
Group size:	10/dose
GLP:	no information
Study period:	Publication dated 2002

Female rats were fed a diet supplemented with 0, 250, or 1000 mg daidzein/kg, starting 2 weeks prior to mating. For male animals, treatment started with the 2-week mating phase. Offspring was sexed at birth and litters reduced so that each dam had 10 offspring (4–6 females/dam). At day 21 postpartum, offspring was weaned and fed the same diets for the remainder of the experiments.
For histomorphological analysis of the ovaries, uterus, and vagina, the entire reproductive tract was dissected out and preserved in 4% paraformaldehyde.

Results

Virgin female rats fed 250 mg and 1000 mg daidzein/kg diets from 2 weeks prior to breeding had a slightly, but not a significantly, reduced number of litters compared to those fed diets without daidzein.

The numbers of male and female offspring were not significantly different in litters exposed prenatally to daidzein in the diet as compared with those receiving no daidzein in the diet.

There were no treatment-related effects on anogenital distances for male and female offspring.
Body weights of female offspring exposed to the high daidzein dose were significantly reduced at all ages investigated. The lower dose resulted in a slight but not significant decrease in body weights.
At necropsy, there were slight but not significant alterations in ovarian and uterine weights and abdominal mammary gland size. Measurements of sex steroid concentration in the blood of adult females revealed that the high daidzein dose resulted in significantly reduced progesterone concentration. However, both doses resulted in slight but not significant decrease in estrogen levels.

Histomorphological analysis of the reproductive tracts of female offspring 50 days of age exposed perinatally to daidzein did not reveal any pathology in the vaginal, uterine and ovarian tissues. Perinatal exposure to 1000 mg daidzein/kg diet neither significantly altered the number or percentage of terminal ductal structures in 50-day old animals, nor caused any pathological lesions, including intraductal proliferations and hyperplastic alveolar nodules in the mammary glands.

Neither daidzein doses had any significant effect on fertility, numbers of male and female offspring, or anogenital distances.

The high, but not the low, Daidzein dose resulted in reduced body weight (154 ± 4 g and 170 ± 6 g, respectively, compared to controls at 189 ± 6, *i.e.* -18.5 %), a fact that may be explained by reduced feed consumption (food consumption reduced by 4.44% and 16.5% at the low and high dose). Circulating progesterone, but not estrogen, levels were statistically significantly reduced with the high, but not low, Daidzein-containing diet.

Compared to controls, both Daidzein doses resulted in slight, but not significant, decreases in ovarian (46 ± 3 g and 43 ± 2 g for the low and high dose respectively compared to control 47 ± 4 g, i.e. -2.1 and -8.5 %) and uterine weights (130 ± 12g and 134 ± 9 g for the low and high dose respectively compared to control 154 ± 17 g, i.e. -15.6 and 12.98 % at the low and high dose, respectively), and mammary gland size (496 ± 43 mm² and 448 ± 23 mm² for the low and high dose respectively compared to control 490 ± 40 g, i.e. +1.2% and - 8.6%) .

Perinatal exposure of female offspring

Perinatal exposure of female offspring to 250 mg daidzein/kg diet did not alter mammary gland development. High dietary daidzein concentrations resulted in reduced feed intake. As a consequence, body weights of female offspring exposed to the high daidzein dose were significantly reduced at all ages investigated. Ovarian and uterine weights and mammary gland size were not significantly altered in 50-day-old female offspring.

Conclusion (Mulon Conseil Submission)

In the developmental toxicology and bioavailability studies, the dietary daidzein exposure was initiated 2 weeks prior to the beginning of mating. The diet was supplemented with 0, 250, or 1000 mg daidzein/kg diet, (10 females/group).
The 1000-mg daidzein/kg diet resulted in significantly reduced body weights, a fact that can be explained on the basis of reduced feed consumption. Circulating progesterone, but not estrogen, levels were statistically reduced with the high daidzein-containing diet only.

Otherwise, none of the daidzein doses had a significant effect on the weights and histomorphology of the female reproductive tract.

SCCS comment
This study is described in the open literature and the original study report is not available to the SCCS. Based on the effects described, the SCCS considers the highest dose of 66 mg/kg bw/d as the LOAEL and 19 mg/kg bw/d as the NOAEL.

Talsness *et al.*, 2015

Guidelines/Guidances:	NTP Modified One-Generation study design
GLP:	FDA GLP
Test item:	daidzein (DZ)
Purity:	/
Vehicle:	2% Corn starch
Positive control:	17α-Ethinyl estradiol (EE)
Purity	/
Vehicle:	Peanut Oil
Route of exposure:	Oral - gavage
Duration of exposure:	GD 6 –GD 21
Doses:	DZ: 0, 5 and 60 mg/kg bw/d; EE: 0.0002 mg/kg bw/d
Experimental animals:	
Species:	Rats
Strain:	Sprague Dawley
Sex:	Gravid female
Animal numbers:	6 groups of 8-10 females (Each treatment group was investigated with its own parallel control group.
Study period:	

Six groups of gravid rats were administered per gavage either 2% cornstarch (10 ml/kg bW) as vehicle, 5 or 60 mg DZ/kg BW/d, or 0.002 mg EE/kg BW/d as a reference control on gestation days 6–21. The respective doses of DZ administered are approximately 5- and 50-fold higher than that recommended by commercial retailers of isoflavone supplements. Based on the uterotrophic assay, EE is estimated to be 40,000-fold more potent than DZ (Bolt *et al.*, 2001).

The following ovarian-related endpoints were investigated during adulthood in female offspring:
- Vaginal Cytology and Evaluation of the Reproductive Cycle
- Ovarian Surface Epithelium Height, Ovarian Histology and Follicle Counting

Results

Ovarian-related endpoints were investigated during adulthood in female offspring. The mean cell height of the ovarian surface epithelium (OSE) was significantly reduced in all treated groups. The reduction in cell height was more pronounced in the 60mg DZ/kg bw/d group than in the 5mg DZ/kg bw/d and 0.002mg EE/kg bw/d groups.

Figure 3:

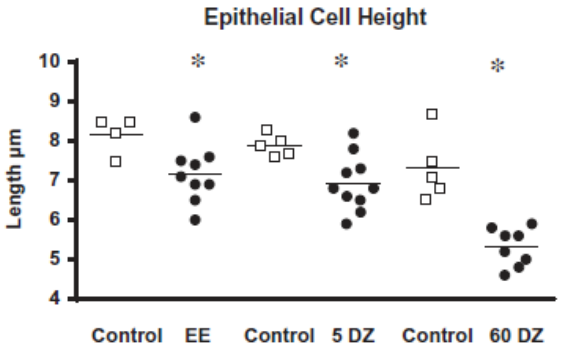


FIGURE 1. Ovarian surface epithelial cell height during estrus of adult female offspring exposed to ethinyl estradiol or daidzein on gestation days 6–21. ●, □, Arithmetic mean epithelial cell height obtained from 60 measurements per ovary; bar shows arithmetic mean for each group. Significance: $*p < .05$, unpaired *t*-test; difference among controls is not significant. EE, 0.002mg ethinyl estradiol/kg BW/d. 5 DZ, 5 mg daidzein/kg BW/d. 60 DZ, 60 mg daidzein/kg BW/d.

Alterations in folliculogenesis included increased follicular atresia, a reduction in secondary and tertiary follicle numbers, and cyst formation were observed among the 60-mg DZ/kg BW/d group.

Figure 4:

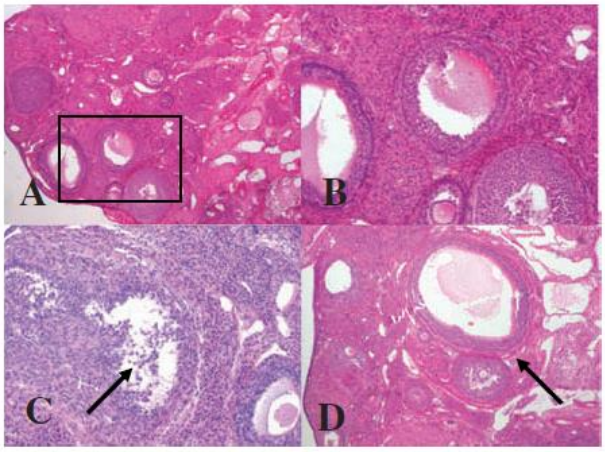


FIGURE 2. Histological sections of ovaries from adult (approximately 16 wk) female offspring in estrus. A, Histological section of an ovary from the control group depicting normal follicular development (magnification 4×). B, Enlargement of section A (magnification 10×). C, Histological section of an ovary from the group exposed to 60 mg DZ/kg BW/d during gestation day 6–21. The arrow is pointing to desquamation of follicular granulosa cells (magnification 10×). D, Histological section of an ovary from the group exposed to 0.002 mg EE/kg BW/d during gestation day 6–21. The arrow is pointing to a cystic follicle (magnification 4×).

There were no significant changes observed in the number of primordial follicles in any of the treatment groups compared to control. In the 5-mg DZ/kg bw/d group, secondary follicles were significantly reduced. In the 60-mg DZ/kg bw/d group, a significant decrease in secondary follicles was also observed, as well as decreased numbers of tertiary follicles and significant increased number of atretic follicles.

A significant rise in the proportion of cycles with an estrus greater than 2d long was observed in both the 60-mg DZ/kg bw/d and 0.002-mg EE/kg bw/d groups ($p<0.05$). There were no marked changes in the length of the estrous cycle.

The morphological changes to the ovarian surface epithelium (OSE) are consistent with an antiproliferative effect, while ovarian folliculogenesis was adversely affected. The OSE consists of a single layer of flat to columnar epithelial cells and proliferates to restore the ovarian surface after ovulation. The OSE and underlying stroma of mice express both ERα and ERβ. The effects of the high-dose DZ (60 mg/kg bw/d) were similar to those observed with 17-α EE at 0.002 mg/kg bw/d.

The histological features of the ovaries reported in this study are consistent with an increased rate of follicular atresia. An increased rate of follicular atresia may have consequences on the length of the reproductive life span, as the number of oocytes is fixed at birth.

The greater prevalence of animals with a longer vaginal estrus suggests that the neuroendocrine regulation of the cycle or the ovarian response to this control is affected. This is also suggested by the cystic ovarian follicles, which might result from a failure of

ovulation and lack of regression of the follicle. Extended estrus and presence of cystic ovarian follicles are both consistent with a delay in ovulation.
The authors also stressed that interspecies differences need to be considered when making conclusions about risks for human health.

SCCS comment
This study is described from the open literature and the original study report is not available to the SCCS. It is not a guideline study and not all parameters usually investigated in guideline studies were addressed. Based on the effects described (reduction in cell height of the ovarian surface epithelium, increased rate of follicular atresia and greater prevalence of animals with a longer vaginal estrus), the SCCS considers 60 mg/kg bw/d as a LO(A)EL and 5 mg/kg bw/d as a NO(A)EL. With this statement SCCS concurs with the Nordic Council report of Ministers (2020) and with the NTP-CERHR (2010) as both consider thtn even if the study by Talsness et al. (2015) may be potentially relevant, due to limitations, it is not sufficiently robust to conclude that daidzein has reproductive effects in laboratory animals.

In addition to these studies, the submission provided information from a reproductive toxicity study available in the public literature (Matulka *et al.*, 2009). In that study, SE5-OH has been administered, which is an equol-rich soy product prepared by bacterial fermentation and contains approximately 0.65% equol, 0.024% daidzein, 0.022% genistein and 0.3 % Glycitein. In that study, the NOAEL for SE5-OH determined for both male and female rats was 1000 mg/kg bw/day (6.5 mg Equol/kg/day), based on a reduction of body weight, implantations, and live births in the F1 and F2 animals at the 1000 mg/kg bw/day dose level. For the developmental part of that study, the study authors concluded that the NOAEL for developmental effects of SE5-OH is 2000 mg/kg bw/day, based on the lack of significant embryo-to-fetal stage effect.

SCCS comment
In the study from Matulka *et al.* (2009), the rats were not exposed to daidzein only but to a new Equol-rich soy product (SE5-OH) which contains approximately 0.65% Equol, 0.024% daidzein, 0.022% genistein, and 0.30% Glycitein. This study cannot be used for MoS calculation because the SCCS considers that only the studies performed with pure daidzein are relevant for safety assessment as a cosmetic ingredient.

SCCS overall comments on reproductive toxicity

Genistein

The evidence from a multigenerational study in rats indicates that genistein produces developmental toxicity in terms of a transient decrease in the F1 and F3 pup body weights following dietary exposure to 5, 100, and 500 ppm via oral administration.

From the relevant toxicological studies, the SCCS regarded it reasonable to consider that a NOAEL for genistein aglycone is between 5 and 100 ppm. A recent report by the Nordic Council of Ministers (2020) has used 100 ppm as a NOAEL from the NCTR study for pregnant women (equivalent to 8.9 mg/kg bw). However, the same report used 100 ppm as a LOAEL for children (equivalent to 20 mg/kg bw) from a study by Li *et al.* (2014).

In view of these studies, the SCCS considered it pragmatic to use 100 ppm as a LOAEL for the calculation of Margin of Safety (MoS) in the current assessment.

Daidzein

Only studies published in the literature were used by SCCS, but the original study reports were not available. Based on the observed effects in the most comprehensive study of Lamartinière *et al.* (2002), the SCCS considers 19 mg/kg bw/d as a NOAEL for the effects on reproduction and fertility. Other studies have investigated more specific effects of daidzein that could have impact on the fertility or development. The study from Talsness *et al.* (2015) focused on the changes in the ovaries of female rats and based on the effects reported by the authors, the SCCS considers 60 mg/kg bw/d as a LO(A)EL and 5 mg/kg bw/d as a NO(A)EL. However, the biological consequences of these effects in terms of adversity are not known. As daidzein has also been associated with an estrogenic mode of action, the endocrine potential that may lead to adverse effects needs to be investigated before conclusions can be drawn about the reprotoxicity of this compound (see section 3.3.10)

3.3.6 Mutagenicity / genotoxicity

As part of the safety assessment, the SCCS carefully examined all the *in vitro* and *in vivo* studies from the published literature until November 2021 on mutagenicity/genotoxicity that were deemed relevant to mutagenicity/genotoxicity of daidzein and genistein (aglycone form). A summary of the studies considered by the SCCS in this Opinion is presented in a tabulated form in Annex-A and B. For this, the SCCS assessed the reliability and relevance of each study using the criteria proposed by Klimisch *et al.* (1997). The relevance of each study was assigned high, limited or low ranking, based on relevance of the test system (genetic endpoint) used, whereas reliability of the study was scored as follows:

1. reliable without restriction
2. reliable with restrictions
3. insufficient reliability
4. reliability cannot be evaluated
5. reliability not evaluated since the study is not relevant and/or not required for the risk assessment.

The reliability and relevance of the studies are described in the revelant Tables in Annex A and B of this opinion

3.3.6.1 Genistein

3.3.6.1.1 Genistein: Bacterial gene mutation studies (Table A1 – Annex A):

Genistein was tested in several Ames test studies of high, limited and low relevance. A high-quality GLP study of genistein on *S. typhimurium* with TA1535, TA1537, TA98 TA100 and Escherichia coli WP2 uvrA was negative. Another study on *S. typhimurium* TA1538, TA98 and TA100 strains showed negative results but was considered of limited relevance. None of the studies were conducted according to GLP.

3.3.6.1.2. Genistein: *In vitro* mammalian cell chromosomal aberrations (CA)/ micronucleus (MN) tests (Table A2 – Annex A):

Genistein was tested for chromosomal aberrations/micronucleus in several studies of high or limited relevance. Results of six of the studies were positive, whereas one study of high relevance was negative. Two other studies were negative but were of low relevance and were not considered in this evaluation. None of the studies were carried out according to GLP.

3.3.6.1.3. Genistein: *In vitro* mammalian cell gene mutation studies (Table A3 – Annex A):

Genistein was tested in mammalian gene mutation tests, in Tk locus, Hprt, as well as the Na^+/K^+ ATPase locus in various cell types, such as L5178Y mouse lymphoma cells, V79 cells, primary SHE cells or in the human lymphoblastoid cells AHH-1. All studies (high or limited relevance) were positive. Two high-quality studies performed with and without S9 mix showed positive effect both with and without metabolic activation.

3.3.6.1.4 Genistein: *In vitro* DNA damage studies (Table A4 – Annex A):

Several studies investigated DNA damage after exposure to genistein by Comet assay**.** Various primary cells (primary human lymphocytes and leucocytes, Human papillary thyroid cancer (PTC), sperm cells, colon cells), and different cell lines were used - such as V79, HT 29, CHO cells. Five studies of high or limited relevance were positive while four were negative. Studies of low quality and relevance were not considered in this evaluation. None of the studies were carried out according to GLP. Therefore, the results can only be considered as part of an overall weight of evidence.

3.3.6.1.5 Genistein: Other *in vitro* studies (Table A5 – Annex A):

Two cell transformation assays of high relevance have been performed on Syrian Hamster Embryo (SHE) cells (Tsutsui et al., 2003; Harvey et al., 2005). Study of Tsutsui et al. (2003) shows that genistein is capable of inducing morphological transformation of SHE cells. In the same study, genistein was also tested for the formation of DNA adducts by ^{32}P-postlabeling with positive results. However, the pH 6.7 version of the SHE assay as described by Harvey et al., (2005) was negative.

3.3.6.1.6. Genistein: *In vivo* chromosome aberration/ micronucleus (Table A6 – Annex A):

In vivo micronucleus tests of high relevance in mice and rats after oral administration of genistein were negative (McClain et al., 2006). Two *in vivo* studies, one of limited and another of low relevance on mice were also negative (Reward et al., 1995; Matsuda et al, 2012).

3.3.6.1.7. Genistein: *In vivo* DNA damage/Comet assay (Table A7 – Annex A)

An *in vivo* Comet assay after oral administration of genistein alone was negative, but co-administration of genistein and $NaNO_2$ significantly increased DNA damage with and without Fpg.

3.3.6.1.8. Genistein: *In vivo* gene mutation test (Table A8 – Annex A)

Several negative *in vivo* mammalian gene mutation studies are available on Big Blue transgenic rats in the liver cII gene (Chen *et al.*, 2005), lacI gene in uterine cells in female (Aidoo *et al.*, 2005), lacI gene in mammary gland cells in female (Manjanatha *et al.*, 2006), in Hprt gene in lymphocytes isolated from the spleen of fema-le (Manjanatha *et al.,* 2006) and in lacI gene of heart tissue (Manjanatha *et al.,* 2005).

3.3.6.1.9 Genistein: Other *in vivo* studies (Table A8 – Annex A)

A $P53^{-/-}$ Mouse Tumorigenesis Assay on male and female mice with genistein in the diet was negative. Histopathology on various organs and tissues showed no difference from

control (Mishra *et al.*, 2002). Genistein was tested positive in a sister chromatid exchange assay after intraperitoneal administration. The study has low relevance (Giri and Lu, 1995).

High dose of genistein administrated subcutaneously was able to affect topoisomerase II *in vivo* in juvenile male Wistar rats (Baechlar et al, 2016). Genistein significantly increased the amount of covalent topoisomerase Ila and β-DNA complexes in the gut. However, no effect on the topoisomerase IIa and β-DNA complex was found after dietary isoflavone exposure. Genistein clearly possesses topoisomerase poisoning properties *in vivo*, whereas an isoflavone-rich diet containing genistein only caused a slight effect whose relevance has to be clarified in further studies.

3.3.6.2. Daidzein

Conclusion from the Mulon Conseil submission on genotoxicity

Daidzein was not mutagenic in the bacterial reverse mutation assay and no relevant genotoxic effects were generally observed in mammalian cells *in vitro*. In humans, purified soy unconjugated isoflavone mixture (including 74 mg of Daidzein) was not genotoxic.
Moreover, S-Equol, the active metabolite of Daidzein via ingestion, did not show significant genotoxic activity in animals.

The two oxidative daidzein metabolites (3',4',7- and 4',6,7-trihydroxyisoflavone) formed by oral route showed genotoxicity expressed *in vitro* in mammalian cells. The clastogenic effect of these two catecholic metabolites may be attributed to oxidation to o-quinones, which are known to be clastogens and could represent a potential hazard *in vivo*. However, as soy isoflavones and their metabolites are rapidly conjugated with glucuronic acid and sulfate *in vivo* by the oral route, it is unlikely that the high concentrations of the free metabolites required for adverse effects are reached in humans even after ingestion of high levels of isoflavones. Furthermore, the negative outcome observed for daidzein *in vivo* is reassuring about the genotoxicity of these catecholic metabolites.

3.3.6.2.1 Daidzein: Bacterial gene mutation studies (Table B1 – Annex B)

Daidzein was tested in a few Ames test studies on *S. typhimurium* TA98, TA100 and TA1538 strains with inconclusive or negative results. The studies were considered of limited or low relevance. None of the studies were conducted according to GLP.

3.3.6.2.2 Daidzein: *in vitro* mammalian cell chromosomal aberrations/ micronucleus studies (Table B2 – Annex B)

Daidzein was tested in several *in vitro* MN/CA tests. In one study of limited relevance Daidzein was tested on Chinese hamster V79 fibroblasts with a weakly positive result. In one study of limited relevance on chromosomal aberration on SHE cell cultures, the results were negative. In one study on L5178Y $tk^{+/-}$ mouse lymphoma cells, which was considered of limited relevance, Daidzein was tested with an inconclusive result.
Two studies on V79 fibroblasts and on HTC rat hepatoma cells were considered of low relevance with inconclusive results.
None of the studies were fully compatible with current OECD TG nor were they conducted according to GLP.

3.3.6.2.3 Daidzein: *in vitro* mammalian cell gene mutation studies (Table B3 – Annex B)

Daidzein was tested in mammalian cell gene mutation tests in Hprt locus on V79 cells with inconclusive result of low relevance and in Na^+/K^+ ATPase locus on V79 and primary SHE cells with a positive result of limited relevance.

3.3.6.2.4 Daidzein: *in vitro* DNA damage studies (Table B4 – Annex B)

Daidzein was tested in the Comet assay or DNA elution assay on different cell types. In one study of high relevance (HT29 cells, -/+Fpg), Daidzein was tested with a negative result. In three studies on leucocytes and sperm cells, positive results were obtained, however the studies were considered of limited relevance. Daidzein was tested with an equivocal result in one study of low relevance on MIA PaCa-2 and HT-29 cells. One study of low relevance on alkaline elution of DNA in V79 cells Daidzein gave a negative result.
None of the studies were conducted according to GLP status.

3.3.6.2.5 Daidzein: other *in vitro* studies (Table B5 – Annex B)

Daidzein was tested in Cell Transformation Assay (CTA) in one study of limited relevance with a positive result (SHE cells). It was also tested for formation of DNA adducts by ^{32}P-postlabeling assay in one study of high relevance with a positive result (SHE cells).
None of the studies were conducted according to GLP status.

3.3.6.2.6 Daidzein: *in vivo* chromosome aberrations (CA)/micronucleus (MN) studies

No relevant studies on the *in vivo* chromosome aberrations CA/MN tests with Daidzein are available in the open literature.

3.3.6.2.7 Daidzein: *in vivo* mammalian cell gene mutation studies (Table B6 – Annex B)

Daidzein was tested for gene mutations in Big Blue transgenic rats (lacI mutagenesis assay) after administration in the diet in two studies of high relevance with negative results (the same animals were exposed but study results were reported in two different publications: one study on uterine cells, and the other on mammary gland cells). Daidzein was also tested in one study of high relevance with a negative result in an Hprt gene mutation assay in lymphocytes in Big Blue transgenic rats after administration in the diet.
None of the studies were conducted according to GLP status.

3.3.6.2.8 Daidzein: *in vivo* Comet and SCE studies (Table B7 – Annex B)

Daidzein was tested in a low relevance study on Comet assay in mice after oral administration with an inconclusive result.

It was also tested in a limited relevance study on Sister Chromatid Exchange assay in mice after intraperitoneal administration with a positive result (Table B8 – Annex B).

Overall SCCS comment on Genotoxicity/Mutagenicity

Genistein

1. Based on the available studies, genistein shows no evidence for mutagenicity in the **bacterial gene mutation test** (Ames tests) (Bartholomew and Ryan, 1980, McClain *et al.,* 2006). In contrast, ***in vitro* mammalian gene mutation** studies using Tk locus, Na+/K+ ATPase or Hprt loci on Mouse lymphoma L5178Y tk+/- cells, human lymphoblastoid cells AHH-1 and L3 or SHE cells show positive results indicating mutagenic potential of genistein *in vitro (*Tsutsui *et al.*, 2003, McClain *et al.* 2006, Morris *et al.* 1998, Zou *et al.* 2012). In consideration of several other ***in vivo* mammalian gene mutation** studies on Big Blue transgenic rats (Chen *et al.*, 2005, Aidoo *et al.*, 2005, Manjanatha *et al.*, 2005, 2006), that were negative, the SCCS considers that genistein does not pose a gene mutation hazard *in vivo*.
2. The available studies of ***in vitro MN/CA*** with Genistein show clastogenic effect (Morris *et al.*, 1998; Di Virgilio *et al.*, 2004. Kulling and Metzler, 1997, Kulling *et al.*, 1999). These data are supported by the induction of DNA breaks by genistein as measured in the **Comet assay *in vitro*** (Salti *et al.*, 2000; Di Virgilio *et al.*, 2004). The results of studies on ***in vivo MN/CA*** with genistein, however, show no clastogenicity (McClain *et al.*, 2006).
3. The available results of a low relevance study on **sister chromatid exchange *in vivo*** with genistein show positive results. Results from **cell transformation assay** were positive in one study and negative in another study. High doses of genistein have shown topoisomerase poisoning properties *in vivo* after subcutaneous administration. These data can, however, be only considered in an overall WoE, which suggests no concern for mutagenicity/genotoxicity of genistein.

Table b. Summary of conclusions on genotoxicity studies with Genistein

Genotoxicity endpoint	Gene mutations		Chromosomal aberrations (aneugenicity, clastogenicity)
	in bacteria	in mammalian cells	Micronucleus test/Chromosomal aberration test
In vitro studies	Negative	Positive	Inconclusive
In vivo studies	Negative		Negative
Overall conclusion on genotoxic hazard	No genotoxicity hazard *in vivo*		No genotoxicity hazard *in vivo*

Daidzein

1. Although the available results of the *in vitro* bacterial gene mutation studies (Sugimura *et al.*, 1977; Nagano *et al.*, 1981; Bartholomew *et al.*, 1980) as well as of the *in vitro* mammalian cell gene mutation studies on V79 cells (Kulling and Metzler, 1997) and SHE cells (Tsutsui *et al.*, 2003) do not allow drawing firm conclusions, the SCCS, after analysis of the high relevance *in vivo* mammalian cell gene mutation studies with negative results

(Aidoo *et al.*, 2005; Manjanatha *et al.*, 2006), considers that Daidzein does not pose a gene mutation hazard *in vivo*.

2. The analysis of available studies on Daidzein in the *in vitro MN/CA* tests with negative result on SHE cells (Tsutsui *et al.*, 2003), inconclusive results on V97 cells (Kulling *et al.*, 1997), L5178Y tk+/− mouse lymphoma cells (Schmitt *et al.*, 2003) or HTC rat hepatoma cells (Lepri *et al.*, 2013), as well as positive result on V79 cells (Di Virgilio *et al.*, 2004), do not allow drawing firm conclusions. No studies on the *in vivo MN/CA* tests with well characterised test item (daidzein) have been found in the open literature.

3. The available, mostly positive, results of the *in vitro* Comet assays on V79 cells (Kulling and Metzler, 1997), isolated human sperm cells or peripheral blood leucocytes (Anderson *et al.*, 1997a; 1997b; Cemeli *et al.*, 2004), HT29 cells (Baechler *et al.* 2014; Gundogdu *et al.*, 2018), MIA PaCa-2 cells (Gundogdu *et al.*, 2018), as well as of the cell transformation assay on SHE cells (Tsutsui *et al.*, 2003) and formation of DNA adducts (Tsutsui *et al.*, 2003), indicate that a DNA damaging effect of Daidzein cannot be excluded. The available results on the *in vivo* Comet assay on mice stomach mucosa cells (Toyoizumi *et al.*, 2010) were considered inconclusive and on the SCE test in mice (Giri and Lu, 1995) were considered positive.

However, as the results of the other DNA damage studies were mostly of limited or low relevance, they have been considered as only supportive in the overall WoE.

Additional micronucleus test on human lymphocytes (the OECD TG 487, 2016) with daidzein (July 2022) was provided by the Applicant in response to the preliminary Opinion.

Test item	Daidzein CAS No. 486-66-8, Amb17933362
Batch	TFS20211018
Cell strain	Human peripheral blood lymphocytes
Culture medium	RPMI 1640
Cytochalasin B	6 µg/mL
Solvent used	DMSO
Stability in solvent	unknown (dilutions were prepared extemporaneously)
Purity	≥ 98%
Correction factor	1.02 (*i.e.* worst case)
Expression of the concentrations	µg/mL of pure Daidzein
Treatment durations	Without S9-mix 4 h + 24 h recovery period (short treatment) **125 – 62.5 – 31.25** – 15.63 µg/mL 24 h without recovery period (continuous treatment) 125 – **62.5 – 31.25 – 15.63** – 7.81 µg/mL With S9-mix 4 h + 24 h recovery period, with 5% S9-mix **125** – **62.5 – 31.25 –** 15.63 µg/mL
Metabolic activation	Aroclor1254-induced rat livers.
Positive controls without S9-mix	mitomycin C 0.15 µg/mL for 4h treatment, for 24h treatment mitomicyn C 0.075 µg/mL griseofulvin 10 µg/mL
with S9-mix	cyclophosphamide 10 µg/mL
GLP compliance	Yes, control of concentration in treatment preparation No
Study date	22.07.2022

Daidzein (batch **TFS20211018**) has been investigated by the *in vitro* mammalian cell micronucleus test on cultured human lymphocytes in the presence and absence of metabolic activation with the maximum concentration, compatible with the solubility of the test item in the test system, or its cytotoxic activity. The study was in compliance with OECD Guideline 487 (2016).

The cytotoxicity assay was carried out using cells taken from one 41 years old female donor. The test item was dissolved in DMSO (Merck, Batch K52795678 110) at a maximum initial concentration of 100 mg/mL which was diluted to obtain a 12.5 mg/mL solution. A final concentration of 125 µg/mL was used for the incubations at 1% DMSO in culture medium.

In the preliminary cytotoxicity assay using a 4-hour treatment without metabolic activation followed by a recovery period, very slight cytotoxicity was observed at the highest concentration (125 µg/mL), with a percentage of cytostasis of 5.2% corresponding to a replication index of 94.8%. This concentration was hence retained as the top concentration to be tested in the main genotoxicity assay.
In the preliminary cytotoxicity assay using a 4-hour treatment with metabolic activation followed by a recovery period, a moderate cytotoxicity was observed at the highest concentration tested (125 µg/mL), with a percentage of cytostasis of 37.2% corresponding to a replication index of 62.8%. This concentration was hence retained as the top concentration to be tested in the main genotoxicity assays.
In the preliminary cytotoxicity assay using a 24-hour continuous treatment without metabolic activation and without recovery period, a moderate to strong cytotoxicity was observed at the 2 highest concentrations tested (125 and 62.5 µg/mL), with percentages of cytostasis of 34 and 48.1% corresponding to replication indices of 66 and 51.9%. The concentration of 125 µg/mL was thus retained as the top concentration to be tested in the main genotoxicity assay.

The genotoxicity test was carried out using pooled cells taken from two 22 years old female donors. Lymphocyte cell division was stimulated in culture by treatment with phytohaemagglutinin (PHA) and the cytokinesis-block method was applied; 2000 binucleated cells/concentration were counted.
In the short-term treatment without metabolic activation followed by a 24-hour recovery period (assay S9- 4h/+ 24h), the test item daidzein induced neither statistically nor biologically significant increases in the number of micronucleated cells at all the concentrations analyzed ranging from 125 to 31.25 µg/mL; 10 or 14 micronucleated binucleated cells were observed per 2000 cells, *vs.* 10 in the solvent control. In the short treatment with metabolic activation followed by a 24-hour recovery period (assay S9+4h/24h), the test item daidzein induced neither statistically nor biologically significant increases in the number of micronucleated cells at all the concentrations analyzed ranging from 125 to 31.25 µg/mL; In particular, 6 to 9 micronucleated binucleated cells were observed per 2000 cells, *vs.* 8 in the solvent control. In the continuous treatment without metabolic activation without recovery period (assay S9- 24h/+0h), the test item daidzein induced neither statistically nor biologically significant increases in the numbers of micronucleated cells at all concentrations analyzed ranging from 62.5 to 15.63 µg/mL; 8 to 18 micronucleated binucleated cells were observed per 2000 cells, *vs.* 10 in the solvent control. The ANOVA test was significant, however, in the absence of clear dose-effect relationship, it has no meaning in terms of genotoxicity.

All positive controls showed significant increases in micronucleus frequency.

The study authors concluded that under the experimental conditions used, no genotoxic activity of daidzein was revealed in presence or in absence of metabolic activation, or with a 24 h treatment in absence of metabolic activation.

Study report Number FSR-IPL 220304,
INSTITUT PASTEUR DE LILLE, Genetic Toxicology Laboratory, 2022

SCCS comment
SCCS agrees that study results show no genotoxic potential of daidzein *in vitro*. Daidzein does not induce increases in the number of micronucleated cells under the experimental conditions used. The SCCS noted increases in MN frequency in the cells after continuous exposure for 24 h, however the increases were not concentration dependent (they were observed at the highest and the lowest concentrations tested), were not significant (less then 2-fold comparing to the control cultures values) and were within the range of historical vehicle control values. Therefore, the slight increases were considered by the SCCS not to be biologically meaningful.

The SCCS overall conclusion on genotoxic hazard of daidzein
Considering all the available data on daidzein genotoxicity the SCCS is of opinion that daidzein does not pose a genotoxicity hazard *in vivo.*

Table c. Summary of conclusions on genotoxicity studies with Daidzein

<table>
<tr><td rowspan="2">Genotoxicity endpoint</td><td colspan="2">Gene mutations</td><td colspan="2">Chromosomal aberrations (aneugenicity, clastogenicity)</td></tr>
<tr><td>in bacteria</td><td>in mammalian cells</td><td>Micronucleus test</td><td>Chromosomal aberration test</td></tr>
<tr><td>In vitro studies</td><td>Positive</td><td>Inconclusive</td><td>Inconclusive</td><td>Inconclusive</td></tr>
<tr><td></td><td></td><td></td><td colspan="2">New valid in vitro micronucleus study carried out with properly characterised test item</td></tr>
<tr><td></td><td></td><td></td><td colspan="2">Negative</td></tr>
<tr><td>In vivo studies</td><td>/</td><td>Negative</td><td>/</td><td>/</td></tr>
<tr><td>Overall conclusion on genotoxic hazard</td><td colspan="2">No genotoxicity potential in vivo</td><td colspan="2">No genotoxicity potential in vitro</td></tr>
</table>

3.3.7 Carcinogenicity

3.3.7.1 Genistein

Genistein has been reported to be positive (Tsutsui et al., 2003) in an *in vitro* Syrian hamster embryo cell transformation (CTA) assay but negative in the CTA assay, tested at 2-4 µg/ml (pH 6.7, maximum concentration was limited by cytotoxicity) (Harvey *et al.*, 2005).

- A 2-year feed study on toxicology and carcinogenesis (December 2007, NTP TR 545) also provides information on the carcinogenic potential of genistein. In one study, 50 male and female Sprague-Dawley rats were exposed to 5, 100, or 500 ppm genistein from conception through weaning via mothers fed on genistein feed. In the second study, groups of 50 male and female rats were given the same feed with 5, 100, or 500 ppm genistein for 20 weeks after birth, followed by untreated feed for the remainder of the two years. In the third study, groups of 50 male and female rats were exposed from conception through weaning, and then given untreated feed for the duration of the study. Control animals received the same feed without genistein. At the end of the study, tissues from >40 sites were examined for every animal. The results indicated that, under the condition of the 2-year feeding study:

    - there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm through continuous exposure to the test compound from conception through termination (F1C). There was some evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on increased incidences of mammary gland adenoma or adenocarcinoma (combined) and pituitary gland neoplasms. The incidence of benign mammary gland fibroadenoma in female rats was significantly decreased in the 500 ppm group (28.9 to 44.3 mg/kg bw/day of genistein).
    - there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm of the test substance from conception through 20 weeks followed by control feed until termination (F1T140). There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on marginally increased incidences of pituitary gland neoplasms.
    - there was no evidence of carcinogenic activity of genistein in male Sprague-Dawley rats exposed to 5, 100, or 500 ppm where offspring of three prior generations of animals exposed to the test compound were exposed from conception through weaning (PND 21), followed by control feed until termination (F3T21). There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on increased incidences of mammary gland adenoma or adenocarcinoma (combined).
    - exposure to genistein was also shown to accelerate the onset of aberrant estrous cycles in female Sprague-Dawley rats whether exposures were continuous or truncated at PND 140 or at weaning in the 500 ppm group. The effects of genistein on estrous cycling and the incidences of hormonally related spontaneous neoplasms of female Sprague-Dawley rats are consistent with an estrogenic mechanism of toxicity (see further analysis of endocrine effects in section 3.3.10).

In brief, none of the studies showed any increased rate of cancer in male rats. There was equivocal evidence of carcinogenic activity of genistein in female Sprague-Dawley rats based on marginal increase in the incidence of pituitary gland neoplasms.

- Barnes (1995) reported that purified genistein, when administered neonatally to rats, caused a delay in the mammary tumour appearance in association with an increased cell differentiation in mammary tissue in rats treated with 7,12-dimethylbenz[a]anthracene, inhibited phorbol ester-induced H_2O_2 production in a model of skin cancer, and inhibited aberrant crypt formation in a model of colonic cancer. In a DMBA model, subcutaneous injections of genistein during the neonatal period (approximately 18.5 mg/kg bw, on postnatal days 2, 4, and 6) or prepubertal period (500 mg genistein/kg bw on postnatal days 16, 18, and 20) significantly decreased the number of tumours developed per rat by nearly a half.

- Neither carcinogenicity nor genotoxicity was observed in mouse skin chronically treated with topical genistein (Huang *et al.*, 2008). The author reported anti-photo-carcinogenic effects of genistein in SKH-1 murine skin.
- A study by Misra *et al.* (2002) used p53 knockout mouse model in tumorigenesis assay in 20 male and 20 female 4- to 6-week old mice that received either distilled water (control), or AIN-76A 76A semi-purified diet (ad libitum), or genistein through an AIN-76A diet containing 0.04% genistein (50-60 mg/kg bw/day). Food intakes and body weights were recorded weekly, and mice were observed daily for any signs of clinical effects. A wide range of tissues from all animals sacrificed or found dead was examined. Both male and female p53 knockout mice in the control group developed a variety of spontaneous tumours and became moribund after around 100 days (75% of males, 80% of females developed lymphomas, 30% of which also developed osteosarcomas and soft tissue sarcomas). Dietary genistein group showed no effect on survival, weight gain, tumour incidence, tumour multiplicity, or tumour spectrum.
- In a 36-month double-blind randomised clinical trial on humans, Marini *et al.* (2009) gave 54 mg genistein/ day (purity of genistein approximately 98%) to 198 subjects and placebo to 191. The average age of volunteers in the genistein group was 53.8 ± 2.9 (placebo: 53.5 ± 2.0). The average time since menopause was 3.6 ± 3.0 years (genistein group); 3.6 ± 2.2 years (placebo group). The study found no statistically significant differences between the groups at any timepoint (baseline, 2 years, 3 years), either with digitised assessment or visual classification. Mammograms and uterine ultrasounds for endometrial thickness showed no reported cases of breast cancer or changes in uterine thickness in either of the groups at the end of the 24 months. In a third-year extension of the trial, the study participants (n=71) who consumed purified genistein, or placebo group (n=67), did not reveal any evidence of breast or uterine carcinogenicity.
- In a 12-month randomised double-blind placebo-controlled study, Morabito *et al.* (2002) gave 54 mg genistein/ day (purity of genistein approximately 98%). The HRT group was given 1 mg/day of 17β-oestradiol combined with 0.44 mg/day norethisterone acetate, Activelle®. Number of subjects was 30, HRT:30, placebo:30; average age 52±3; HRT: 52±5; placebo: 51±4. Time since menopause (years) was 7 ± 6; HRT:7 ± 3; placebo:6 ± 5. The study found no statistically significant changes in mammography exams at 1 year in any of the groups.

The data from these studies suggest that purified genistein does not exert adverse estrogenic effects on breast tissue when consumed at a dose of 54 mg/day (Taylor *et al.*, 2009).

- Epidemiological data indicate that Asians who consume a diet high in soy have a much lower risk of breast and prostate cancer than individuals living in the West (Misra *et al.*, 2002).
- The meta-analysis of 18 published epidemiological studies from Trock, 2006 (as cited in Taylor report - 2009) supports the hypothesis that soy isoflavones may reduce breast cancer risk. Across all investigations, high soy intake was associated with an odds ratio of 0.86 (95% confidence interval 0.75– 0.99%) for the development of breast cancer. Case-control data further support the hypothesis that high isoflavone, and in particular genistein intake, may be associated with reduced breast cancer risk. Another study by Verheus, 2007 (as cited in Taylor report - 2009) (n = 766), found that plasma genistein levels were inversely correlated with the subsequent incidence of breast cancer in both pre- and postmenopausal Dutch women (see Annex-A Table).

3.3.7.2 Daidzein

Human or animal studies on carcinogenicity of daidzein have not been performed. The effects of isoflavone intake from food supplements on effects of endocrine sensitive tissues (uterus, thyroid and breast) has been reviewed by EFSA (2015). In the more recent report from VKM (2017), a risk assessment of isoflavones from soy was not limited to these three sensitive tissues and considered more recent literature. According to EFSA (2015), in adults, it can be considered that exposure through moderate and regular soy consumption (< 1mg/day) could have some effects but is unlikely to induce adverse effects on the uterus, thyroid and breast endpoints.

With respect to Cancer Risk in pre- and post-menopausal women, (VKM) stated:

Cancer risk

Most studied have reported that isoflavones seem to reduce the risk of cancer. However, a few studies have also indicated the opposite tendency. In a Randomized Clinical Trial (RCT), a significantly higher rate of endometrial hyperplasia without atypia was reported in healthy Italian women after 150 mg/day of isoflavones in tablets (genistein:daidzein:glycitein %: 40-45:40-45:10-20) after 5 years (6 vs. 0 cases) (Unfer *et al.*, 2004). However, no cases of endometrial hyperplasia with atypia or endometrial carcinoma were observed. EFSA (2015) mentioned some methodological weaknesses of this study; a considerable number (up to 25%) of specimens of endometrium were neither obtained nor assessable at each time point and that these samples were not consistently obtained from the same participants at each time point, and that the effects observed were indicative of a possible estrogenic but not a carcinogenic effect.

A prospective study reported that genistein and daidzein calculated from a food frequency questionnaire (FFQ) were dose-dependently associated with increased risk of hepatocellular carcinoma in Japanese women, with multivariate hazard ratios for highest vs. lowest tertile of 3.19 (95% CI 1.13-9.00, Ptrend = 0.03) and 3.90 (95% CI 1.30-11.69, Ptrend = 0.01), respectively (Kurahashi *et al.*, 2009).

A retrospective case-control study found that soy food and isoflavone intake from food estimated from FFQ for the highest quartile of intake (Q4) vs. the lowest (Q1) was generally associated with decreased risk of colorectal cancer in Korea (Shin *et al.*, 2015), however, the middle (second and third) quartiles of intake of total soy products were associated with a non-significantly elevated colon cancer risk in women (Q2: OR: 1.27, 95% CI 0.86-1.88), Q3: OR: 1.37, 95% CI 0.92-2.04). The same non-significant tendencies of reduced risk associated with Q4 and increased risk associated with Q2 and Q3 vs. Q1 was seen with total isoflavones. However, a meta-analysis of 4 cohort and 7 case-control studies found reduced risk in women or no association in men between soy intake and colorectal cancer (Yan *et al.*, 2010).

Another retrospective case-control study found that post-menopausal Canadian women had a positive association between ER-PR- breast cancer and adult total isoflavone intake from foods (≥497 µg/day) (highest vs. lowest tertile: OR: 1.50, 95% CI 1.05-2.15, Ptrend = 0.04), indicating increased risk for this breast cancer subtype with total isoflavone intake (Anderson *et al.*, 2013). Also, in women not stratified by menopause status there was a positive association between ER-PR- breast cancer and the highest tertile of adult total isoflavone intake (≥497 µg/day) (OR: 1.38, 95% CI 1.05-1.81, Ptrend = 0.01). However,

EFSA (2015) concluded, based on a weight of evidence approach those adverse effects on mammary gland have not been seen in either humans or in animals.

VKM (2017) concluded

In the meta-analysis of 92 RCTs on post-menopausal women using phytoestrogen (isoflavones, lignans and coumestans) supplements for treatment of climacteric syndrome (Tempfer *et al.*, 2009), the rates of hormone-related side-effects such as endometrial hyperplasia, endometrial cancer and breast cancer were not significantly different between groups.

The relevance of the few studies that found increased risk of cancer from a very high dose of isoflavone supplements or in comparisons of dietary intake of soy food products in mostly Asian populations is difficult to interpret in relation to the intake of isoflavone supplements in Norwegian peri- and post-menopausal women.

With respect to cancer in men, VKM (2017) stated:
A large number of studies were also found on effects of soy food products or isoflavones on non-healthy men, i.e., patients with prostate cancer or with increased PCA at risk for prostate cancer, hepatocellular carcinoma or bladder cancer, men with adenomatous colorectal polyps, hypercholesterolic men and men with metabolic syndrome (see Table 9.3 in Appendix 9.3 of VKM, 2017). Also in these studies, effects on sex hormone levels were reported (considered beneficial in men with or at risk for prostate cancer). Two studies in Chinese men, of uncertain relevance for this risk assessment, reported an increase in relative risk of bladder cancer associated with estimated dietary soy food, soy protein or isoflavone intake (Sun *et al.*, 2002). Shin *et al.* (2015) reported a decreased risk of colorectal cancer associated with the highest quartile of dietary soy isoflavone intake (Q4) vs. the lowest quartile (Q1), but an increased risk was found associated with Q2 and Q3. A meta-analysis of colorectal cancer found no significant association, positive or negative, with soy consumption from food in men (Yan *et al.*, 2010).

SCCS overall comments on carcinogenicity
Most studied have reported that isoflavones seem to reduce cancer risk. However, a few studies have also indicated the opposite tendency.
Genistein
The available evidence from published studies suggests that genistein is not genotoxic *in vivo*, and does not exhibit a carcinogenic potential

Daidzein
Carcinogenicity data are not available for daidzein. However, there is huge amount of literature investigating possible associations between intake of soy isoflavones and various cancer types. These studies have been summarised by various organisations, the most recent ones being EFSA (2015), VKM (2017) and the report from the Nordic Council of ministers from 2020. The *in vivo* relevance of the few studies that found increased risk of cancer of a very high dose of isoflavone supplements, or in occasional comparisons of dietary intake of soy food products in mostly Asian populations, is difficult to interpret in relation to the intake of isoflavone supplements in Norwegian pre- and post-menopausal women as well as with respect to cancer in men (VKM, 2017).

3.3.8 Photo-induced toxicity

3.3.8.1 Phototoxicity / photo-irritation and photosensitisation

According to the submitter, the Photopatch test assesses the potential side effects (skin erythema and oedema reactions) that may occur after applying a cosmetic product and after exposure to UVA/visible irradiation to evaluate whether the topical product is safe for consumer use.
Twenty-five healthy adult volunteers of both sexes, with no history of allergic skin reactions and not under treatment with steroids, were included in the test. The product has been applied as it is by using the Finn Chamber, an 8 mm diameter aluminium disk. The cosmetic product was applied in 2 different sites (CONTROL SITE and TEST SITE) and left in contact with the skin surface for 48 hours. Finn chamber assures an occlusive application of the product. Any irritating reactions are recorded after the removal of the Finn Chambers from both the sites. Then, only one application site (TEST SITE) is irradiated at a dose of 10 J/cm 2 (320-400 nm with a solar simulator).

Skin reactions are evaluated 15 minutes, 1, 24, 48 hours after patch removal in the CONTROL SITE and 15 minutes and 48 hours after UVA/visible irradiation in the TEST SITE. The presence of positive responses of the same intensity on the two sides excludes any role of the UV rays in the appearance of the reaction.

Potential skin irritation and photo-irritation of the product has been assessed according to the amended Draize classification.
On the basis of the data obtained, we deem the cosmetic product has been assessed as non-irritating and non-photo-irritating (after exposure to UVA/visible irradiation).

Ref.: Individual company submission

SCCS comment
The original study report was not provided. The study appears to be based on a finished cosmetic product of unknown composition, and therefore it cannot be used for the SCCS evaluation of potential phototoxicity of genistein or daidzein.

3.3.8.2 Photomutagenicity / photoclastogenicity

/

3.3.9 Human data

According to the submitter, the Patch test assesses the potential side effects (skin erythema and oedema reactions) that may occur after applying a cosmetic product to evaluate whether the topical product is safe for consumer use.
Twenty-five adult healthy volunteers of both sexes on sensitive skin, with no history of allergic skin reactions, and not under treatment with steroids were included in the Patch test. The product was applied as it is by a Finn Chamber fixed to the skin of the arm. The samples were maintained in situ for 48 hours and the affected area has not been cleansed for the entire duration of the test. The evaluation of the reactions was carried out 15 min.,1 hour, and 24 hours after removal of the Finn Chambers.

According to the amended Draize scale (< 0.5 is the limit under which the product is classified as not irritating), the product was demonstrated as non-irritating for human skin. At the described conditions, the cosmetic product was well tolerated and assessed as non-irritating on sensitive skin.

Ref.: Individual company submission

SCCS comment
The original study report was not provided. The study appears to be based on a finished cosmetic product of unknown composition, and therefore it cannot be used for the SCCS evaluation of potential skin irritancy of genistein or daidzein.

3.3.10 Special Investigations: Assessment of Endocrine Disrupting Potential

The ability of isoflavones to interact with oestrogen receptors is attributed to their structural analogy with 17β-oestradiol. The two types of oestrogen receptors have different biological actions. Oestradiol receptor alpha (ERα) is associated with cell proliferation-while oestradiol receptor beta (ERβ) has pro-apoptotic and pro-differentiating effects. Isoflavones can bind to both oestradiol receptors but have a higher affinity for ERβ. In addition to effects of isoflavones on ERα and ERβ, interactions are also known to occur with the oestrogen-related receptors ERRα, ERRβ or ERRγ (Suetsugi *et al.*, 2003) and with GRP 30, identified as a further oestrogen receptor involved in proliferation of breast cancer cell lines *in vitro* (Maggiolini *et al.*, 2004).

3.3.10.1 Endocrine-related Adverse Effects of Genistein

Genistein has been reported to bind with ER alpha and ER beta, although it also has an antiestrogenic action (Dang et al., 2003). Phytoestrogens are known to have a higher affinity for ERβ; for example, genistein has an expected EC50 around 1.9–6.1 nM for ERβ and 16.2–18.8 for ERα (Islam et al., 2015; Tiosano et al., 2014). Genistein also seems to have a different response depending on the dosage used. At <10 μM, it is reported to increase proliferation in PC3 cells, whereas at >10 μM, it increased cytotoxic effects that resulted in a decrease in cell viability and migration. This disparity of results complicates the understanding of the impact of genistein on PCa progression (Terzioglu-Usak et al., 2019). At higher concentrations, genistein promotes lipogenesis through the PPAR gamma pathway, and the ER-independent pathway (Dang et al., 2003). At low concentrations, genistein acts as an estrogen and has an inhibitory effect on lipogenesis. There are also sexual differences in the effect of genistein on adipose deposition and insulin resistance, an effect that involves ER beta (Penza et al., 2006).

Genistein exposure has been shown to alter folliculogenesis in rats *in vivo* (Medigovic *et al.*, 2012; Zhuang *et al.*, 2010), but the results have varied depending on age, strain, and dose. Genistein exposure (50 mg/ kg) decreased healthy primordial, primary, and secondary follicles but increased the amount of antral follicles in 18-day old Wistar rats, suggesting that genistein exposure accelerates follicle recruitment (Medigovic *et al.*, 2012). Additionally, the same study showed that genistein exposure (50 mg/kg) increased the number of atretic secondary and antral follicles (Medigovic *et al.*, 2012), suggesting that genistein-induced accelerated follicles may not survive to produce viable oocytes. Conversely, other studies indicate that genistein exposure (160 mg/kg) increases primordial follicles and decreases antral follicles in 3-month-old Sprague-Dawley rats (Zhuang *et al.*, 2010).

Dietary exposure to genistein in C57BL/6J mice postweaning (peripubertal) could result in earlier puberty in females assessed by vaginal opening, estrous cyclicity, corpus luteum and mammary gland development (Li *et al.*, 2014). Newly weaned female mice were fed with 0, 5, 100, or 500 ppm genistein diets. Decreased age at vaginal opening, increased length on estrus stage, and accelerated mammary gland development were detected in 100 and 500 ppm genistein-treated groups. Increased presence of corpus luteum was found in 5 ppm genistein-treated group at 6 weeks old only. Increased expression of epithelial-specific genes but not that of ERα and ERβ was detected in 500 ppm genistein-treated mammary glands at 5 weeks old. No significant adverse effect on embryo implantation was observed. In female mice, post-weaning dietary genistein consumption advanced puberty by decreasing the age of the vaginal opening, increased the length of the estrus stage, and accelerated mammary gland development.

Pihlajamaa *et al.*, (2011) determined that genistein was not merely an AR agonist but also a potential selective androgen receptor modulator (SARM), since it has a tissue-specific AR response. As such, by giving transgenic male mice a daily dose of 10 mg/kg of genistein for five days, they observed an antiandrogenic response in the testis, prostate, and brain. However, in the case of castrated males, the treatment induced the activation of AR in the prostate and brain only. These results showed that genistein could act as a partial agonist/antagonist in the prostate, depending on the presence of circulating androgens (Pihlajamaa *et al.*, 2011).

In view of the potential endocrine-mediated adverse effects of genistein, the SCCS assessed the currently available evidence in accordance with the Guidance jointly published by the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) (2018).

Level 1: Existing data and existing or new non-test information

- Physical and chemical properties, e.g. MW, reactivity, volatility, biodegradability
- All available (eco) toxicological data from standardised or non-standardised tests.
- Read-across, chemical categories, QSARs and other *in silico* predictions, and ADME model predictions

The Mulon Conseil submission has detailed a study by Roncaglioni (2019) that used *in silico* modelling approach to predict estrogenic and androgenic activity of phytoestrogens using ToxCast data and models. The models included ToxCast Pathway Models based on Area Under the Curve (AUC) values from *in vitro* assays for androgen and estrogen receptors; COMPARA (Consensus) comprising a combination of predictive models based on androgen data; and CERAPP Potency Level for estrogen data from Mansouri *et al.* (2016). ToxCast data from *in vitro* assays as well as predictive *in silico* models for estrogenic and androgenic activity indicate a moderate estrogenic mechanism for genistein (aglycone) as agonist, and a moderate binding ability that is slightly higher than for daidzein. These findings are consistent with those reported by Bovee *et al.* (2004), who assessed estrogenic binding potency of genistein with a yeast bioassay stably expressing human estrogen receptors (hERα) and (hERβ). ToxCast data did not indicate an androgenic mechanism for genistein (Mulon Conseil Submission).

Level 2: *In vitro* assays providing data about selected endocrine mechanism(s)/ pathways(s) (Mammalian and non-mammalian methods)

In vitro studies have suggested that genistein can inhibit the enzymes aromatase (involved in estrogen production), 5α-reductase (involved in testosterone metabolism), and 17β-hydroxysteroid dehydrogenase Type I (involved in the biosynthetic pathway from cholesterol to the sex steroids). However, the effects were not consistently reproduced in whole-animal studies. Two studies in male rats fed phytoestrogens found no effect on brain aromatase activity, while one of the studies reported unspecified changes in 5α-reductase activity in the amygdala and preoptic area (Whitten and Patisaul, 2001).

It has also been reported that genistein inhibits CYP1A1, an enzyme, which degrades 17β-estradiol, in a mouse hepatoma cell culture (Bouker and Hilakivi-Clarke, 2000).

Rozman *et al.* (2006) has summarised the studies reporting relative ER-binding of genistein in *in vitro* assays (see Annex-A, Table A10). Although *in vitro* estrogenicity assays are not necessarily predictive of *in vivo* effects (UK Committee on Toxicity, 2003), the results consistently indicate weak estrogenic activity of genistein, compared to 17β-estradiol.

In estrogen dependent cells, phytoestrogens have also been observed to both stimulate and inhibit proliferation. It has been suggested that proliferation, which was observed at lower concentrations of phytoestrogens (<10 μM [equivalent to ~2700 μg/L using molecular weight of genistein]), was mediated through receptor responses, since proliferation was not stimulated by phytoestrogens in cells lacking ERs (reviewed by UK Committee on Toxicity, 2003).

Oral exposure studies in rats have reported inconsistent results, with one study demonstrating an increase in uterine weight following oral exposure of rats to ≥150 ppm [~14 mg/kg bw/day] genistein through diet, but other studies indicating no effect on uterine weight with genistein doses up to 750 ppm in feed [~124 mg/kg bw/day] (Santell *et al.,* 1997). Uterine weight increase was observed in most studies in which rats were exposed to ≥2 mg/kg bw/day genistein by s.c. or i.p. injection (Noteboom and Gorski, 1963).

The potency of genistein in inducing increases in uterine weight was, however, much lower than that of 17β-estradiol or diethylstilbestrol (see Table d below)

Harris *et al.* (2002) tested for selective affinity of various phytoestrogens to human, rat or mouse ERs, and reported that most compounds were non-selective, which was consistent with the observation that the ligand-binding domains of both ERs are highly conserved across species.

Matsumura *et al.* (2005) determined the ER-binding of isoflavones in human breast cancer cells, using radiolabelled oestrogen [2,4,6,7-3H]oestrogen at 16 x 10^{-10} M. Genistein inhibited [^{3}H]oestrogen binding by 50% at 1000- fold molar excess.

In experiments with different isoflavones and their combinations, genistein appeared to have the maximum affinity to both receptors, with approximately 60-fold higher binding preference for ERβ (Zhao *et al.*, 2009).

The Table (reviewed by the UK Committee on Toxicity, 2003) shows a comparison of the ER-binding potencies of isoflavones in human, mouse and rat. In all species, the oestrogenic binding potency of isoflavones is much weaker than that of oestradiol, and higher molar concentrations are needed to achieve 50% inhibition of oestradiol binding to ERα and β. In the case of genistein, the concentration must be ≥ 100 fold higher for ERα, and ≥ 2 fold higher for ERβ. The relative binding affinities (RBAs) presented in Table d illustrate how isoflavones preferentially bind ERβ.

Table d. The ER binding potency of isoflavones in different species

Compound	ERα		ERβ		Ratio of RBAs ERβ/ERα	Reference
	IC_{50} or [EC_{50}] (μM)	RBA (%)	IC_{50} or [EC_{50}] (μM)	RBA (%)		
Human						
17β-oestradiol	0.0020	*100*	0.0023	*100*	0.86	Harris et al., 2002
Diethylstilbestrol	0.0014	*142*	0.0011	*209*	1.27	
Genistein	0.3340	*0.60*	0.0066	*34.8*	50.60	
Daidzein	>5.0000	*<0.04*	0.4100	*0.56*	>12.19	
17β-oestradiol	0.0043	*100*	0.0057	*100*	0.75	Mueller et al., 2004
Diethylstilbestrol	0.0046	*93*	0.0046	*124*	1.00	
Genistein	0.3000	*1.43*	0.0150	*38.0*	20.00	
Equol	1.5000	*0.29*	0.2000	*2.85*	7.50	
17β-oestradiol	[0.021 x 10^{-3}]	100	[0.11 x 10^{-3}]	100	0.19	Muthyala et al., 2004
Genistein	[0.0800]	0.02	[0.0066]	7.40	12.00	
Daidzein	[0.2500]	0.01	[0.1000]	0.04	2.50	
Equol	[0.2000]	0.20	[0.0740]	1.60	2.70	
17β-oestradiol		100				Kwok and Cheung, 2010
Diethylstilbestrol		100				
Genistein		1.50				
Mouse						
17β-oestradiol	0.0021	*100*	0.0025	*100*	0.84	Harris et al., 2002
Diethylstilbestrol	0.0002	*1050*	0.0023	*109*	0.09	
Genistein	0.4000	*0.53*	0.0046	*54.3*	86.95	
Daidzein	4.9930	*0.04*	0.1670	*1.50*	29.89	
Rat						
17β-oestradiol	0.0018	*100*	0.0018	*100*	1.00	Harris et al., 2002
Diethylstilbestrol	0.0006	*300*	0.0011	*164*	0.54	
Genistein	0.2820	*0.64*	0.0058	*31.0*	48.62	
Daidzein	5.1790	*0.03*	0.3450	*0.52*	15.01	
17β-oestradiol	0.0250	100	0.0320	100	0.78	Zhao et al., 2009
Genistein	4.7350	0.53	0.0790	41.1	60.00	
Daidzein	26.6500	0.09	1.7380	1.87	14.27	
Equol	5.8800	0.43	0.5820	5.57	10.09	
G + D	9.8960	0.26	0.1570	20.6	62.87	
17β-oestradiol	0.0009	100				Branham et al., 2002[a]
Genistein	0.2000	0.45				
Daidzein	4.0000	0.02				
Equol	0.6000	0.15				

IC50 – molar concentration of compound leading to a 50% inhibition of 17β-oestradiol binding to ER
EC50 – molar concentration of compound producing response equal to 50% of that observed with 17β-oestradiol

RBA – the Relative Binding Affinity of the compound as a percentage of the binding affinity of 17β-oestradiol (100%) calculated as (IC50/EC50 of 17β-oestradiol)/(IC50/EC50 of test compound) x 100.

Italicised values have been calculated for this statement based on IC50/EC50 values reported by authors.

The ER type not stated, presumably ERα

The UK Committee on Toxicity (2003) noted that genistein binds weakly to sex hormone-binding globulin and concluded that phytoestrogens are unlikely to prevent binding of estrogen or androgens at genistein levels found in blood (<5 μM [<1351 μg/L]). *In vitro*

studies demonstrated that genistein (≥5 µM [<1351 µg/L]) increases synthesis of sex hormone-binding globulin (UK Committee on Toxicity, 2003). However, studies in humans given isoflavones reported inconsistent effects on sex hormone-binding globulin synthesis (Whitten and Patisaul, 2001; UK Committee on Toxicity, 2003).

Bovee *et al.* (2004) assessed the estrogenic binding potency of genistein with a yeast bioassay stably expressing human estrogen receptors (hERα) and (hERβ). Ranking of the *in vitro* estrogenic potency with ERα was: 17β-estradiol ⩾ 8-prenylnaringenin > coumestrol > zearalenone ⩾ genistein ⩾ genistin > naringenin. Ranking of the estrogenic potency with ERβ was: 17β-estradiol ⩾ coumestrol > genistein > zearalenone > 8-prenylnaringen ⩾ daidzein > naringenin > genistin ⩾ daidzin. Genistein (aglycone form) showed a moderate estrogen mechanism as agonist and a moderate binding ability.

A study by Pfeiffer *et al.* (2005) examined the effects of genistein and other isoflavones on *in vitro* glucuronidation of 17β-estradiol. Microsomes were obtained from the liver of a 63-year-old male and incubated with 17β-estradiol alone or together with genistein. Formation of estradiol 3-glucuronide (catalyzed by UGT1A1) and estradiol 17-glucuronide (catalyzed by UGT2B7) were measured by HPLC. Genistein inhibited formation of estradiol 3-glucuronide (by ~80%) but had no effect on the formation of estradiol 17-glucuronide. Results were confirmed using genetically engineered Sf-9 insect cells expressing UGT1A1, which is involved in the formation of the 3- glucuronide.

I) Estrogen (OECD TG 493) or androgen receptor binding affinity (US EPA TG OPPTS 890.1150)

NONE

II) Estrogen receptor transactivation (OECD TG 455, ISO 19040-3), yeast estrogen screen (ISO 19040-1 & 2)

The estrogenic activities of genistein were evaluated by Skledar *et al.* (2020) using a transactivation assay (OECD TG 455, ISO 19040-3) using the human estrogen receptor alpha (hERα) mediated luciferase expression in Hela9903 cell line used for activity evaluation of hERα agonists and antagonists. Supra-maximal luciferase activity was seen for most of the flavonoids tested at concentrations below 1 µM. At concentrations above 1 µM, non-specific interactions were observed. Due to the supra-maximal luciferase activity, sigmoid dose-response curves, necessary for EC50 determinations, could not be constructed for most of the flavonoids tested, the concentrations corresponding to 10% (PC10 equivalent to 0.02 µM $\pm$ 0.003) and 50% (PC50 equivalent to 0.10 µM $\pm$ 0.02) of the maximum activity of the positive control, (1 nM E2), were used for quantitative determination of estrogenic activities. Due to the reported supra-maximal increases in luminescence signals observed >1 µM, the authors questioned whether this method was suitable for determination of estrogenic activities of the phytoestrogens.

III) Androgen receptor transactivation (OECD TG 458)

The inhibitory activities of genistein on the firefly *Photinus pyralis* (FLuc) and pansy *Renilla reniformis* (Rluc) luciferase enzymes *in vitro* were determined by Kenda *et al.* (2021) in lysates of the AR-EcoScreen cell line. This cell line was stably transfected with the Fluc and Rluc reporter-genes and used in OECD TG 458 for compound testing for (anti)androgen activity. Genistein showed a significant inhibition (71.2%) of Fluc at 100 µM. Genistin (the glycosated form) was inactive against Fluc. The IC50 value for Fluc inhibition was determined for genistein which was 36.89 µM, while the IC50 for the resveratrol was almost eight-fold higher at 4.94 µM.

IV) Steroidogenesis *in vitro* (OECD TG 456)

Receptor mediated endocrine effects were assessed by Kolle *et al.* (2012) using the yeast (*Saccharomyces cerevisiae*), stably transformed with the human estrogen receptor (hER) and human androgen receptor (hAR) gene. Effects on steroid hormone biosynthesis of genistein were assessed using the human cell adrenocortical cell system line H295R in the steroidogenesis assay with seven concentrations of genistein (0.1, 0.3, 1, 3, 10, 30 and 100 µM) and plasma samples were analysed for metabolome profile after a single *in vivo* dose of 1000 mg/kg bw. Estrogen agonism was observed in the yeast based on the assays at LOAEC of 10 µM. The H295R cell line-based assay was used to evaluate the potential interference with the hormone biosynthesis (estradiol and testosterone). Based on the decision matrix described in OECD TG 456 the LOAECs of the three experiments for the seven concentrations of genistein tested were for estradiol and testosterone 1, 1, 3 µM and 10, 10, 10 µM respectively. Genistein pattern ranking of metabolome analysis was, estrogenicity (weak), anti-prolactin (weak), antiglucocorticoid in adrenals (weak), gonadotropin-releasing hormone agonism (weak).

Strajhara *et al.* (2017) tested chemicals (including genistein) interfering with the production of key adrenal steroids, using H295R adrenocortical–cells cultivated according to the OECD 456 test guideline. H295R cells were incubated for 48 h with vehicle (0.1% DMSO) or genistein at 10 µM concentration to be tested for potential disruption of adrenal steroidogenesis. Steroid profiles were determined before and after incubation with reference compounds and genistein. Changes in steroid levels were measured by LC–MS. Data were expressed as a fold change relative to the solvent control. Genistein (10 µM) led to enhanced pregnenolone (2.17), 17a-hydroxypregnenolone (5.86) and dehydro-epiandrosterone (7.66), but reduced progesterone (0.19), 17a-hydroxyprogesterone (0.35), corticosteroids (0.05 – 0.42) and 11 ß HSD-dependent androgens (0.12).

Haggard *et al.* (2018) reported that genistein increased E2 in the human adrenocarcinoma (H295R) cell-based assay OECD interlaboratory validation, but failed to increase E2 in the ToxCast high throughput HT-H295RHT assay. The HT-H295R assay is comprised of 4 main experimental components: (1) H295R cell culture and treatment; (2) cell viability assay using the MTT tetrazolium reduction assay; (3) quantification of steroid hormones in the media from exposed H295R cells; and (4) statistical analysis of steroid hormone concentrations. Genistein did produce a strong pathway positive, based on the significant effects on OH pregnenolone, progesterone, OH progesterone, deoxycorticosterone, 11-deoxycortisol, cortisol, androstenedione, and Testosterone, with a significant, high adjusted max median Mahalanobis distance (31.8), value employed to characterise the magnitude of change for 11 steroid hormones produced by H295R cells. One concentration (11.11 µM) appeared to significantly increase estrone and estradiol, but did not meet the minimum criteria for a positive result (2 consecutive concentrations with significant results, or the highest non-cytotoxic concentration with significant results).

V) Aromatase Assay (US EPA TG OPPTS 890.1200)

NONE

VI) Thyroid disruption assays (e.g. thyroperoxidase inhibition, transthyretin binding)

Daidzein and genistein are inhibitors of TPO, albeit at much lower potency compared to the well-known inhibitors methimazole and 6-propylthiouracil. (Paul *et al.*, 2014, 2016).

See also Level 5 *in vivo*

VII) Retinoid receptor transactivation (RXR) assays

Bargues *et al.* (2017) isolated and cultured cortical astrocytes from dissected cerebral cortices of neonatal mice (C57BL/6 J) preincubated with genistein (5 mM) for 24 hours, prior to the addition of Amyloid-b (Ab) (5 mM), which resulted in a ApoE release to the culture medium in a concentration dependent manner. This effect is mediated by activation of PPARg as its inhibition significantly prevents the increase in genistein induced ApoE, release upregulated by activation of the retinoid X receptor moiety of the RXR/PPAR dimeric receptor. Treatment of an Alzheimer's disease mouse model with genistein was associated with a lowering of Ab levels in brain, in the number and the area of amyloid plaques (confirmed *in vivo* by positron emission tomography) as well as in microglia.

VIII) Other hormone receptor assays as appropriate

IX) High-Throughput Screens

Level 3: *In vivo* assays on endocrine mechanism(s)/pathway(s)(a)

I) Uterotrophic assay (OECD TG 440)

Ohta *et al.* (2012) treated OVX 6 mice at age 8 weeks by subcutaneous (sc) injection or oral gavage (0, 20, 60, 200 and 600 mg/kg bw) at 24 hr intervals for 7 consecutive days in compliance with the OECD Test Guideline No. 440 (OECD, 2007). 17-α -ethynyl estradiol (EE) was used as a reference control (6 µg/kg bw for oral route and 0.2 µg/kg bw for subcutaneous route). Genistein by oral route showed significant agonistic effects at 200 and 600 mg/kg in a dose dependent manner up to the uterotrophic level of 0.2 µg/kg EE sc. The calculated agonistic and antagonistic LOELs were 200 mg/kg bw and 60 mg/kg bw respectively. Molar equivalent dose of genistin (the glucoside form), was equipotent to that of genistein in both agonistic and antagonistic activity, confirming that the glucoside was fully hydrolysed to aglycone in this bioassay. Genistein did not induce U-shaped dose-response because the uterine effects at maximal dose (limited by MTD) were smaller than the reference EE.

II) Hershberger assay (OECD TG 441)

Stroheker *et al.* (2003) fed pregnant female Wistar rats with the control diet until delivery (PND 21). The rats were weaned and castrated before the treatments - from PND 21 to PND 28, to allow the rats to recover from surgery; (a) the castrated rats were fed the L5 (control diet), DO4 (genistein 3.9 x 10^{-3}% and daidzein 2.3 x 10^{-3}%) or DO3 (genistein 6.3 x 10^{-3}% and daidzein 3.0 x 10^{-3}%) diet. (b) Rats were fed with the L5 or DO3 diet. After castration, they were treated for ten days, from 28 to 38 days of age, without or with testosterone propionate injected subcutaneously (0.1, 0.2, 0.4, or 0.8 mg/kg b.w./day). (c) Rats were fed with L5 or DO3 diet. Beside testosterone propionate injected subcutaneously, rats were also gavaged from PND 28 to 38 with vinclozolin (0, 25, 50 or 100 mg/kg b.w./day).

No significant effect of dietary phytoestrogens was observed on the relative weights of the seminal vesicles, prostate and bulbocavernosus/levator ani (BC/LA) muscle after feeding on L5 diet or L5/DO3 for 10 days.

Level 4: *In vivo* assays on adverse effects on endocrine relevant endpoints

•Repeated dose 28-day study (OECD TG 407)

The study by Okazaki *et al.* (2002) used seven-week-old Crj:CD(SD)IGS rats and assigned them to one of four groups, each consisting of ten males and ten females. Genistein was administered once daily by gavage at doses of 0 (control), 120, 400 or 1000 mg/kg body weight per day for detecting endocrine-related effects of endocrine-disrupting chemicals based on the existing TG 407.

Male rats were killed 1 day after the 28th administration. For females, killing was delayed for up to 4 days after the 28th administration until the animal entered the diestrus stage of the estrous cycle, determined by observing vaginal smear values. No genistein-related changes were evident in either sex regarding clinical signs, detailed clinical observations, functional tests or grip strength. No statistically significant differences in body weight and food consumption were recorded between values for the control group and those of any dose group in either sex. None of the females exhibited an apparent abnormal estrous cycle. Blood chemistry showed significant increases in total protein in both sexes in the 1000 mg/kg group and in triglycerides in the 400 and 1000 mg/kg group females. A significant decrease was observed in the albumin/globulin ratio in females of the 1000 mg/kg group. In males, a significant increase in prolactin, but not of T3, T4, TSH, testosterone, estradiol, to approximately double that the control mean, was observed in the 1000 mg/kg group. In males, a significant increase in the liver weight relative to body weight, but not pituitary, thyroids, kidneys adrenals, was recorded in the 400 and 1000 mg/kg groups. In the vagina, slight or mild vacuolation and mucinification of the epithelium (vacuole or cleft formation featuring cell debris) was observed in 2/10 animals in each of the 400 and 1000 mg/kg groups. No statistically significant differences were recorded either in the number or morphology of the sperm between the control group and any of the dose groups. Changes were not observed in male and other female genital organs, including the oviducts, ovaries and mammary glands, although mammary gland were shown to be relatively sensitive with ethinylestradiol.

Level 5: *In vivo* assays (more comprehensive data on adverse effects on endocrine relevant endpoints over more extensive parts of the life cycle of the organism)(b)

•Extended one-generation reproductive toxicity study (OECD TG 443)(e)

•Two-Generation reproduction toxicity study (OECD TG 416 most recent update)

Hebron *et al.* (2000) exposed Sprague–Dawley rats to genistein aglycone in soy-free feed, 5K96 meal (genistein (0.54 mg/kg) and daidzein (0.48 mg/kg) contents), fortified at 0, 5, 100, and 500 ppm starting in utero through 20 weeks. After weaning, 48 pups were placed on the same genistein-supplemented chow received originally by the mother and were maintained on this food source until the time of termination at postnatal day (PND) 140.

Serum levels in rats terminated at PND 140 represented total genistein obtained after enzymatic deconjugation, because the glucuronide conjugate is the predominant form present in rat serum (98%) with the remainder being aglycone. Total genistein in the serum of male and female rats fed soy-free chow was 0.02 µM. The serum and thyroid levels of genistein, determined using isotope dilution LC-ES/MS, increased in a dose-dependent manner, 0.06/0.10, 0.59/0.94, 6,00/7.94 µM in males/females at 5, 100, 500 ppm respectively.

The intracellular concentrations of total genistein were up to 1920 nM and up to 350 nM for the aglycone. These concentrations of genistein aglycone are of the same order as those shown to inactivate rat TPO *in vitro* (10– 1000 nM). The distribution of unconjugated and conjugated forms of genistein in the thyroid was different from that in the blood (18-28% aglycone in the thyroid vs 2% aglycone in serum of adult male and female rats).

Analysis of sera from all rats showed no significant differences in the levels of T3/T4 and TSH hormones relative to untreated rats. The Authors reported that based on the concentrations of total genistein present in serum, rats fed the control and low-dose (5 ppm) diets are similar to human adults consuming a typical Western diet (0.1 μM). Rats fed the 100 ppm genistein diet are similar to human adults consuming a typical Asian diet (0.1–1.2 μM) or soy nutritional supplements (0.5– 0.9 μM). Rats fed the 500 ppm genistein diet were similar to infants on soy formulas (2–7 mM).

Delclos *et al.* (2001) conducted a dose range-finding study as a prelude to a multigeneration bioassay to assess potential toxicities associated with genistein consumption. Genistein was administered in a soy- and alfalfa-free diet at 0, 5, 25, 100, 250, 625, or 1250 ppm (genistein and daidzein, limit of detection 0.5 ppm) to pregnant dams starting on gestation day 7 and continuing throughout pregnancy. Dietary exposure of the dams continued through lactation, and pups were maintained on the same dosed feed as their mothers after weaning until sacrifice at PND 50. Body weight and feed consumption of the treated dams prior to parturition showed a decreasing trend with a significant reduction at the highest dose. Genistein treatment had no significant effects on gestation duration, litter size, proportion of live pups, or sex. Mean live pups' weight was decreased in the 1250 ppm dose group and pups of both sexes had significantly decreased body weights relative to controls at the time of sacrifice. Genistein had no significant effect either on anogenital distance measured on PND 2 in either sex, or on the age at preputial separation in males and age of vaginal opening in females. No significant treatment-related differences were detected in any of the clinical chemistry or hematological parameters measured. The organ weight effects in the pups were decreased ventral prostate weight in males at the 1250 ppm dose and a trend toward higher pituitary gland to body weight ratios in both sexes. Histopathologic examination of female pups revealed ductal/alveolar hyperplasia of the mammary glands at 250 to 1250 ppm. Abnormal cellular maturation in the vagina was observed at 625 and 1250 ppm, and abnormal ovarian antral follicles were observed at 1250 ppm. No gross abnormalities, including retained or small testes, retention of Mullerian duct remnants, and hypospadias were detected during necropsy. Ductal/alveolar hyperplasia and hypertrophy also occurred in males, with significant effects seen, minimal, at 25 ppm and mild above. In males, aberrant or delayed spermatogenesis in the seminiferous tubules relative to controls was observed at 1250 ppm. There was increased prevalence of hypospermia in the head of the epididymis in the 1250 ppm dose group although testicular spermatid head counts and epididymal spermatozoa counts did not show significant differences between all treated and control groups. Both sexes showed an increase in the incidence and/or severity of renal tubal mineralisation at doses of 250 ppm and above. Dietary genistein thus produced effects in multiple estrogen-sensitive tissues in males and females that are generally consistent with its estrogenic activity.

The study by Lewis *et al.* (2003) allocated mated female rats to three groups of 20 and allowed to litter. Pups were dosed genistein subcutaneously at 0.2 or 4 mg/kg (equivalent to 4 or 40 mg/kg by oral route, bioequivalent selected from the results of a previous study on comparative exposure by different routes) each day from PND 1 (the day of birth) to PND 6, and orally (4 or 40 mg/kg) each day from PND 7 to 21. At PND 22 one male and one female pup from each litter were killed and serum samples were taken for the analysis of testosterone, LH, and FSH in males, and estradiol, LH, FSH, and progesterone in females. There were no consistent treatment-related effects on hormone levels in males or female offspring at day 22. Blood progesterone concentration was reduced in mature females following 40 mg/kg genistein. Genistein at both doses had no effect on basal or GnRH stimulated LH secretion from the pituitary. Anogenital distance was not different in immature pups when assessed at day 2 postpartum and showed no biologically significant difference from control values at day 22. In female pups, the uterus weight at 40 mg/kg

bw was two-fold higher than that of the control pups at day 22 but returned to control values in mature females at 12 weeks of age. The time of vaginal opening was four days earlier in females in the high-dose group and in most developing female pups. At 40 mg/kg bw for the animals smeared from the time of vaginal opening, 13/20 showed permanent estrus (persistent cornification) with 19/20 of the animals smeared from day 43 showing this pattern. The time of preputial separation in males was not affected by administration of genistein. Testis weights were unaffected by either dose of genistein. The no observed adverse effect level (NOAEL) of genistein was considered to be between 4 and 40 mg/kg/day based on the hormonally induced functional changes at the higher dose due to the expected endocrine effects.

McClain *et al.* (2006) reported a feeding study with genistein in rats with 5, 50, and 500 mg/kg/day over 4, 13, and 52 weeks with an interim sacrifice after 26 weeks for histopathology. At 500 mg/kg/day there was an increase in uterus weight after 4 and 13 weeks of treatment. These changes are most probably within the range of physiological variation and without toxicological relevance since there were no histopathological changes up to 13 weeks in contrast to the observations after 26 and 52 weeks of treatment. Additionally, after 52 weeks, increased weights of the ovaries and prostate were recorded at 500 mg/kg/day. Furthermore, histopathology revealed treatment-related changes after 26 and 52 weeks of the ovaries, uterus, vagina, mammary gland, prostate, and epididymides at 500 mg/kg/day, and for some of these organs already at 50 mg/kg/day.

For genistein, in the F1-generation there were histopathological alterations in some endocrine dependent tissues (e.g., ovaries, uterus, vagina, mammary gland, testes), in sexual development (time to vaginal opening) and in fertility parameters (number of life pups) at dose levels clearly lower than those leading to slight effects in the ‘‘enhanced TG 407’’ test.

When comparing the NOELs/LOELs of genistein for different exposure durations there was no apparent difference after 4 and 13 weeks. The treatment-related findings would be expected to occur with a compound with intrinsic estrogenic activity. The no observed adverse effect level (NOAEL) of genistein is considered to be between 5 and 50 mg/kg/day based on the hormonally induced functional changes at the higher dose due to the expected endocrine effects.

In a clinical trial by Bitto *et al.* (2010), genistein aglycone (54 mg) or placebo was given daily to osteopenic post-menopausal women for 24 months. A sub-cohort then continued therapy for an additional 12 months to evaluate the thyroid (THR α and THR β) and retinoid (RAR α, RAR γ, and RXR α) hormone receptors, mRNAs and the levels of thyroid peroxidase (TPO), thyroglobulin (TG), and thyroid microsomal antigen (TMA) autoantibodies in all the genistein aglycone (n 71) and placebo subjects (n 67). Serum T3, T4, and TSH levels were also studied in 40 genistein and 37 placebo subjects from the 3-yr subgroup to determine any long-term effects of pure genistein administration on thyroid related markers. TSH levels were in the normal range (0.27– 4.2 μg/ml) and did not change over time. At a genistein concentration of about 1 μM normal variation in serum free T3 and T4 values were noted in the treated group after 3 years of treatment, but again these were in the normal range (1.80–4.60 pg/ml) and (9.3–17 pg/ml) respectively. No significant alteration of thyroid-specific autoantibodies was observed during these 3 years in either genistein- and placebo-treated subjects. No significant change was observed in THRα, THRβ, RAR, RARα, RARγ, and RXRα expression between the two groups after 3 yr.

This study reveals that chronic administration of pure genistein aglycone (54 mg/d) does not affect thyroid function in postmenopausal women after 3 years of treatment.

SCCS comment on endocrine disrupting potential of genistein
Several studies have shown genistein to be an endocrine active substance, with a potential to cause endocrine-related effects at high doses. Whilst a weak binding affinity to estrogen receptor in vitro has been clearly demonstrated, the evidence for any significant endocrine-related effects in vivo has been limited to studies on rodents at high doses. With the collective view of the available information, the SCCS considers that the toxicological point of departure (PoD) selected for this assessment (see section 3.4) also adequately covers the endocrine-related effects of genistein.

3.3.10.2 Endocrine-related Adverse Effects of Daidzein

In the Nordic Council of Ministers report (2020), in which the authors assessed the risk of isoflavones and in particular genistein and daidzein in pregnant women or children, a total of 12 human studies on the association between exposure to soy/isoflavones and effects on puberty in girls (11 studies) and boys (4 studies) ≤18 years were identified. Risks for adults were not in the scope of that report. In conclusion, based on the identified human studies:

- no straight conclusion can be made for an association between exposure to dietary soy or isoflavones and risk of precocious puberty based on the identified studies for either girls or boys;
- no association was found between early exposure to dietary soy or isoflavones and later risk of breast cancer;
- although there may seem to be a theoretical correlation between in utero isoflavone exposure and risk of hypospadias, it has not been confirmed in the identified human studies.
- only two studies on adverse effects of isoflavones on thyroid function relevant for children and pregnant women (unborn children) were identified. The study by Li *et al.* (2011) included Chinese women, who may respond differently to isoflavone exposure compared to Western populations due to the differences in metabolism. Based on the study by Milerová *et al.* (2006), adverse effects of isoflavones on thyroid function when iodine intake is insufficient cannot be excluded. Statistically significant Spearman's correlations with serum daidzein was observed with TSH.

***In silico* analysis**

From Mulon Conseil Submission:

The selected compounds were investigated by searching them on the US EPA dashboard (https://comptox.epa.gov/dashboard) by inspecting the ToxCast Models in the Bioassays tab. For each compound available in the database there are the following information (if available):

- ToxCast Pathway Model (AUC) – Androgen and ToxCast Pathway Model (AUC) – Estrogen. These contains the Area Under the Curve (AUC) value derived by the combination of the *in vitro* assays available in ToxCast for AR (11 assays) and ER (18 assays) respectively.

- CERAPP Potency Level (From Literature) – Estrogen. Obtained as described in the paper by Mansouri *et al.* 2016 from literature sources. To reduce the variability that increased with the disparate literature sources, the chemicals with quantitative information were categorised into five potency activity classes: inactive, very weak,

weak, moderate, and strong. These five classes were used to evaluate the quantitative predictions. The following thresholds were applied to the concentration–response values:

- Strong: Activity concentration below 0.09 µM.
- Moderate: Activity concentration between 0.09 and 0.18 µM.
- Weak: Activity concentration between 0.18 and 20 µM.
- Very Weak: Activity concentration between 20 and 800 µM.
- Inactive: Activity concentration higher than 800 µM.

The five classes were assigned scores from 0 (inactive) to 1 (strong) with 0.25 increments. Then, for each chemical, the arithmetic mean of the scores of the merged entries from different literature sources was calculated. A new class was assigned to the merged entries according to the following thresholds.

- Strong: Average score > 0.75
- Moderate: 0.5 < Average score between ≤ 0.75
- Weak: 0.25 < Average score ≤ 0.5
- Very weak: 0 < Average score ≤ 0.25
- Inactive: Average score = 0

CHEMISTRY DASHBOARD ToxCast: MODELS

	MODEL	AGONIST	ANTAGONIST	BINDING
17beta-Estradiol	ToxCast Pathway Model (AUC) - Estrogen	0,935	1,53E-02	-
17beta-Estradiol	CERAPP Potency Level (From Literature) - Estrogen	Active (Strong)	-	Active (Moderate)
DAIDZEIN	ToxCast Pathway Model (AUC) - Estrogen	0,44	0	-
DAIDZEIN	CERAPP Potency Level (From Literature) - Estrogen	Active (Moderate)	-	Active (Weak)
DAIDZIN	ToxCast Pathway Model (AUC) - Estrogen	-	-	-
DAIDZIN	CERAPP Potency Level (From Literature) - Estrogen	-	-	-
Equol	ToxCast Pathway Model (AUC) - Estrogen	-	-	-
Equol	CERAPP Potency Level (From Literature) - Estrogen	Active (NaN)	-	Active (NaN)
GENISTEIN	ToxCast Pathway Model (AUC) - Estrogen	0,538	0	-
GENISTEIN	CERAPP Potency Level (From Literature) - Estrogen	Active (Moderate)	Active (Weak)	Active (Moderate)
Genistin	ToxCast Pathway Model (AUC) - Estrogen	-	-	-
Genistin	CERAPP Potency Level (From Literature) - Estrogen	Active (NaN)	-	Active (NaN)
17beta-Estradiol	ToxCast Pathway Model (AUC) - Androgen	1,25	5,19E-02	-
DAIDZEIN	ToxCast Pathway Model (AUC) - Androgen	0	0	-
DAIDZIN	ToxCast Pathway Model (AUC) - Androgen	-	-	-
Equol	ToxCast Pathway Model (AUC) - Androgen	-	-	-
GENISTEIN	ToxCast Pathway Model (AUC) - Androgen	0	0	-
Genistin	ToxCast Pathway Model (AUC) - Androgen	-	-	-

In vitro

From Mulon Conseil Submission

Two main estrogen receptors (ER), that is ERα (NR3A1) and ERβ (NR3A2), have been identified in rats, mice, primates and humans. These ER subtypes have different roles in gene regulation, cancer biology and therapy. ERα activation in the breast and uterus has been shown to enhance cell proliferation, necessary for growth and tissue maintenance but may also play a role in the unlimited growth of, in particular, ERα-dependent breast tumours. Whereas ERβ has been shown to counteract the ERα- mediated stimulation of cell proliferation.

Phytoestrogens show tissue-specific effects that may also result from differences in coactivators and corepressors activated upon activation of the two ERs in different tissues and/or the possible crosstalk of the ERs with other nuclear receptors. Furthermore, the actual mode of action of a phytoestrogen, either as an agonist or an antagonist, may also depend on the level of endogenous estrogens present (Rietjens & al - 2017).

The potential hazards of concern are those expected from the interaction of isoflavones with endocrine pathways. The roles played by estrogen receptors in enhancing cell proliferation in the mammary gland or in the uterus and the interaction of isoflavones with thyroid functioning led in some historical cases to endocrine-responsive cancers and endocrine dysfunction in these tissues being identified as the most relevant endpoints for hazard assessment. From these observations, many studies were performed to examine the mechanisms concerned (EFSA - 2015).

- ER-mediated effects

In vitro assays include receptor binding studies, ERα and ERβ-dependent reporter gene assays and cell proliferation assays using estrogen-sensitive human cell lines derived from three different female estrogen-sensitive tissues, including breast (MCF-7/BOS and T47D), endometrial (ECC-1) and ovarian BG-cells. Most research on the binding of phytoestrogens to ERα and ERβ, and the activation of ERα- and ERβ- mediated gene expression has been conducted with the major soy isoflavones, genistein and daidzein. Usually, isoflavones can bind to both estradiol receptors, but have a higher affinity for ERβ. The ultimate biological activity is more complex since it requires consideration of factors others than receptor binding, including the recruitment of co-regulator proteins in the nuclear pathway as well as non-classical membrane estrogen receptors, but limited information is available on the interaction between isoflavones and these processes.

Studies were performed to assess the estrogenic binding potency of daidzein: in its aglycone form, it shows a moderate activity as agonist and a weak binding ability; it presents an estrogenic activity slightly lower than genistein. These results confirm those found in 2004 by Bovee & al. On the other hand, daidzein does not show androgenic activity.

Equol: the gastrointestinal metabolite of daidzein, shows some estrogen activities but the metabolite is weaker than the parent daidzein. There is no available data for the glycoside form, Daidzin, which is the substance present in plants.

- Other effects highlighted *in vitro*

There are hormone-independent actions of isoflavones, including inhibition of tyrosine kinase activity, inhibition of protein kinase C, inhibition of DNA topoisomerase II, antioxidant activity, anti-angiogenic effects and inhibition of breast cancer resistance protein (BCRP), a cellular efflux protein. These effects are obtained with isolated compounds *in vitro* at doses typically exceeding 10 µM, whereas the ER-mediated effects occur at concentrations of 0.1–1 µM (EFSA - 2015).

***In vivo* animal data (level 3)**

In the EFSA report published in 2015**,** and which focused on the risks of isoflavones from food for peri- and post-menopausal women as well as the target organs of interest (breast, uterus, thyroid), 4 studies for daidzein, 5 for Equol, equivalent to level 3 of the OECD framework were reported. The assessment was limited to isoflavones ingested as food supplements at doses used in the human studies available in the published scientific literature and following a request for information from relevant interested parties.

Genistein and daidzein did not induce histopathological changes such as hypertrophy, hyperplasia or squamous metaplasia in the uterus, whereas rats treated with racemic equol at doses of 10 mg/kg bw/day or higher for 90 days demonstrated a significant increase in

uterine wall thickness and section surface. Rats treated with the same dose for 35 days did not show these histopathological changes.

In rabbits, isoflavones from a red clover extract and daidzein alone did have an effect on uterine weight.

In rats, isoflavones at doses between 10 and 100 mg/kg bw/day consistently caused an increase in uterine weight. This effect appeared to be dependent on the duration of the exposure. Although the results from isoflavone treatments are not completely consistent across all species, experimental designs and endpoints, the data show that sufficiently high doses of isoflavones and racemic equol can elicit uterotrophic effects in animals. Concerning the latter, it should be noted that S-equol does not act as does the racemic mixture of synthetic R- and S-equol (Shinkaruk *et al.*, 2010).

The authors of Nordic council of minister Report (2020) have also identified few *in vivo* studies that were considered relevant. However, only the study by Talsness *et al.* (2015) has reported some effects on the ovaries but due to limitations, the authors concluded that it is not sufficient to change the conclusion of the NTPCERHR (2010) that data on daidzein are insufficient for evaluation of reproductive effects in laboratory animals.

The Nordic Council of Ministries report did not address effects of daidzein on adults (except pregnant women), therefore, the SCCS has performed a new literature search to identify *in vivo* studies investigating effects of daidzein that could be related to an ED mode of action and be used as key study to derive the POD for the MoS calculation. The keys word combination of daidzein and endocrine was used on medline to identify studies published after 2011.

Several *in vivo* studies have recently investigated some more specific effects of daidzein that could be related to an endocrine mode of action. Only the studies in which daidzein was used as an individual substance and not as a mixture of phytoestrogens or isoflavones are described in more details in this section. In these studies, daidzein was administered either subcutaneously or by the oral route. These studies are also summarised in Annex B table B8.

Subcutaneous administration

- ***Effects on male reproductive function***

In the study from Ajdžanović *et al.* (2020), 16 month-aged male Wistar rats were exposed subcutaneously to daidzein at the dose of 30 mg/kg/day during 3 weeks. Rats were divided into sham operated (SO; n=8), orchidectomized (Orx; n=8), estradiol treated orchidectomized (Orx + E; n= 8) and daidzein treated, orchidectomized (Orx + D; n=8). Estradiol was used as a positive control. The objective of this study was to evaluate whether daidzein could be used as a substitute of estradiol for the treatment of ageing-caused androgen deprivation and to perform structural and hormonal analysis of the adrenal cortex, after estradiol or daidzein supplementation in a rat model of the andropause.

Daidzein treatment significantly increased volumes of the zona glomerulosa cell and nuclei, but decreased circulating aldosterone levels. Daidzein significantly decreased both the adrenal and circulating levels of corticosterone but significantly increased the circulating level of dehydroepiandrosterone (DHEA). Given the coherence of its effects and relative safety, the authors concluded that daidzein could be used for the treatment of ageing-caused androgen deprivation and the hypothalamo-pituitary-adrenal axis hyperfunction/related metabolic issues in males.

SCCS comment
This study was performed on a specific rat model of andropause; under these conditions, 30mg/kg could be considered as a LOEL.

In the study from Trifunovic et al (2018), the authors have investigated the effects of daidzein on the morphofunctional and histological parameters of the hypothalamic–pituitary–adrenal (HPA) after 21 days of subcutaneous administration of 30 mg/kg /day daidzein to orchidectomized 2 months male Wistar rats (similar protocol as the previous study).

Daidzein decreased the volume density of CRH neurons within the paraventricular nucleus as well as (corticotropin-releasing hormone) CRH immunofluorescence in the median eminence. The total number of adrenocorticotropic hormone (ACTH) cells was decreased, while ACTH cell volume and the intensity of ACTH fluorescence were increased. Both ACTH and corticosterone blood levels were increased.

The results demonstrate that volume density of CRH neurons; total number and volume of ACTH cells, as well as stress hormones levels, are vulnerable to the effects of daidzein.

SCCS comment
This study was performed on a specific rat model of andropause. The protocol seems similar to the previous study but there is no indication of a treated control group with estradiol. Moreover, some inconsistencies were noted (body weights of the rats were very different, increased corticosterone level in the study from Trifunovis and decrease in the study from Ajdžanović *et al. (2020)*; under these conditions, 30 mg/kg could be considered as a LOEL. In the study from Nestorovic *et al.* (2018), the authors aimed to examine the potential of the principal soy isoflavones, genistein and daidzein, or isoflavone rich soy extract to recover pituitary castration cells in orchidectomized adult male rats in comparison with the effects of estradiol. The protocol of exposure was again very similar to the previous studies. daidzein (30 mg/kg/day sc) decreased the cell volume of gonadotropic cells but increased their number and numerical density. Orchidectomy increased all examined stereological parameters and relative intensity of fluorescence (RIF). Compared to Orx, estradiol increased the volume of pars distalis, but reversed RIF and all morphometric parameters of gonadotropes to the level of SO rats, except their number. Treatments with purified isoflavones and soy extract decreased RIF to the control SO level, expressing an estradiol-like effect. However, the histological appearance and morphometrical features of gonadotropes did not follow this pattern. Genistein increased the volume of pars distalis, decreased the volume density of LH-labeled cells and raised the number of gonadotropes. Daidzein decreased the cell volume of gonadotropic cells but increased their number and numerical density. Soy extract induced an increase in number and numerical density of FSH-containing cells. The authors concluded that soy phytoestrogens do not fully reverse the Orx-induced changes in pituitary castration cells.

SCCS comment
This study was performed on a specific rat model of andropause; under these conditions, 30 mg/kg could be considered as a LOEL.

In the study from Retana-Marquez *et al.* (2016), the authors evaluated the effects of mesquite pod and Leucaena extracts on several aspects of behaviour and reproductive physiology of the male rat. The effects of the extracts were compared with those of

estradiol (E2) and of daidzein (DAI) and genistein (GEN). DAI was administered subcutaneously to adult male Wistar rats (three months of age, n=10/group) at the dose of 5 mg/kg/day during 51 days. Mount latencies in males treated with Leucaena extract, DAI and E2 were higher than in control males in some of the days of treatment [F5, 55=3.653; p = 0.0032], although there were no differences among days of treatment. Intromission latency increased in almost all groups, except in the Leucaena extract group, and it increased with days of treatment [F5, 55 = 7.801; p = 0.0001]. Ejaculation latencies increased in males from all the experimental groups, compared with control. This effect was stronger on days 40 and 50 [F5, 55 = 4.039; p = 0.0036]. The percentage of tubules with TUNEL-labelled germ cells increased in DAI treated males, compared with testes from control males. Sperm count and sperm viability and testosterone levels were decreased in DAI-treated rats. The results indicate that mesquite pod and Leucaena extracts disrupt male sexual behaviour in a similar way to DAI and GEN, but less than E2.

SCCS comment
Under the conditions of this study, 5 mg/kg could be considered as a LOEL.

- ***Effects on female reproductive function***

In the study from Jaric *et al.* (2017), the authors aimed to investigate effects of daidzein on the uterine function in ovary-intact middle-aged rats. Daidzein was administered subcutaneously to female Wistar rats (12 months old) at the dose of 35 mg/kg/day during 4 weeks. Genistein was used as a positive control. DAI did not change the uterine wet weight and stereological features of the main uterine compartments as well as LAC and VEGF gene expression. Absence of hyperplastic changes were illustrated by an increase in caspase-3 immunoexpression, associated with reduced PCNA expression. DAI up-regulated only the expression of ERβ, while the expression levels of ERα and PR remained unaffected. Also, DAI inhibited the activation of Akt due to down-regulation of phosphorylated and total form of Akt protein expression. In conclusion, compared to GEN, DAI did not promote events associated with the endometrial cell proliferation.

SCCS comment
In this study, 35 mg/kg could be considered as a NOAEL.

In the study from Sergio *et al.* (2019), the authors aimed to investigate different female reproductive variables of leucaena and daidzein in ovariectomized or ovary intact rats. Daidzein was administered subcutaneously to ovariectomized or ovary intact Wistar rats (3 months old) at the dose of 5 mg/kg during 30 days. Estradiol was used as a positive control. In intact females, daidzein disrupted the estrous cycle and female sexual behaviour, decreased the number of follicles and corpora lutea, increased uterine and vaginal epithelium in proestrus and diestrus periods, increased uterine and vaginal relative weights during diestrus, and decreased serum progesterone during proestrus. All these effects were similar to those of leucaena but lower than E2-induced effects. In OVX females, daidzein decreased body weight, induced lordosis, stimulated vaginal epithelium cornification, increased vaginal weight, and augmented vaginal epithelium thickness. Again, these effects were similar to the effects of leucaena but lower than the effects observed with E2. These results indicate that, in gonadally intact females, daidzein can produce antiestrogenic effects in sexual behaviour but estrogenic effects on uterine and vaginal weight and epithelia, without modifying serum levels of E2. In OVX females, in

total absence of endogenous E2, daidzein induced estrogenic effects on vaginal weight and epithelia, as well as on sexual behaviour.

SCCS comment
In this study, 5 mg/kg could be considered as a LOAEL.

Oral administration

- ***On male reproductive function***

In the study from Mohamad *et al.* (2019), the authors aimed to investigate the effects of Pueraria Mirifica and daidzein against benign prostate hyperplasia (BPH). Daidzein was administered at 10 and 100 mg/kg during 30 days to SD male rats suffering testosterone induced prostate hyperplasia. Prostate hyperplasia was induced by subcutaneous administration of testosterone (3 mg/kg) for 30 days in all groups except the control group. The rats were treated with vehicle Tween-20 (0.2% v/v, p.o.) or finasteride (1 mg/kg, p.o.), p. mirifica (10, 100 or 1000 mg/kg, p.o.), daidzein (10 or 100 mg/kg, p.o.) or genistein (10 or 100 mg/kg, p.o.) before administration of corn oil or testosterone (3 mg/kg); the results showed that orally administered, daidzein increased testosterone levels in testosterone-induced prostate hyperplasia by 11%. However, levels of FSH, LH, triglyceride and HDL were not affected. The zinc content increased significantly and the zinc transporter gen of ZnT4 and ZIP4 highly expressed, suggesting that daidzein plays an essential role in modulating prostate zinc homeostasis. Similarly, the expression of IL-6, AR and ER was significantly reduced, indicating functioning in regulation of prostate growth and that it plays an anti-inflammatory role in preventing BPH.

SCCS comment
This study was performed to investigate effects of p. mirifica in a rat model of testosterone induced prostate hyperplasia. The effects of daidzein reported in this study were limited and 10 mg/kg can be considered as a LOEL.

- ***On female reproductive function***

In the study from Zhang *et al.* (2018), SD pregnant female rats were exposed from Day 6 of gestation to delivery to daidzein at the dose of 50 mg/kg feed via supplemented food. Daidzein supplementation significantly increased the total litter weight and the total viable newborn weight. Daidzein supplementation acutely elevated the concentrations of serum estrogen, progesterone and insulin-like growth factor-1 after the maternal rats' delivery. IgA and IgG were also significantly higher in the DAI than in the CON maternal rats. Daidzein significantly increased the total antioxidant capacity (T-AOC) in maternal rats' sera and in newborns and elevated the concentration of superoxide dismutase (SOD) in both the maternal rats' sera and their ovaries. Daidzein supplementation significantly elevated the expression levels of estrogen receptor beta (ERβ) and NR5A2 genes in maternal rats' ovaries and downregulated the expression level of prolactin receptor (PRLR) in newborns. These results suggest that dietary daidzein supplementation improves reproductive performance and fetal development in rats, which is associated with changes in serum hormones, tissue antioxidant capacity, and expression levels of reproductive-related genes, both in maternal rats and their offspring.

SCCS comment
Based on the results of this study, positive effects of daidzein administered during pregnancy were observed on the female reproductive performance.

In the study from Jeminiwa *et al.* (2020), male Long-Evans rats at 21, 35, and 75 days of age were maintained either on a casein-control diet, soybean meal (SBM), or control diet supplemented with daidzein (200 ppm) and genistein (G + D) for 14 days. Feeding of all isoflavone-containing diets decreased ($P < 0.05$) testicular T concentrations, and more so in the G + D diet group. Interestingly, Esr1 and androgen receptor protein and pituitary Fshβ with Lhβ subunit protein were increased ($P < 0.05$) by the feeding of genistein and G + D diets, but not by the feeding of the daidzein diet. However, daidzein and genistein both caused a concentration dependent inhibition ($P < 0.05$) of T secretion by Leydig cells *in vitro* with IC50 of 184 nM and 36 nM, respectively.

SCCS comment
Only minor effects of daidzein-supplemented diets were reported in this study.

***In vivo* Studies (level 4)**

See section on reproductive toxicity.

SCCS comment on the ED Properties of daidzein
The SCCS has based its assessment on the studies described above and on previous reports published by EFSA (2015) and from the Nordic Council of Ministers (2020).

SCCS overall comments on Endocrine Disrupting Potential of Genistein and Daidzein

Genistein and daidzein have a weak binding affinity to estrogen receptor *in vitro*, whereas significant endocrine-related effects *in vivo* observed in rodents have been reported at high doses only. With the collective view of the available information, the SCCS considers that the toxicological point of departure (PoD) selected for the safety assessment of genistein (see section 3.4) also adequately covers its endocrine-related effects.

3.4. SAFETY EVALUATION (including calculation of the MoS)

Genistein

From the NTP study detailed in section 3.3.5.1, the SCCS identified a LOAEL of 100 ppm (equivalent to 7.4 mg/kg bw/day for male and 10.2 mg/kg bw/day for female rat) as the toxicological point of departure (PoD).

Furthermore, the SCCS identified a NOAEL of 4 mg/kg bw/day as the toxicological PoD for the subcutaneous route from the study by Lewis (2003) that is also detailed in section 3.3.5.1.

MoS calculation based on PoD by the **oral** route:

Amount of product applied daily (g/day)	7.82
Typical body weight of human (kg)	60
Amount of product applied daily (mg/kg bw)	130
Concentration of genistein (%)	0.007
Amount of genistein applied daily (mg/kg bw)	0.0091
Absorption through the skin (%)	50
Systemic exposure dose (SED) (mg/kg bw/d)	0.0046
LOAEL 100 ppm (7.4 mg/kg bw) (mg/kg bw/d)	7.4
Adjusted NOAEL (LOAEL to NOAEL) (mg/kg bw/d)	2.5
Bioavailability (%)	25
Adjusted NOAEL (mg/kg bw/d)	0.617
Margin of Safety ($NOAEL_{adj}$/SED)	**135**

MoS calculation based on PoD by the **subcutaneous** route:

Amount of product applied daily (g/day)	7.82
Typical body weight of human (kg)	60
Amount of product applied daily (mg/kg bw)	130
Concentration of genistein (%)	0.007
Amount of genistein applied daily (mg/kg bw)	0.0091
Absorption through the skin (%)	50
Systemic exposure dose (SED) (mg/kg bw/d)	0.0046
No observed adverse effect level (NOAEL mg/kg bw/d)	4.00
Bioavailability (%)	100
Margin of Safety (NOAEL/SED)	**876**

Because of the large uncertainties associated with oral absorption of genistein in humans, and rapid conjugation of the absorbed genistein aglycone, the SCCS considers that the use of data from subcutaneous studies in this case is also reliable and justified than the data from oral studies, for which 25% oral bioavailability is applied. The SCCS considers that the calculation of margin of safety (MoS) for genistein based on the data from subcutaneous route provides a more reliable indicator of safety, which is also supported by the very conservative estimate based on data from the oral route.

Daidzein

The toxicokinetic data show that metabolism of daidzein is different depending on the route of exposure. After ingestion, gut microflora can metabolise daidzein to equol, which is the isoflavone-derived metabolite with the highest estrogenic and antioxidant activity. The conversion of daidzein into equol takes place in the intestine via the action of reductase enzymes belonging to incompletely characterised members of the gut microbiota. There is however no evidence that equol is produced at a similar level after skin application of daidzein.

Therefore, as for genistein, because of the large uncertainties associated with oral absorption of daidzein in humans, the SCCS considers that the use of data from subcutaneous studies in this case is also more reliable and justified than the data from oral studies. The SCCS considers that the calculation of margin of safety (MoS) for daidzein based on the data from subcutaneous route would provide a more reliable indicator of

safety, which is also supported by the very conservative estimate based on data from the oral route.

Moreover, a significant skin penetration of daidzein would be considered because the SCCS considered appropriate to use the default value of 50% skin penetration (the submitter had proposed 100%).

Two PoDs are derived from the most sensitive endpoint reported in relevant studies:

- A LOEL of 5 mg/kg for the subcutaneous route, derived from Retana–Marquez et al (2016); as the effects reported were not directly associated with the reduction of fertility performances in rats, 5 mg/kg could be considered as a NOAEL.
- A LOEL of 5 mg/kg for the oral route, derived from Talsness *et al.* (2015); as the effects reported were not directly associated with the reduction of fertility performances in rats, 5 mg/kg could be considered as a NOAEL.

1. MoS calculation based on the oral POD

Based on the analysis of the studies described above, the LOEL of 5 mg/kg derived from Talsness et al (2015) is considered as the POD for the calculation of the Mos. As the effects reported were not directly associated to reduce fertility performances in the female rats, this LOEL could be considered as a NOAEL.

Leave-on products with 0.02% Daidzein

Amount of product applied daily:	g/day	=	7.82
Typical body weight of human:	kg	=	60
Amount of product applied daily:	mg/kg bw	=	130
Concentration of Daidzein:	%	=	0.02
Amount of Daidzein applied daily	mg/kg bw	=	0.026
Absorption through the skin	%	=	50
Systemic exposure dose (SED)	mg/kg bw	=	0.013
No Observed Adverse Effect Level (developmental study*, oral, rat)	NOAEL mg/kg bw/d	=	5
Bioavailability oral route	%	=	25
Systemic POD	POD_{sys} mg/kg bw/d	=	5
Margin of Safety adjusted	PODsys/SED	=	96

* ***Talsness et al., 2015***

2. MoS calculation based on the subcutaneous POD

Based on the analysis of the studies described above, the LOEL of 5 mg/kg derived from Retana–Marquez et al (2016) is considered as the POD for the calculation of the MoS. As the effects reported were not directly associated to reduce fertility performances in male rats, this LOEL could be considered as a NOAEL.

Leave-on products with 0.02% Daidzein

Amount of product applied daily:	g/day	=	7.82
Typical body weight of human:	kg	=	60
Amount of product applied daily:	mg/kg bw	=	130
Concentration of Daidzein:	%	=	0.02
Amount of Daidzein applied daily	mg/kg bw	=	0.026
Absorption through the skin	%	=	50
Systemic exposure dose (SED)	mg/kg bw	=	0.013
Low observed adverse effect level (90-day, sc, rat*)	NOAEL mg/kg bw/d	=	5
Bioavailability	%	=	100%
Systemic POD	POD_{sys} mg/kg bw/d	=	5
Margin of Safety	PODsys/SED	=	385

**** Retana Marquez, 2016***

SCCS conclusion
Although the Margin of Safety (MoS) calculated on the basis of oral exposure to daidzein comes out at marginally below 100 (96), it is much higher than 100 (385) when exposure via subcutaneous route is taken into account. Taking into view that the value of 25% used for oral bioavailability is very conservative, and that the subcutaneous route represents the worst exposure scenario, the SCCS considers that the exposure to daidzein in leave-on products at the concentration of 0.02% as safe.

3.5. DISCUSSION

Physicochemical properties

The data and information provided on physicochemical properties of genistein and daidzein is incomplete in certain aspects. This has been highlighted in relevant sections. Full reports of the chemical characterisation in terms of identity, and purity/impurity profiles, in representative batches of genistein and daidzein must be provided, along with documentation of the validity of the analytical methodologies used. The identity and concentration of any impurities of concern that may be present must also be provided.

The aqueous solubility of the isoflavone aglycones is generally very low and is pH dependent due to the acidic nature of the phenolic groups. The solubility values in aqueous media have been reported to be <0.1 g/L, which indicates that both genistein and daidzein are practically insoluble in water.

Data on the stability of genistein and daidzein under the experimental conditions of the reported studies, and under the conditions of use, needs to be provided along with information on any hydrolysis products.

Exposure assessment and Toxicokinetics

Genistein and daidzein are isoflavones used in formulations of leave-on cosmetic products. Both isoflavones belong to polyphenolic category of compounds. The majority of the isoflavone cosmetic ingredients are derived from soybean plants (Glycine max L.). In plants, both genistein and daidzein are largely present in glucoside forms but in cosmetic products they are used in aglycone forms.

In CosIng database, the functions of both genistein and daidzein are reported as 'skin conditioning - Miscellaneous'.

Dermal penetration

The dermal/percutaneous absorption of genistein and daidzein has been reported in studies from open literature. Original study reports were not available for the SCCS evaluation, but the study descriptions indicated that they were not performed according to the SCCS basic criteria for dermal absorption studies (SCCS Notes of Guidance, 2021). The studies, however, indicated that both genistein and daidzein become systemically available after topical application *in vivo* in humans.

In the absence of studies on dermal/percutaneous absorption that are in accordance with the SCCS requirements, the SCCS will use a default value of 50% for dermal absorption of both genistein and daidzein in safety assessments.

Toxicokinetics

The available information on toxicokinetic and metabolism in test animals and humans indicates that isoflavones (including genistein and daidzein) are absorbed and biodistributed in the form of glucuronide conjugates, with only a small proportion as aglycone.

After absorption via the oral route, the glycoside form of genistein is rapidly and extensively metabolised enzymatically (in the gut and liver), and by microorganisms (in the intestine), and therefore the concentration of genistein (aglycone) in plasma in humans is very low. This is an important consideration for safety assessment because the conjugated form has relatively little biological activity.

The available information also indicates that ADME of isoflavones is different between rodents and humans and therefore oral bioavailability values from rodents cannot be extrapolated to humans as such. In humans, studies in volunteers provide some information on the oral bioavailability, based on the percentage recovery in urine of the administrated dose. However, there are limitations in the derivation of oral bioavailability based on this approach because urinary isoflavone concentrations are poorly correlated with maximal serum concentrations, indicating the limitations of urinary measurements as a predictor of systemic bioavailability.

For genistein, the recovery in urine of the administrated dose is reported to vary between 7.7% and 30%. From collective consideration of the available information, the SCCS will use a value of 25% for oral bioavailability of genistein.

Information on human toxicokinetics of daidzein (aglycone) is limited, most studies refer to the isoflavone or glycosidic form of daidzein, which is of limited relevance for the safety

evaluation of daidzein in aglycone form used in cosmetic applications. Based on the data available on the toxicokinetics of daidzein, species differences between rodents and humans have been observed. In humans, it seems that daidzein is well absorbed, but there are no data allowing the derivation of a value for oral bioavailability. After systemic absorption, daidzein undergoes extensive first-pass metabolism, which accounts for its low bioavailability in humans. Because of the insufficient data on oral bioavailability, and on the first-pass metabolism in the gut, the SCCS will use a value of 25% to account for oral bioavailability when MoS calculation is based on oral studies.

Apart from the oral studies, data from studies using subcutaneous administration are also available for both genistein and daidzein, which allow the identification of a point of departure for risk assessment. Due to the uncertainties associated with the oral absorption percentage of daidzein in humans, and rapid conjugation of the absorbed genistein (aglycone), the SCCS considers that the use of data from subcutaneous studies is also justified in these cases, since this provides more reliable information than the data from oral studies.

Toxicological Evaluation

Since this Opinion relates specifically to genistein (CAS No 446-72-0, EC No 207-174-9) and daidzein (CAS No 486-66-8, EC No 207-635-4), the SCCS has considered only those studies of relevance that had used aglycone form of the two substances for this Opinion.

Irritation and corrosivity

Adequate studies on skin and eye irritation have not been provided. However, the SCCS considers that the proposed low level of use (0.007% genistein; 0.02% daidzein) in a final cosmetic product is unlikely to cause skin/eye irritation.

Skin sensitisation

No data on skin sensitisation have been provided, and therefore adequate information on the skin sensitisation of genistein and daidzein should be provided

Acute toxicity

The available information indicates that the acute toxicity of both genistein and daidzein is low.

Repeated dose toxicity

For genistein, a number of repeated dose toxicological studies are available. These have been described in sections 3.3.4, section 3.3.10.1 (under level 5), and in the Annex-A Tables.

For daidzein, one 28-day oral study in rats and one 700day oral study in pigs were described from the open literature but the original study reports were not available to the SCCS. These studies indicate that 100 mg/kg bw/d can be considered as NOAEL in rats after 28-day repeated oral administration of daidzein and 200 mg/kg bw/d in pigs after 70 days exposure to daidzein/kg diet.

Reproductive toxicity

Genistein
The evidence from a multigenerational study in rats indicates that genistein produces developmental toxicity in terms of a transient decrease in the F1 and F3 pup body weights following dietary exposure to 5, 100, and 500 ppm via oral administration.

From the relevant toxicological studies, the SCCS regarded it reasonable to consider that a NOAEL for genistein aglycone is between 5 and 100 ppm. A recent report by the Nordic Council of Ministers (2020) has used 100 ppm as a NOAEL from the NCTR study for pregnant women (equivalent to 8.9 mg/kg bw). However, the same report used 100 ppm as a LOAEL for children (equivalent to 20 mg/kg bw) from a study by Li *et al.* (2014). In view of these, the SCCS considered it pragmatic to use 100 ppm as a LOAEL for the calculation of Margin of Safety (MoS) for genistein in the current assessment.
Daidzein
Only studies published in the literature were used by SCCS, but the original study reports were not available. Based on the observed effects in the most comprehensive study of Lamartinière *et al.* (2002), the SCCS considers 19 mg/kg bw/d as a NOAEL for the effects on reproduction and fertility. Other studies have investigated more specific effects of daidzein that could have impact on the fertility or development. The study from Talsness *et al.*, 2015 focused on the changes in the ovaries of female rats and based on the effects reported by the authors, the SCCS considers 60 mg/kg bw/d as a LO(A)EL and 5 mg/kg bw/d as a NO(A)EL. However, the biological consequences of these effects in terms of adversity are not known. As daidzein has also been associated with an estrogenic mode of action, endocrine potential that may lead to adverse effects needs to be investigated before conclusions can be drawn about the reprotoxicity of this compound (see section 3.3.10)

Mutagenicity / genotoxicity

Genistein

Based on the available studies, genistein shows no evidence for mutagenicity in the bacterial gene mutation test (Ames tests). In contrast, *in vitro* mammalian gene mutation studies using Tk locus, Na+/K+ ATPase or Hprt loci on Mouse lymphoma L5178Y tk+/- cells, human lymphoblastoid cells AHH-1 and L3 or SHE cells show positive results indicating mutagenic potential of genistein *in vitro*. In consideration of several other *in vivo* mammalian gene mutation studies on Big Blue transgenic rats that were negative, the SCCS considers that genistein does not pose a gene mutation hazard *in vivo*.

Studies of in vitro micronucleus/chromosomal aberration with Genistein show clastogenic effect. This is supported by the induction of DNA breaks by genistein as measured in the Comet assay in vitro. The results of studies on in vivo micronucleus/chromosomal aberrations with genistein, however, show no clastogenicity (McClain et al., 2006).

The available results of a low relevance study on sister chromatid exchange *in vivo* with genistein show positive results. Two studies of cell transformation assay were performed, one with positive and another one with negative results. High doses of genistein have shown topoisomerase poisoning properties *in vivo* after subcutaneous administration. These data, however, can only be considered in an overall weight of evidence, which suggests no concern for mutagenicity/genotoxicity of genistein.

Daidzein

Although the available results of the *in vitro* bacterial gene mutation studies as well as of the *in vitro* mammalian cell gene mutation studies do not allow drawing firm conclusions, the SCCS, after analysis of the high relevance *in vivo* mammalian cell gene mutation studies with negative results, considers that daidzein does not pose a gene mutation hazard *in vivo*.

The analysis of the *in vitro* micronucleus/chromosomal aberration studies was inconclusive, and no valid *in vivo* micronucleus/chromosomal aberration study was found in the open literature. However, a new valid *in vitro* micronucleus study on cultured human lymphocytes has been provided by the applicant, showing no mutagenic effect of daidzein.

Considering all the available data on daidzein genotoxicity the SCCS is of opinion that daidzein has no genotoxicity hazard *in vivo.*

Carcinogenicity

Most studies have reported that isoflavones seem to reduce the risk of cancer. However, a few studies have also indicated the opposite tendency.

Genistein
The available evidence from published studies suggests that genistein is not genotoxic *in vivo* and does not exhibit a carcinogenic potential *in vivo*.

Daidzein
Carcinogenicity data are not available for daidzein. However, there is huge amount of literature investigating possible associations between intake of soy isoflavones and various cancer types. These studies have been summarised by various organisations, the most recent ones being EFSA (2015), VKM (2017) and the report from the Nordic Council of ministers from 2020. The relevance of the few studies that found increased risk of cancer after intake of a very high dose of isoflavone supplements, or in occasional comparisons of dietary intake of soy food products in mostly Asian populations, is difficult to interpret in relation to intake of isoflavone supplements in Norwegian pre- and post-menopausal women as well as with respect to cancer in men (VKM, 2017).

Photo-induced toxicity

The original study report of a phtototoxicity study performed was not provided. However, the study appeared to be based on a finished cosmetic product of unknown composition, and therefore it cannot be used for the SCCS evaluation of potential phototoxicity of genistein or daidzein.

Results of a patch test on human volunteers were provided. A full report on the study was not available, but it appeared to be based on a finished cosmetic product of unknown composition, and therefore could not be used for the SCCS evaluation of potential skin irritancy of genistein or daidzein.

Special investigation: assessment of endocrine disrupting potential (including human data)

The ability of isoflavones to interact with oestrogen receptors is attributed to their structural analogy with 17β-oestradiol. The two types of oestrogen receptors have different biological actions. Oestradiol receptor alpha (ERα) is associated with cell proliferation while oestradiol receptor beta (ERβ) has pro-apoptotic and pro-differentiating effects. Isoflavones can bind to both oestradiol receptors, but have a higher affinity for ERβ. In addition to effects of isoflavones on ERα and ERβ, interactions are also known to occur with the oestrogen-related receptors ERRα, ERRβ or ERRγ and with GRP 30, identified as a further oestrogen receptor involved in proliferation of breast cancer cell lines *in vitro*.

Both genistein and daidzein have weak binding affinities to estrogen receptor *in vitro*, whereas any significant endocrine-related effects *in vivo* observed in rodents have been reported only at high doses. With the collective view of the available information, the SCCS considers that the toxicological point of departures (PoDs) selected for this assessment (see section 3.4) also adequately cover the endocrine-related effects of genistein and daidzein.

4 CONCLUSION

1. *In light of the data provided and taking under consideration the concerns related to potential endocrine disrupting properties of phytoestrogens,*

   (c) does the SCCS consider genistein safe when used in cosmetic products up to a maximum concentration of 0.007%?

   (d) does the SCCS consider daidzein safe when used in cosmetic products up to a maximum concentration of 0.02%?

   From the safety assessment based on the available relevant data on the aglycone form of genistein and daidzein, and in consideration of the potential endocrine disrupting properties of phytoestrogens, the SCCS considers that:

   (c) the use of genistein (CAS No 446-72-0, EC No 207-174-9) in cosmetic products up to a maximum concentration of 0.007% is safe.

   (d) the use of daidzein (CAS No 486-66-8, EC No 207-635-4) in cosmetic products up to a maximum concentration of 0.02% is safe.

2. *Alternatively, according to the SCCS what is the maximum concentration of genistein and daidzein that is considered safe for individual and combined use in cosmetic products?*

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3. *Does the SCCS have any further scientific concerns with regard to the use of genistein and daidzein or other related phytoestrogens in cosmetic products?*

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5 MINORITY OPINION

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7. GLOSSARY OF TERMS

See SCCS/1628/21, 11th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 181.

8. LIST OF ABBREVIATIONS

See SCCS/1628/21, 11th Revision of the SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation – from page 181.

ANNEX A - GENISTEIN

Table A1: Bacterial gene mutation assays (Ames test)

Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance	Result	Reliability/ Comments	Relevance of the result	Ref ID_ authors, year
S. typhimurium TA1538, TA98 and TA100	0, 1, 10, 50, 100, 500 µg/plate S9 Aroclor 1254-induced rat livers	Genistein supplied by Dr. B. Knuckles, USDA Western Regional Research Laboratories (Albany, CA).	**Negative** 2-aminoanthracene (2-anthramine) and quercitin, were mutagenic to all 3 strains of S. typhimurium	**2** Quite low concentrations, 3 test strains used, quercetin is not a typical positive control used with S9-mix	**Limited**	Bartholomew and Ryan, 1980 Lack of mutagenicity of some phytoestrogens in the Salmonella/mammalian microsome assay. Mutation Research, 1980, 78: 317-321.
S. typhimurium TA98 and TA100	No data on concentrations used.	Genistein provided by Dr. Tomio Takeuchi, Institute of Microbial Chemistry, Tokyo.	**Negative**	4 No access to original data; no data on concentrations, number of revertants missing, no data on positive or negative controls, on historical controls	Low	Sugimura, T., M. Nagao, T. Matsushima, T. Yahagi, Y. Seino, A. Shirai, M. Sawamura, S. Natori, K. Yoshihira, M. Fukuoka and M. Kuroyanagi (1977) Mutagenicity of flavone derivatives, Proc. Jpn. Acad., 53B, 194-197. Reported also in: Nagao M, Morita N, Yahagi T, Shimizu M, Kuroyanagi M, Fukuoka M, Yoshihira K, Natori S, Fujino T, and Sugimura T. Mutagenicities of 61 flavonoids and 11 related compounds. Environmental Mutagenesis 1981, 3: 401-419.

Bacterial gene mutation test in Salmonella strains in accordance with the OECD test guideline 471.	Reverse mutation assay in Salmonella typhimurium strains TA1535, TA153, TA98 TA100 and Escherichia coli WP2 uvrA. Triplicate plates treated with conc up to 3300 µg/plate in a first experiment using the plate test methods and with up to 1000 µg/plate in a second experiment using pre-incubation method (due to excessive precipitation of Genistein at the higher dose levels). Treatments were performed both in the presence and absence of rat liver S9.	Genistein (batch 15574B-42-2) was a beige powder with a purity of 99.5% and was stored in a refrigerator (below 4 C); protected from light, under nitrogen.	**Negative** **Both + and – S9-mix** Genistein did not show any genotoxic activity in the reverse mutation assay (Ames test) using either the standard plate incorporation method or the pre-incubation method and either in the presence or absence of metabolic activation	**1** Performed according to OECD TG, Not GLP study	**High**	McClain *et al.* (2006)
Bacterial gene mutation test in Salmonella strains	Ames test in Salmonella typhimurium (strains TA98 and TA100) after exposure to genistein under acidic conditions (after a nitrite treatment).	Genistein from Wako Pure Chemical Industries (Osaka, Japan)	**Negative** When Genistein was applied alone, no mutagenicity was observed in the Ames test at 10 µM per plate either in the presence or absence of S9 metabolic activation	**3** Results are of limited value because only one dose tested that was also not sufficiently high (10 µM/plate), and only two S. typhimurium tester strains (TA98 and TA100) were used	**Low**	Masuda *et al.* (2012).

Table A2: *In vitro* mammalian cell chromosomal aberrations/ micronucleus test

Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance	Result	Reliability/ Comments	Relevance of the result	Ref ID_ authors, year
Micronucleus (MN) test without CytB, CREST staining Male Chinese hamster V79 lung fibroblasts Cytotoxicity with DMSO	5, 10, 18, 25, 50, 75uM, Positive controls MMS at 50ug/ml and vincristine (VCR) at 10 nM. A solvent (DMSO) and a medium control were run in parallel for each assay. The cells were treated during 18 h (1.5 cell cycles)	Genistein purchased from Sigma Aldrich Chemical Co., phytoestrogens were prepared in DMSO and stored at −20 ◦C in the dark. Vincristine (VCR), methyl methane sulfonate (MMS), neutral red, acridine orange, propidium iodide and DAPI	**Positive** Genistein caused a clear dose-related induction of MN within the range of 5–25_M; MN rates were declining at higher genistein concentrations. This was probably due to cytotoxicity of genistein since reduced neutral red uptake and MTT formation with an IC50 of about 75_M occurred.	**2** No historical controls	**Limited**	Di Virgilio *et al.*, Toxicology Letters 151 (2004) 151–162
Micronucleus (MN) test without CytB and CREST staining Male Chinese hamster V79 lung fibroblasts Cytotoxicity with sulforhodamine B	5, 10, 18, 25 uM, exposure for 6 h then kept in fresh medium for 6-24 hr. The cells were plated on sterile microscope glass slides in a quadriperm vial (30,000 cells/mL DMEM, 5 mL corresponding to 150,000 cells per slide). 2000 cells per slide were examined for MN (DAPI and PI staining) and CREST signals. Three independent experiments.	Genistein (research grade) from Fluka Chemical Co.	**Positive** Results showed that cells treated with concentrations of Genistein exceeding 25 μM presented signs of toxicity. Genistein induced significant increase in the number of MN in V79 cells. Only CREST-negative MNs were induced Nitroquinoline-N-oxide (0.5 μM, 24 h) induced 4-fold increase in MN frequency.	**3** only 6 h of exposure, no S9-mix used, no data on historical controls	Low	Kulling and Metzler, 1997

Micronucleus assay cultured human peripheral blood lymphocytes	Genistein exposure for 6h at the single concentration of 25µM. After sampling, for analysis, 100 metaphase cells were examined for genotoxicity while the cytotoxicity was assessed by counting the mitotic index in ‰, i.e. number of metaphases per 1000 cells. Three independent experiments	Genistein (research grade) from Fluka Chemical Co	**Positive** Genistein induced structural chromosome aberrations in Human lymphocytes after 6-hour treatment at the lonely concentration studied of 25 µM that induced a mean of ca 55 %reduction of Mitotic Index	**3** Single concentration	**Low**	Kulling *et al.*, (1999).
Micronucleus assay with CYT B in the human lymphoblastoid cells AHH-1 and L3	Cells were exposed to 0.0, 1.0, 5.0, 10.0 and 20.0 µg/mL of Genistein for 24 h.	Genistein, obtained from Sigma St. Louis, MO, was.diluted in DMSO Sigma.	**Positive** Exposure to Genistein resulted in the induction of micronuclei in both cell lines. Differences in p53 functional status affected the results as evidenced by the significant difference in the slopes of the two concentration–response lines ($p \leq 0.0001$). A higher percentage of micronuclei per 500 cells were detected in the L3 cell line than in AHH-	**2** Non GLP Triplicate experiment Positive controls not reported	**Limited**	Morris *et al.* (1998)
In vitro chromosomal aberrations	The effects of Genistein studied in mouse splenocytes in culture.	Genistein	**Positive** Genistein (25 µM) induced the production of large numbers of micronuclei. No clastogenic effect was noted at 12.5 or 2.5µM. noclastogenic effect was noted	**2**	**Low**	Record *et al.* (1995)

In vitro chromosomal aberrations	The effects of Genistein studied in HeLa S3 cells	Genistein	**Positive** The study shows that genistein at 100 μM in HeLa S3 cells in vitro causes abnormal cell division and cleavage furrow regression, resulting in the generation of binucleated cells and hence polyploidy. Moreover, it affects the formation of the central spindle, which is essential for completion of cytokinesis. Its impairment is accompanied by aberrant chromosome segregation, such as a chromosome bridge and lagging chromosomes	**2**	**Limited**	Nakayama *et al.* (2014) (EFSA 2015)
Chromosomal aberrations Primary SHE cell cultures were established from 13-day-gestation fetuses	Genistein (12.5, 25, 50 μM) 24 h treatment Three hours before harvest, colcemid was administered and metaphase chromosomes prepared. 100 metaphases were scored per experimental group.	Genistein from Indofine Chemical Company, Inc. (Somerville, NJ), and was of purity >98% (HPLC and GC/MS)	**Positive** Cytotoxicity: cytotoxic in the highest concentration, only a few metaphases	**2** No positive control but coumestrol used in the same study induced also a high % of aberrant metaphases. Only 100 metaphases were	**Limited**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320
Micronucleus study on protective effect of the ability of soy isoflavones (genistein and daidzein) HTC hepatoma cells	For the genotoxicity evaluation, the cells were treated with 10 μM of genistein. antigenotoxicity, treatment at 0.1, 1 or 10 μM along with either doxorubicine at 0.2 μM	Genistein, CAS 446-72-0, from Acros Organics	**Negative** Genistein at a single dose level of 10 uM, did not induce significant increases in micronuclei. EFSA 201-	**3** limited validity no change in the number of binucleated cells was observed in cultures treated with the test	**Low**	Lepri *et al.* (2013),

from the Cell Bank of Rio de Janeiro (RJCB—Brazil)	or 2-aminoanthrace at 13 µM. 1000 binucleated cells were scored, coded slides 3 experiments (1,000 cells per treatment). Cytotoxicity MTT 24 h.			compound or in solvent-treated cultures owing to selection of an inadequate, i.e. not sufficiently high, dose level. No S9 fraction		
In vitro cytokinesis block micronucleus assay for MCF-7 cells	Genistein dissolved in DMSO or etanol, 24h exposure without S9mix, concentrations 10^{-7}, 10^{-8} M, 2 µg/mL cytochalasin B, Positive control BaP	Genistein was obtained from LC Laboratories (Woburn, MA, USA)	**Negative** Genistein did not induce micronucleated cells	**3** Important experimental details are missing, only 2 concentrations used	**Low**	Nasri and Pohjanvirta, 2021
Micronucleus test L5178Y tk+/− mouse lymphoma cells (clone 3.7.2c)	Genistein only 30 µM Exposure for 5h plus 20h incubation, without S9-mix. Staining with acridine orange. N=3 experiments testing metabolites (on average 82 micronuclei per 1000 cells in 30 determinations).	Genistein (Sigma Chemie GmbH)	**Inconclusive** Genistein did not induce increased frequency of micronuclei up to 100 µM	**2** Study not according to OECD TG and GLP. Only 5 h of exposure without S9-mix was used.	**Limited**	Schmitt E, Metzler M, Jonas R, Dekant W, Stopper H. Genotoxic activity of four metabolites of the soy isoflavone daidzein. Mutation Research **2003**, 542: 43–48

Table A3: *In vitro* mammalian cell gene mutation assays

Test system/ Test object	Exposure conditions (concentration/ duration/metabolic activation	Information on the characteristics of the test substance	Result	Reliability/ Comments	Relevance of the result	Ref ID_ authors, year
Hypoxanthine guanine phosphoribosyltransferase (HPRT) assay Male Chinese hamster V79 lung fibroblasts	The cells were seeded into a 250-mL cell culture flask and incubated for 24 hr. Then the medium was removed and the cells were incubated with GEN for 3 hr in FCS-free DMEM. 6-day expression period Mutation frequency represents the number of 6-TG-resistant mutants per 10^6 viable cells.	Genistein (research grade) from Fluka Chemical Co.	**Negative/Inconclusive** 10 and 25 µM genistein did not cause a significant effect. Concentrations used gave 80 and 60% viability NQO at 0.5 µAM led to a ~8-fold increase in number of mutants.	**3** not according to GLP, only 2 concentrations with low cytotoxicity, only 3 h of exposure, no S9-mix used, no data on historical controls)	**Low**	Kulling and Metzler (1997)
Mammalian cell gene mutation test Mouse lymphoma L5178Y tk+/ conducted in accordance with the OECD guideline TG 476	Four independent exp: two in the absence of S9 (exposure times of 3 and 24 hours) and two in presence of S9 (exposure time 3 hours). In the absence of S9, cells were treated with up to 60 and 7.5µg/ml. In the presence of S9-	Genistein (off white powder, 99.8% purity), batch DIV-132	**Positive** **Both + and –S9-mix** Genistein significantly increased resistant mutant colonies both in the presence and absence of metabolic activation (S9). These were predominantly small colonies, indicating that Genistein acts as a clastogen	**1** OECD TG Not a GLP study, no data on historical controls provided.	**High**	McClain *et al.* (2006). * **

	mix, the experiments were carried out up to a maximum concentration of 6.5 and 7.5 µg/ml.					
Gene mutations in the human lymphoblastoid cells AHH-1 tk+/− (p53+/−) and L3 (p53+/−),	Cells were exposed to 0.0, 1.0, 5.0, 10.0 and 20.0 µg/mL of Genistein for 24h. The mutant fraction at the tk locus determined by resistance to TFT and at Hprt locus by 6-TG.	Genistein, obtained from Sigma St. Louis, MO, was diluted in DMSO Sigma.	**Positive** Genistein induced gene mutation in both cell lines (the cells differ in the functional status of the tumour suppressor gene, p53).	**2** Non GLP Triplicate experiment Positive controls not reported	**Limited**	Morris *et al.* (1998)
The mouse lymphoma assay, Tk locus.	Exposure with genistein concentrations of 2.5–20 µg/ml for 3 or 24 hours in the absence of S9 metabolic activation.	Genistein	**Positive** The results obtained showed statistically significant and dose-related increases in mutation frequencies at both treatment times. The authors concluded that the induced mutations were mainly of hemizygous type caused by loss of heterozygosity at the TK locus. the relative total growth in the solvent control was rather low,	2 The relative total growth in the solvent control was rather low, thus limiting the strength of the test	**Limited**	Zou *et al.* (2012) EFSA 2015
Gene mutations at the Na+/K+ ATPase or hprt loci SHE cell cultures were established from 13-day-gestation fetuses	Cells treated with genistein and other phytoestrogens or B[a]P for 48h gene mutations at the Na+/K+ ATPase or hprt loci (resistance to Oua or TG).	Genistein from Indofine Chemical Company, Inc. (Somerville, NJ), and were of purity >98% (HPLC GC /MS)	**Positive** At 12.5 and 25 µM positive, dose response, 50 µM was too toxic; mutation frequencies were increased at the Na+/K+ ATPase locus as well as Hprt	**2** Study not according to OECD TG and GLP. Not clear how the MF was calculated in both tests, no data were provided on negative historical controls. B[a]P, a known indirect mutagen was	**Limited**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer

	Expression time: 4 days; plus 7 days for colony formation			apparently used without S9-mix.		**2003**, 105: 312–320

Table A4: *In vitro* DNA damage (e.g. Comet assay, Alkaline elution)

Test system/ Test object	**Exposure conditions (concentration/ duration/metabolic activation**	**Information on the characteristics of the test substance**	**Result**	**Reliability/ Comments**	**Relevance of the result**	**Ref ID_authors_year**
DNA breaks by the alkaline filter elution (Kohn *et al.*, 1981).	3 x 10^6 V79 cells per vial plated in 650-mL cell culture flasks were grown for 48 h and then exposed to daidzein for 6 h. Eluted fractions from filters were analysed for DNA by the Hoechst 33258 fluorometric method.	Genistein (research grade) from Fluka Chemical Co.	**Positive** No detailed data were provided in the paper. Information provided only in abstract but not mentioning in the paper.	**3** Insufficient reliability	Low No information available.	Kulling and Metzler (1997)
DNA breaks by the comet assay Male Chinese hamster V79 lung fibroblasts Cytotoxicity with DMSO	3h exposure, 100, 250 and 500 uM, Genistein Positive controls H2O2 Neutral red and MTT – 24h treatment	Genistein purchased from Sigma Aldrich Chemical Co., phytoestrogens were prepared in DMSO and stored at −20 ◦C in the dark.	**Negative** DNA breakage only at concentration inducing cytotoxic response after 24h treatment. No cytotoxicity test for 3h exposure.	**2** Reliabille with restriction Not OECD test guideline	**Limited**	Di Virgilio *et al.*, Toxicology Letters 151 (2004) 151–162 Referred In VKM report, 2017.

DNA damage by the comet assay in leukocytes from mucopolysaccharidosis IVA patients	Five MPS IVA patients with a mean age of 20.2 years, Leucocytes were treated 6h with 10, 30 and 50µM genistein using PBS buffer and DMSO. The proportion of blood and buffer +DMSO/genistein solution used was 3:1.	The isoflavone (SigmaAldrich®; batch 071M1606V) was dissolved in DMSO (Sigma Aldrich®) and a stock solution (280µM of genistein and 0.05% of DMSO) was frozen (−20 °C) until blood samples incubation	**Positive** Increase in DNA damage	**3** Blood from patients and not healthy controls No positive control	**Low**	Negretto *et al.* (2014)
In vitro alkaline Comet assay human lymphocytes	Purified genistein, human lymphocytes treated at 10 to 50 µM. The test compound was applied directly onto gelatinised slides.	Purified genistein, from Sigma (St. Louis, MO)	**Positive** Dose-related and statistically significant increases in DNA breakage (increased tail length in the comets). The authors concluded that the clastogenic activity of both compounds was caused by their pro-oxidant activity, mediated by copper and not by iron and zinc, as supported by the action of copper chelators. In addition, the authors asserted that these compounds have antioxidant activity.	**2**	**Limited**	Ullah *et al.* (2009) EFSA 2015 (VKM report, 2017).

Antineoplastic action in primary. Thyroid tissues were.	Human papillary thyroid cancer (PTC) cells treated with Genistein (1-10-50-100 µM). Cell viability, proliferation, DNA primary damage and chromosomal damage were evaluated.	Purified genistein, from Sigma (St. Louis, MO)	**Negative** Antiproliferative effect was induced by the highest doses. Comet assay did not show DNA damage at any of the times (4 and 24 h) and doses tested. A reduction of hydrogen peroxide-induced DNA primary damage in primary thyrocytes from PTC cells pretreated with Genistein was observed. Data suggests that Genistein exerts antineoplastic action, does not induce genotoxic effects while reduces oxidative-induced DNA damage in primary thyrocytes from PTC cells	**2**	**Limited**	Ullah *et al.* (2009)
Comet assay HT29 clon 19 And Primary colon cells	30 min exposure with genistein 12.5-100 M (HT29 cells), 12.5-50 µM primary colon mucosa cells In some experiments End III for detection of oxidised bases was used 3 experiments	Genistein synthesised by authors	**Positive** Increase in strand breaks Dose response in HT29 cells (12.5-100 µM), **Negative** In primary colon mucosa cells no SBS, no DNA oxidation (12.5-50 µM)	1 No historical controls, short exposure	**High/Limited**	Pool-Zobel BL, H. Adlercreutz, M. Glei, U.M. Liegibel, J. Sittlingon, I. Rowland, K. Wahala, G. Rechkemmer, Isoflavonoids and lignans have different potentials to modulate oxidative genetic damage in human colon cells, Carcinogenesis 21 (2000) 1247-1252.

Comet assay on human sperm cells Isolated peripheral blood leukocytes (1 male and 1 female donors)	Genistein concentrations: 0, 1, 10, 25, 50, 100, µM Lymphocytes treated for 0.5 h: 2 separate studies Sperm cells treated for 1h: 3 separate studies COMET 3.0 (Kinetic Imaging Ltd., Liverpool, UK) was used to measure comet parameters.	Genistein from Sigma Chemical Co. (Gillingham, Dorset, UK)	**Positive** Increased DNA damage from 50 µM in both donors in female human lymphocytes Decreased % head DNA in human sperm both after H2O2 as well as genistein The effect of genistein stronger in sperm compare to lymphocytes	**2** Only 30 mint treatment Tail moment for the lymphocytes was used.	**Limited**	Anderson D, Dobrzynska MM, and Basaran N. Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the comet assay. Teratogenesis, Carcinogenesis, and Mutagenesis, **1997**, 17:29–43
Comet assay Isolated lymphocytes from a healthy donor. Semen sample from another healthy donor.	Comet: lymphocytes treated for 30 min at 37°C, semen treated for 1 hr at 37°C to genistein Viability checked by Trypan blue exclusion. Analysis with Komet 4.0; Kinetic Imaging, Liverpool, U.K.). Tail moment values for lymphocytes and % head DNA for sperm were determined.	Genistein (CAS 446-72-0) From Sigma Aldrich	**Positive** Genistein, as well as H2O2, induced significant increases in tail moments in the lymphocytes and significant decreases in % head DNA in human sperm.	**2** Short treatment Tail moment	**Limited**	Cemeli E, Schmid TE, and Anderson D. Modulation by flavonoids of DNA damage induced by estrogen-like compounds. Environmental and Molecular Mutagenesis **2004**, 44: 420 –426

In vitro alkaline Comet assay HT-29 cells	HT-29 colon cancer cells treated with 2 to 200 µM for 1 or 48 hours.	Genistein was obtained as a generous gift from the National Cancer Institute (NCI) (R.K. Varma).	**Positive** Dose-related increases in DNA breakage from 10 to 100 µM following 1 hour of treatment. After a 48-hour treatment, marked DNA breakage was only observed at 100–200 µM. The authors concluded that DNA breakage induced by genistein in HT-29 colon cancer cells was the result of poisoning of topo II through the stabilisation of the cleavable complex	**2**	**Limited**	Salti *et al.* (2000) Also in EFSA, VKM report, 2017).
In vitro alkaline Comet assay	Thyroid tissues were treated with Genistein (1-10-50-100 µM). Exposure 4 and 24h. Cell viability, proliferation, DNA primary damage and chromosomal damage were evaluated.		**Negative** An antiproliferative effect was induced by the highest doses of Genistein. Comet assay did not show any genotoxic effect in terms of primary DNA damage at all the times (4 and 24 h) and tested doses. A reduction of hydrogen peroxide-induced DNA primary damage in primary thyrocytes from PTC cells pretreated with Genistein was observed. Data suggest that Genistein does not induce genotoxic effects while reduces oxidative-induced DNA damage in primary thyrocytes from PTC cells.	**2**	**2**	Ferrari et al – 2019 Genotoxicity Evaluation of the Soybean Isoflavone Genistein in Human Papillary Thyroid Cancer Cells. Nutrition and Cancer, Volume 71, 2019 doi.org/10.1080/01635581.2019.1604004

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Table A5: Other *in vitro* assays

Test system/Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance	Result	Reliability/ Comments	Relevance of the result	Ref ID_ authors_year
Inhibitory effect of genistein on DNA topoisomerases I and II (topo I and II) isolated from HCT116 human colon carcinoma	Potential inhibitory activity of three soy isoflavones, daidzein, genistein and glycitein, and their glycosides, daidzin, genistin and glycitin, on purified DNA topoisomerases I and II (topo I and II) from human placenta which generate DNA single- (ss) and double-strand (ds) breaks respectively. The catalytic activity of both topo I and II was evaluated by detecting supercoiled plasmid DNA (form I) in its nicked state (form II).	Genistein purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA;	**Positive for TopoII** **Topo I:** Neither genistein nor other isoflavones influenced the topo I nicking activity at 100 uM and greater. **TopoII** Genistein at 100 uM inhibited the nicking activity of topo II, (not daidzein) Genistein interacts directly with the enzyme by the interaction of GEN with DNA double-strand breaks through its thermal transition. Hypothesisis: genistein induces arrest of cell proliferation.	**2** Conclusion is mostly based on thermal profiles of the transition of ds DNA to ss DNA with or without genistein. However, the evidence that genistein suppresses growth of HCT116 cells in a dose-related way indicate a true cell cycle-inhibitory effect.	**Low**	Mizushina *et al.* (2013)

Cell transformation assay (CTA) SHE cell cultures were established from 13-day-gestation fetuses	Cells treated with genistein and other phytoestrogens or B[a]P for 48 hr.	Phytoestrogens, including genistein obtained from Indofine Chemical Company, Inc. (Somerville, NJ), and were of purity >98% (HPLC and gas chromatography/mass spectrometry)	**Positive** Significant at 12.5 and 25 µM, 50 µM was too cytotoxic CTA - positive control B[a]P at 4 µM had less transformed colonies than genistein at 25 µM.	**1**	**High**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320
Cell transformation assay (CTA) standard 7 day exposure SHE assay	Genistein tested (together with metaproterenol hemisulfate, p-anisidine, resorcinol, rotenone and benzo[a] Pyrene) at 2-4 µg/ml (pH 6.7, maximum concentration was limited by cytotoxicity). Treatment continuous 7 days	Genistein (100% pure), were purchased from Sigma.	**Negative** Positive control BaP was positive	**1**	**High**	Harvey, J. S., Howe, J. R., Lynch, A. M., & Rees, R. W. (2005). The results of five coded compounds: genistein, metaproterenol, rotenone, p-anisidine and resorcinol tested in the pH 6.7 Syrian hamster embryo cell morphological transformation assay. Mutagenesis, 20(1), 51–56. https://doi.org/10.1093/mutage/gei009.

DNA adducts by ^{32}P-postlabeling SHE cell cultures were established from 13-day-gestation fetuses	Genistein 12.5, 25 and 50 µM for 24 h co-chromatography	Genistein from Indofine Chemical Company, Inc. (Somerville, NJ), and was of purity >98% (HPLC and GC/MS)	**Positive** DNA adducts induced by genistein in concentration response.	**1**	**High/limited**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320

Table A6: *In vivo* chromosome aberrations/ micronucleus test (OECD TG 474)

Test system/Test object	**Exposure conditions (concentration/duration)**	**Information on the characteristics of the test substance**	**Result**	**Reliability/ Comments**	**Relevance of the result**	**Ref ID_ authors_year**
In vivo micronucleus test in mice and rats	3 independent *in vivo* bone marrow micronucleus tests performed in Moro mice treated orally for 14 days at doses 0.2, 2 and 20 mg/kg bw/day, in RAIf rats (500, 1000 and 2000 mg/kg in aqueous carboxymethylcellulose 0.5%) and Wistar rats treated orally once at single dose 2000 mg/kg (with harvests at 24 and 48 h). Blood levels of total Genistein measured in the micronucleus test conducted in male and female Wistar were of 40,313 and 24,940 nmol/L, respectively	Mice exp and RAIf rats: Genistein was a faint brownish, fine crystalline powder batch: 604 01B-1995 (98.33% purity) Exp with Wistar rats Genistein was a solid white powder with a 97.7% purity (batch 6241/GST/20/M30/34/001)	**Negative** Genistein did not induce micronucleated cells in 3 independent in vivo bone marrow micronucleus tests performed.	**1**	**High**	McClain e*t al.* (2006) * **
In vivo micronucleus test in mice	The effects of Genistein was studied in Female C57BL/6J mice (approx. 8 wk of age) dosed orally with 20 mg Genistein/kg body weight/day for 5 days (approximately equivalent to a 70 kg human consuming 2.8 kg soybeans/day).	Genistein	**Negative** There was no observable increase in the micronucleus frequency even though the plasma Genistein levels in the treated animals were found to be 9.2 ± 2.0 µM in treated animals	2 One treatment dose was used.	**Limited**	Record *et al.* (1995)

In vivo induction of chromosomal aberration in male mice	The genotoxic properties of Genistein were investigated when administered alone or after a nitrite treatment under acidic conditions in an in vivo micronucleus test in peripheral blood cells of ICR male mice.	Genistein from Wako Pure Chemical Industries (Osaka, Japan)	**Negative** no genotoxicity was observed when Genistein was applied alone to male mice at a single dose of 2 mmol/kg bw by oral gavage for 24 or 48 hours.	**3** The use of a single dose level, not sufficiently high to cause any reduction in the ratio of mature to immature erythrocytes. the number of cells scored was low	**Low**	Masuda *et al.* (2012).

Table A7: *In vivo* DNA damage/Comet assay

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance	Result	Reliability / Comments	Relevance of the result	Ref ID_ authors_year
Comet assay with Fpg modification on stomach mucosa cells Effects of co-administratio of genistein with NaNO2	Genistein dissolved in saline – oral admin at dose of 1 mg/kg bw (mice N=5 per group) and 10 mg/kg NaNO2 FPG-modified comet assay 3h with genistein alone and 1, 3 and 6h after co-administration with NaNO2 Tail moment Measurement of nuclear 8-oxodG SOD, histopatology	Genistein from LKT Laboratories Inc. (St. Paul, MN, USA).	**Negative** Single administration 3h exposure negative. **Positive** Co-administration of genistein and NaNO2 significantly increased DNA damage with and without FPG	**3** Only one, rather low dose of genistein was used, only 3 h exposure time used, no positive control substance used but positive response demonstrated with co-exposure, tail moment	**Low**	Toyoizumi T, Sekiguchi H, Takabayashi F, Deguchi Y, Masuda S, Kinae N. Induction effect of coadministration of soybean isoflavones and sodium nitrite on DNA damage in mouse stomach. Food and Chemical Toxicology **2010**, 48: 2585–2591

Table A8: *in vivo* gene mutation and other assays

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance	Result	Reliability/ Comments	Relevance of the result	Ref ID_ authors_year
p53-/- Mouse Tumorigenesis Assay, male female mice	40 male and 40 female p53-/- mice (4 to 6 weeks old; Taconic Transgenics, Germantown, NY) 20 control diet and 20 mice with diet containing 0.04% genistein (»50 to 60 mg/kg bw/day) for appr 100 days Tissues (brain, salivary gland, nasal structures, mandibular and mesenteric lymph nodes, pancreas, pituitary, adrenal gland, thyroid, parathyroid, trachea/esophagus, thymus, liver, gall bladder, spleen, lung, heart, right abdomi- nal skin with mammary glands, stomach, jejunum, colon, duo- denum, ileum, cecum, rectum, urinary bladder, kidney, femur, testicle/epididymis, seminal vesicle, bladder/prostate or ovary, uterine horn, sampled for histopathology	Genistein was a gift from Dr. Rao Vishnuvajjala (Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI), and was >97% pure.	**Negative** No difference from control mice, no gender differences	**2** No positive control or historical controls	**Limited**	Misra *et al.* (2002) * **
In vivo gene mutation in rat Female Big Blue rats	To investigate whether endogenous estrogen and supplemental Genistein affect DMBA-induced mutagenesis in rat liver Female Big Blue rats were treated with 80 mg DMBA/kg body weight. Some of the rats also received a supplement of 1,000 ppm Genistein (around 20 mg/day). Sixteen weeks after the carcinogen treatment, the rats were sacrificed, their livers were removed, and mutant frequencies (MFs) and types of mutations were determined in the liver cII gene.		**Negative for genistein** DMBA signif increased the MFs in liver for both the intact and ovariectomized rats. Molecular analysis of the mutants showed that DMBA induced chemical-specific types	**1**	High	Chen *et al.* (2005)

			of mutations in the liver cII gene The results suggest that endogenous ovarian hormones have an inhibitory effect on liver mutagenesis by DMBA, whereas dietary Genistein does not modulate spontaneous or DMBA-induced mutagenesis in either intact or ovariectomized rats.			
Gene mutations in female (3-wk-old) Big Blue transgenic rats lacI Mutagenesis Assay in **uterine** cells	Different treatment groups (N=5 rats for mutagenesis study and N=10 rats for carcinogenesis study per group) and fed diets containing 250 ppm or 1,000 ppm genistein, a combination of 1,000 ppm daidzein and 1,000 ppm genistein until termination of the experiments. At the same time, 2 sets of rats were gavaged with a single dose (1 ml) of 80 mg DMBA/kg, and the other 2 sets were gavaged with an equal volume of sesame oil. At 9 wk of age, 1 set of rats from DMBA treatment or vehicle control was bilaterally ovariectomised. The uteri were harvested and weighed for lacI Mutagenesis Assay at 23 wk of age (16 wk following DMBA treatment) and at 27 wk (20 wk following DMBA treatment) for carcinogenesis study.	Genistein (lot 1-FSS-31–1) from Toronto Research Chemicals (Toronto, Canada).	**Negative** **for genistein** genistein (or daidzeinI + genistein) in diet at 250 or 1000 ppm did not significantly modify the mutagenicity of DMBA or spontaneous MF in the uterus. In rats fed either daidzein diet alone the	**1**	**High**	Aidoo A, Bishop ME, Shelton SD, Lyn-Cook LE, Chen T and Manjanatha MG. Effects of Daidzein, Genistein, and 17β-Estradiol on 7,12-Dimethylbenz[a]anthracene-Induced Mutagenicity and Uterine Dysplasia in Ovariectomized Rats. Nutrition and Cancer **2005**, 53, 1: 82-90

	Proliferating Cell Nuclear Antigen/apoptosis analysis in uterine tissues and histopathological analysis were performed. Based on consumption calculations, a dose of 1,000 ppm daidzein or genistein, the dose fed to represents ~20 mg of daily ingested isoflavones per rat; (estimated daily dose of 20–50 mg/day consumed per person in Asian populations).		lacI MF in the uterus was not statistically significant compared with the MF in the rats fed the control diet. With the exception of a reduced DMBA lacI MF seen in the rats fed the isoflavone mixture, dietary daidzein or genistein resulted in variable changes in lacI MFs in both OVX and INT rats; however, the responses were not statistically significant.			
Gene mutations in female (3-wk-old) Big Blue transgenic rats lacI Mutagenesis Assay in **mammary gland** cells	Different treatment groups (N=5 rats for mutagenesis study and N=10 rats for carcinogenesis study per group), and fed diets containing 250 ppm or 1,000 ppm genistein, a combination of 1,000 ppm daidzein and 1,000 ppm genistein until termination of the experiments. At post-natal day 50 (PND50), 2 sets of rats were gavaged with a single dose (1 ml) of 80 mg DMBA/kg, and the other 2 sets were gavaged with an equal volume of sesame oil. After 2 weeks post-DMBA treatment, rats were divided into two groups, and were either bilaterally	Genistein (lot 6-ECGW-83-2) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	**Negative for genistein** Genistein in diet at 250 or 1000 ppm did not significantly induced mutation, neither modified the mutagenicity of DMBA or spontaneous	**1**	**High**	Manjanatha MG, Shelton S, Bishop ME, Lyn-Cook LE and Aidoo S. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz[a]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic

	ovariectomized (OVX), or left untreated (INT). The mammary glands were harvested and weighed for lacI Mutagenesis Assay at 23 wk of age (16 wk following DMBA treatment) and at 27 wk (20 wk following DMBA treatment) for carcinogenesis study. Proliferating Cell Nuclear Antigen/apoptosis analysis in mammary gland tissues and histopathological analysis were performed.		MF in the mammary glands			rats. Carcinogenesis **2006**, 27, 12: pp.2555–2564
Gene mutations in female (3-wk-old) Big Blue transgenic rats ***Hprt gene*** mutagenesis assay in lymphocytes isolated from the spleen and cultured for 2 days	Different treatment groups (N=5 rats for mutagenesis study and N=10 rats for carcinogenesis study per group), and fed diets containing 250 ppm or 1,000 ppm genistein, a combination of 1,000 ppm daidzein and 1,000 ppm genistein until termination of the experiments. At post-natal day 50 (PND50), 2 sets of rats were gavaged with a single dose (1 ml) of 80 mg DMBA/kg, and the other 2 sets were gavaged with an equal volume of sesame oil. After 2 weeks post-DMBA treatment, rats were divided into two groups, and were either bilaterally ovariectomized (OVX), or left untreated (INT). The mammary glands were harvested and weighed for lacI Mutagenesis Assay at 23 wk of age (16 wk following DMBA treatment) and at 27 wk (20 wk following DMBA treatment) for carcinogenesis study. Proliferating Cell Nuclear Antigen/apoptosis analysis in lymphocytes from spleen and histopathological analysis	Genistein (lot 6-ECGW-83-2) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	**Negative** **for genistein** Genistein in diet at 250 or 1000 ppm did not significantly induced mutation, neither modified the mutagenicity of DMBA or spontaneous MF in lymphocytes.	**1**	**High**	Manjanatha MG, Shelton S, Bishop ME, Lyn-Cook LE and Aidoo S. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz[a]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic rats. Carcinogenesis **2006**, 27, 12: pp.2555–2564
In vivo gene mutation in rat on Big Blue rats	The study examined whether or not dietary Genistein (or 17 β-estradiol, E2) modulates the lacI mutant frequency (MF) in the heart of ovariectomized (OVX)	Genistein (lot 6-ECGW-83-2) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic	**Negative** Genistein and E2 supplementation alone	**1**	High	Manjanatha *et al.* (2005).

	Big Blue rats exposed to the model carcinogen DMBA. Female rats were administered 80 mg/kg DMBA or vehicle by gavage and were chronically fed with diets containing 0, 250, or 1000 µg/g Genistein or 5µg/g E2. Sixteen weeks after carcinogen treatment, the animals were sacrificed, and the hearts were removed and processed for determining the frequency and types of mutations in the heart tissue.	resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	resulted in non-significant increases in MF. Genistein in the diet had no significant effect on DMBA mutagenicity			
Sister chromatid exchange test in bone marrow Female ICR mice DNA adducts 32P postlabeling In liver and mammary glands	Two groups of 5 mice were pretreated i.p. with: (i) DMSO (solvent controls); (ii) genistein (iii) daidzein; or (iv) a combination of daidzein and genistein. One of the two identically pretreated groups later received DMBA (50 mg/kg) treatment and the other received only the solvent, DMSO. Genistein: 10 or 20 mg/kg daily for 6 days with the last dose given just before 5-bromodeoxyuridine (BrdU) treatment, or 50 mg/kg at 12 h intervals for 3 days with the 5th dose given just before BrdU treatment.	Genistein from Indofine Chemical Company, Inc. (Somerville, NJ).	**Positive** 20 mg/kg genistein or daidzein alone, or a combination of daidzein and genistein (total 100 mg/kg) significantly increased SCE by 22%, 27%, and 42%, respectively. Among the DMBA-treated groups, pretreatment with genistein alone, but not with daidzein alone or a combination of daidzein and genistein caused a significant decrease (17%, P = 0.005) in DMBA induced SCE.	**2** No historical control values provided, what hampers evaluation of the results.	**Limited** SCE test is not recommended for regulatory purposes.	A K Giri [1], L J Lu Genetic damage and the inhibition of 7,12-dimethylbenz[a]anthracene-induced genetic damage by the phytoestrogens, genistein and daidzein, in female ICR mice Cancer Lett . 1995 Aug 16;95(1-2):125-33. doi: 10.1016/0304-3835(95)03877-y.

			Pretreatment with genistein reduced DNA adducts in both organs			
Genistein is able to affect topoisomease e II in vivo. Juvenile male wistar rats	Wistar rats received either a single dose of genistein subcutaneously (s.c.; 10 mg/kg BW) or a lifelong isoflavone-rich diet encompassing in utero, lactation phase and 10 days of oral consumption, whereas genistein was mainly taken up as glycosides (25–50 mg/kg BW of covalent topoisomerase II–DNA-complexes in the duodenum and colon were measured using the "Isolation of in vivo complexes ofenzyme to DNA" (ICE)-bioassay	Genistein, 99% purity from LC Laboratories Woburn, MA, USA	Genistein significantly increased the amount of covalent topoisomerase IIa and b-DNA complexes in the gut, showing more persistent effects in the colon than in the duodenum. Slight increase of topoisomerase IIa–DNA-complexes in the colon. The differences between the exposure routes might be attributed to the higher serum concentration of the genistein aglycon after subcutaneous treatment probably due to circumvention of first-pass metabolism compared to oral			Baechlar *et al*. (2016)

			consumption of an isoflavone-rich diet.			

* more than one assay is investigated/indicates when papers belong to more than one Table
** both in vitro and in vivo assays are investigated

Table A9: NTP TR 539 Multigenerational Reproductive study in rats - (Table 67 on Developmental Toxicity Studies in Orally-Exposed Rats from Rozman *et al.*, 2006)

Effect levels, mg/kg bw/day								
Genistein doses and study design	**Most sensitive endpoints and generation**	**NOEL/ NOAEL**	**LOEL/ LOAEL**	**BMD10 [a]**	**BMDL10**	**BMD 1 SD**	**BMDL1 SD**	**Reference**
Sprague-Dawley dams were fed diet containing 0 or 5 ppm genistein from GD 17 throughout the lactation period up to PND 70 in offspring. **[Exposure in offspring estimated at ~0.68 mg/kg bw/day over the lifetime.]**	Changes in ovarian histology at PND 21 and 70		0.68b					Awoniyi *et al.* (1998)
Long-Evans, 0 or 15 mg/kg bw by gavage on GD 14 to PND 21.	Uterine histomorphometry endpoints	15						Hughes *et al.* (2004)
Pregnant Sprague-Dawley rats were fed diets containing 0, 20, or 100 ppm genistein **[0, 20, or 87 mg/kg bw/day]**.	↑Anogenital distance	20	87	54	34	54	34	Casanova *et al.* (1999)
	↓Weight at vaginal opening,	20	87	55	36	59	36	
	↑Uterus weight on PND 21	20	87	5	3	24	17	
	↑Relative testis weight on PND 21	20	87	171	103	64	38	
	↑Relative testis weight on PND 56	20	87	180	81	118	54	
	↓Vental prostate weight	20	87					
Sprague-Dawley, 0, 5, 25, 100, 250, 625, and 1250 ppm through diet during pregnancy and lactation and until PND 50	↓Dams delivering litters; delayed eye opening	Pregnancy: 34	83					Delclos *et al.* (2001)

in offspring. **[Mean doses: 0.31, 1.7, 5.7, 15, 34, 83 mg/kg bw/day in pregnant dams; 0.56, 2.8, 11, 30, 73, 138 in lactating dams; ~0.6, 3.0, 12, 30, 72, and 180 mg/kg bw/day in pups after weaning.]**

	Lactation: 73	138				
	Pup: 72	180				
Accelerated vaginal opening	Pregnancy: 34	83	85	83	32	26
	Lactation: 73	138	141	138	68	55
	Pup: 72	180	184	180	67	55
↓Relative ventral prostate weight at PND 50	Pregnancy: 34	83	32	22	37	25
	Lactation: 73	138	68	48	79	53
	Pup: 72	180	67	47	78	53
↑Relative vaginal weight	Pregnancy:		6	17	80	35
	Lactation:		13	36	132	78
	Pup:		13	36	173	77
Histopathology in ovaries, uterus, and vagina at PND 50	Pregnancy: 34	83				
	Lactation: 73	138				
	Pup: 72	180				
Prostate inflammation	Pregnancy: 34	83				
	Lactation: 73	138				
	Pup: 72	180				

	Alveolar proliferation in mammary of females at PND 50	Pregnancy: 5.7	15					
		Lactation: 15	30					
		Pup: 12	30					
	Hypertrophy of mammary alveoli and ducts in males at PND 50c	Pregnancy: 1.7	5.7					
		Lactation: 2.8	11					
		Pup: 3.0	12					
	↓Postnatal body weights (females)	Pregnancy: 34	83	6	48	28	17	
		Lactation: 73	138	13	102	59	36	
		Pup: 72	180	13	102	58	36	
Sprague-Dawley 0, 5, 100, 500 ppm (males: 0, 0.3, 7, 35 mg/kg bw/day; females: 0, 0.4, 9, 44 mg/kg bw/day; females during lactation: 0.7, 15, and78 mg/kg bw/day) in diet, multi-generational design.	↓Live pups (F2 females)	Sire: 7	35	9	7	32	23	NCTR, 2005
		Dam: 9	44	12	9	41	29	
	↓Pup weight at birth, F5 (no exposure)	–	Sire: 0.3	183	37	169	38	
			Dam: 0.4	236	47	217	47	
	↓Anogenital distance in males and F1 females	Sire: 7	35	82	40	57	28	
		Dam: 9	44	46	46	54	30	
	↓Pup weight during lactation (F1 males)	Sire: 0.3	7	27	20	30	22	
		Dam: 0.4	9	35	26	39	28	

	↓Body weight at vaginal opening	Sire: 7	35	20	11	35	26	
		Dam: 9	44	25	15	44	33	
	Disrupted estrous cycles following vaginal opening	Sire: 7	35					
		Dam: 9	44					
	Mammary gland hyperplasia in males (F1, F2, F3)	Sire: 0.3	7					
		Dam: 0.4	9					
	Accelerated vaginal opening (F1)	Sire: 7	35	36	29	37	35	
		Dam: 9	44	46	38	47	44	
CD®SD IGS, 0 or 1250 ppm in diet **[mean 147 mg/kg bw/day]** from GD 15 to PND 11.	Decreased litter size, disrupted estrous cycles, endometrial, vaginal and mammary hyperplasia, and atretic ovarian follicles.	–	147					Takagi *et al.* (2004)
Sprague-Dawley, 0 or 5 (n = 16) ppm in feed **[0.12 mg/kg bw/day]** in feed from GD 17 to PND 21.	Transient decreases in serum LH and testosterone on PND 21 and ↓testis and epididymis weight in adulthood	–	0.12b					Roberts *et al.* (2000)
Sprague-Dawley, 0, 5, 100, or 500 ppm in diet during pregnancy and lactation; half of offspring were given control diets at weaning and evaluated in adulthood; multigenerational design. [Intakes assumed to be similar to those in NCTR,	↑Serum testosterone levels in F1 males	Male: 7	35					Dalu *et al.* (2002)
		Female: 9	44					

2005) of which this study was a part.]								
Sprague-Dawley, 0, 300, or 800 ppm genistein in diet during pregnancy and lactation and up to PND 90 in offspring; **[mean exposures: 25 and 53 mg/kg bw/day in dams and 30 and 84 mg/kg bw/day in pups.]**	↓Birth weight of female offspring	–	25	812 ppm	765 ppm	751 ppm	378 ppm	You *et al.* (2002a)
	Accelerated vaginal opening	–	Dam: 25					
			Pup: 30					
	Lower body weights during lactation (values for females given)	Dam: 25	53	50	25	50	27	
		Pup: 30	84	60	30	60	32	
CD®SD IGS, 0, 20, 200, or 1000 ppm in diet **[mean: 1.7, 18, and 90 mg/kg bw/day]** from GD 15 to PND 10.	↓Body weight gain in males on PND 21–42	18	90					Masutomi *et al.* (2003)
Sprague-Dawley, 0, 25, or 250 ppm in diet (2.2 and 22 mg/kg bw/day) during gestation and lactation, male offspring were fed same diets as dams from PND 21–70.	↑Serum testosterone	–	2.2	13	6	29	14	Fritz *et al.* (2002b)
Long-Evans, 0, 5, or 300 ppm in feed during pregnancy and lactation **[~mean of 3 and 150 mg/kg bw/day, although there is some uncertainty due to an apparent error by authors.]**	↓Testis size, delayed preputial separation, and compromised mating performance	–	3b					Wisniewski *et al.* (2003)
	↑Prostate weight on PND 70	–	3b	240	70	282	142	
	↓Plasma testosterone on PND 70	–	3b	76	27	302	66	

Sprague-Dawley, 0, 12.5, 25, 50, or 100 mg/kg bw/day by gavage on PND 1–5.	Lower body weights of males at week 18	–	12.5	78	52	112	73	Nagao *et al.* (2001)
	Lower body weights of females at week 9	–	12.5	107	74	102	69	
	↓Epididymal weight	–	12.5	217	92	299	124	
	↓Pregnant females	–	12.5	20	15	91	63	
	Polyovular follicles	–	12.5					
Sprague-Dawley, 0, 250, or 1000 ppm in feed **[37 and 147 mg/kg bw/day]** on PND 21–35.	↓5α-reductase activity in prostate	–	37					Fritz *et al.* (2002a)
	↓Bud perimeter of the type 1 lateral prostate lobe	37	147					
Sprague-Dawley, 0, 250, or 1000 ppm in feed **[37 and 147 mg/kg bw/day]** on PND 21–35.	No adverse testicular effects	147						Fritz *et al.* (2003)
Strain not indicated, 0, 0.2, or 2 mg/kg bw/day by s.c. injection during PND 1–6 and 4 and 40 mg/kg bw/day by gavage on PND 7–21 (s.c. doses were determined to be equivalent to gavage doses of 4 and 20 mg/kg bw/day); one part of the study examining SDN-POA dosed animals during the same period with s.c. and oral doses equivalent to 4 and 40 mg/kg bw/day by oral exposure.	Advanced vaginal opening, persistent vaginal cornification, and ↓serum progesterone	4	20–40					Lewis *et al.* (2003)
	↑SDN POA volume in females	4	40					

Sprague-Dawley, 0, 25, or 250 ppm in diet **[~0, 2.2, and 22 mg/kg bw/day]** during pregnancy and lactation.	No adverse effects on chemically-induced tumorigenesis or reproductive development in males or females (apparently non-adverse changes in proportion of mammary cells)	22						Fritz *et al.* *(*1998)
Sprague-Dawley, 0, 300, or 800 ppm in diet during gestation and lactation.	Apparently non-adverse changes in proportion of mammary cells							You *et al.* (2002b)
Sprague-Dawley, 0 or 500 mg/kg bw by s.c. injection on PND 2, 4, and 6.	No adverse effects on chemically-induced tumorigenesis; (apparently non-adverse changes in proportion of mammary cells)	500						Lamartiniere *et al.* (1995a, b)
Sprague-Dawley, 0 or 500 mg/kg bw by s.c. injection PND 16, 18, 20.	Apparently non-adverse changes in proportion of mammary cells)	500						Murrill *et al.* (1996)
Sprague-Dawley, 0, 25, 250, or1250 ppm (0, 2, 20, and 100 mg/kg bw/day) in diet from GD 7, during gestation and lactation, until PND 77 in offspring.	Increased saline ingestion in both males and females	20	100					Flynn *et al.* (2000a)
	↓Pup birth weight	20	100	102	73	97	68	
Sprague-Dawley, 0, 5, 100, and 500 ppm **[0, 0.31, 5.7, 34 mg/kg bw/day]** in diet through gestation and lactation and in offspring until PND 140	↑Calbindin-positive cells in SDN-POA in males	–	0.3					Scallet *et al.* (2004)

↑, ↓ Significant increase, decrease.
[a]See the footnote to Table 33 for an explanation of the use of benchmark dose in this report. When doses were given in ppm, benchmark doses were calculated in ppm and converted to mg/kg bw/day using author or CERHR estimates and interpolation.
[b]The Expert Panel has limited confidence in the accuracy of the dose determination in this study.
[c]Mammary gland hypertrophy is not a clear adverse outcome.

Table A9a: Other studies considered relevant to this assessment

Author(s)	Genistein dose	Test animal	Observed effects
Marraudino *et al.* (2021)	50 mg/kg bw	Mice of both sexes treated with genistein from PND1 to PND8.	The offspring adult females showed an advanced pubertal onset and an altered estrous cycle, and males showed a decrease of testicle weight and fecal testosterone concentration. Adult females also showed increase in body weight and altered plasma concentrations of metabolic hormones (leptin, ghrelin, triiodothyronine). The authors considered the results to indicate impairment of reproductive system and obesogenic effect in female mice
Kaur *et al.* (2020)	250 mg/kg feed weight	California mice (Peromyscus californicus) exposed offspring through the maternal diet to genistein (two weeks prior to breeding, throughout gestation, and lactation.	At 90-day age, the offspring were assessed for various behaviours, gut microbiota, and fecal metabolome. The results indicated that genistein-exposed individuals were more likely to exhibit certain behaviours and showed socio-communicative disturbances. The exposed females had increased number of metabolites involved in carbohydrate metabolism and synthesis. Males showed alterations in lysine degradation and phenylalanine and tyrosine metabolism. The authors regarded the findings to indicate concern that developmental exposure to genistein might affect the microbiome-gut-brain axis.
Kaur *et al.* (2021)	250 mg/kg feed weight	California mice developmentally exposed to genistein	The adult offspring subjected to behavioural and metabolic tests, and euthanised. Hypothalamic punches used for sequencing small RNAs. The mice exposed to genistein showed one or more repetitive behaviours, such as altered aspects of ultrasonic and audible vocalisations, and sex-dependent differences in differentially expressed miR/small RNAs in female and male mice. The study concluded that developmental exposure to genistein affects hypothalamic miR/small RNA expression patterns, and such changes correlate with EDC-induced behavioural and metabolic alterations. The authors suggested that miR146 may be an important mediator and biomarker of EDC exposure in mammals, including humans.
Ponti *et al.* (2019)	50 mg/kg bw	investigated hypothalamic and mesencephalic dopaminergic system (identified with tyrosine hydroxylase immunohistochemistry).	Administration of genistein to mice of both sexes during the first week of postnatal period specifically affected tyrosine hydroxylase immunohistochemistry in the hypothalamic subpopulation of neurons, abolishing their sexual dimorphism.
Zhang *et al.* (2013)		In vitro study	estradiol- or environmental endocrine disruptor-induced proliferation of human neuroblastoma cells is effectively abolished by genistein, likely in a cell cycle- and Akt pathway-dependent manner
Zhang *et al.* (2014)	50 mg/kg bw/day	administered genistein, DEHP and their mixtures to prepubertal male Sprague-Dawley rats by gavage from PND 22 to PND35 with vehicle control. On PND90, general morphometry (body weight, AGD, organ weight, and organ coefficient), testicular redox state, and testicular histology were studied.	The results indicated that DEHP could cause dose-dependent decrease in sex organs weight, organ coefficient, and testicular antioxidative ability, whereas coadministration with genistein partially alleviates the effects.

Zhang *et al.* (2017)	10 µmol/L	used primary prepubertal Sertoli cells isolated from 22-day-old Sprague Dawley rats to treat with genistein along with MEHP alone or in combination with genistein. Cell proliferation inhibition rate, apoptosis and necrosis rate, and cellular redox state were evaluated.	The results indicated that, whilst MEHP could significantly increase cell proliferation inhibition rate, apoptosis rate, necrosis rate, and intracellular reactive oxidative species level, coadministration of genistein could partially alleviate the MEHP-induced prepubertal Sertoli cells oxidative injuries.
Song *et al.* (2012)		In vitro assay based on human recombinant TPO (hrTPO),	Inhibition of thyroperoxidase (TPO) by genistein, the enzyme that catalyses the transfer of iodine to thyroglobulin during thyroid hormone (TH) synthesis.
Ross *et al.* (2011)	500 mg/kg diet	Female mice were fed diets supplemented with genistein or control diets before breeding and throughout gestation. Urethras from embryonic day 17.5 male fetuses were harvested, and RNA was prepared, amplified, labelled and hybridised on whole genome microarrays.	The results indicated that gestational exposure to genistein altered the urethral expression of 277 genes ($p < 0.008$) and contributes to hypospadias by altering pathways of tissue morphogenesis, cell proliferation and cell survival. In particular, genes in the MAPK and TGF-β signaling pathways and those controlled by FOXO, HOX and ER transcription factors are disrupted.
Ball *et al.* (2010)	5 mg/kg feed weight	fed dams with genistein-containing feed during both gestation and lactation, during gestation only, during lactation only, or during neither of the periods.	Genistein exposure via maternal diet decreased body mass in the male offspring of the dams fed genistein during both gestation and lactation, during lactation only, but not during gestation only. Anogenital distance was decreased when exposure was during both gestation and lactation, but not when exposure was for one of the time periods. Spatial learning (Morris water maze) was also impaired in male rats exposed to genistein during both gestation and lactation, but not in during only one of the time periods. According to the authors, the data showed that exposure to genistein through the maternal diet significantly impacts growth in male offspring if exposure is during lactation, whereas effects on reproductive development and spatial learning required exposure throughout the pre- and postnatal periods.

Table A10

In Vitro Assays for ER binding affinity of genistein (as percent 17 β-estradiol potency) **(from Rozman *et al.*, 2006)**	
Uterine cytosol from Sprague-Dawley rats fed phytoestrogen-free diet [a]	1
Uterine cytosol from rat or sheepb,[c]	0.45-2
ERs from mouse uterine cytosol [d]	0.87
Liver cytosol [b]	0.1
MCF7 breast cancer cells [b]	0.1-2
hER-transfected yeast [b]	0.05
Synthesized human ERα protein [b,c]	5
Synthesized rat ERβ protein [b,c]	36
Human ERα-transfected baculovirus–Sf9 insect cell system [c,e]	0.7
hERβ-transfected baculovirus–Sf9 insect cell system [c,e]	13
Estrogen-dependent pituitary tumor cells [f]	0.88*
ER-mediated protein induction	
pS2 (estrogen-regulated gene response) in MCF7 breast cancer cells [b,c]	0.001-0.1
Exoprotein: MCF7 breast cancer cells [b]	0.01
Alkaline phosphatase activity in Ishikawa-Var I human endometrial adenocarcinoma cells [g]	0.084
BG1Luc4E2 cell line [c]	0.001
Human ER-galactosidase reporter-transfected yeast [b]	0.01–0.05
hER-Chloramphenicol acetyltransferase reporter-transfected Le42	0.04

TATA-Luciferase-reporter transfected T47D human breast adenocarcinoma cells [h]	0.006
Human ERα-TATA-luciferase reporter-transfected human embryonal kidney 293 cells[c]	0.025
Human ERβ-TATA- luciferase reporter-transfected human embryonal kidney 293 cells[c]	0.8
ERα-luciferase reporter-transfected HepG2 human hepatoma cells[c]	1
ERβ-luciferase reporter-transfected HepG2 human hepatoma cells[c]	30
Cell proliferation	
MCF7 breast cancer cells [b]	0.01-0.08

[The use of 17β-estradiol as a reference compound was not always explicit.]
[*] relative to diethylstoilbestrol
References quoted in Rozamn *et al.* (2006): [a]Santell *et al.* (1997); [b]Reviewed in Whitten and Patisaul (2001); [c]Reviewed in Chen and Rogan (2004); [d]Zhang *et al.* (1999a); [e]Kuiper *et al.* (1998); [f]Stahl *et al.* (1998); [g]Markiewicz *et al.* (1993); [h]Legler *et al.* (1999).

ANNEX B – DAIDZEIN

Table B1: Bacterial gene mutation assays (Ames test)

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	ormation on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments by SCCS	Relevance of the result as evaluated by SCCS	Authors_year
1	*S. typhimurium* TA98 and TA100	No data on concentrations used. Probably daidzein was tested at concentrations of up to 200 µg/plate (Environmental Mutagenesis 3:401-419, 1981). Mutagenic activity expressed in number of revertants per nmole.	Daidzein provided by Dr. Tomio Takeuchi, Institute of Microbial Chemistry, Tokyo. No other information on the test item.	**Negative**	**4** Communication from proceedings; results from testing only 2 bacterial strains; no reliable data on chemical analysis of the test material; no data on concentrations used, no detailed data on number of revertants, no data on positive or negative controls used, and on historical controls	**Low**	Sugimura TM, Nagao T, Matsushima T, Yahagi Y, Seino A, Shirai M, Sawamura S, Natori K, Fukuoka YM and Kuroyanagi M. Mutagenicity of flavone derivatives, Proc. Jpn. Acad., **1977**, 53B, 194-197. Reported also in: Nagao M, Morita N, Yahagi T, Shimizu M, Kuroyanagi M, Fukuoka M, Yoshihira K, Natori S, Fujino T, and Sugimura T. Mutagenicities of 61 flavonoids and 11 related compounds. Environmental Mutagenesis **1981**, 3: 401-419.
2	*S. typhimurium* TA98, TA100 TA1538	0, 1, 10, 50, 100 µg/plate	Daidzein provided by Dr. B. Knuckles, USDA Western Regional Research Laboratories (Albany, CA).	**Negative** 2-aminoanthracene (2-anthramine) and quercitin, were mutagenic to all 3 strains of S. typhimurium	**2** Only 3 bacterial strains used, relatively low concentrations used, quercetin is not a typical	**Limited**	Bartholomew RM and Ryan DS. Lack of mutagenicity of some phytoestrogens in the Salmonella/mammalian microsome assay. Mutation Research, **1980**, 78: 317-321.

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	ormation on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments by SCCS	Relevance of the result as evaluated by SCCS	Authors_year
			No other information on the test item.		positive control used with S9-mix		

Table B2: In vitro mammalian cell micronucleus test/ chromosomal aberrations

	Test system/ Test object	Exposure conditions (concentration/duration /metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	Micronucleus (MN) test without CytB and with CREST staining Chinese hamster V79 lung fibroblasts Cytotoxicity assessed with sulforhodamine B	Cytotoxicity: up to 100 µM MN test: 100 µM Exposure for 6 h then cells kept in fresh medium for additional 6-24 hr. The cells were plated on sterile microscope glass slides in a quadriperm vial (30,000 cells/mL DMEM, 5 mL corresponding to 150,000 cells per slide). 2000 cells per slide were examined for MN (DAPI and PI staining) and CREST signals. Three independent experiments.	Daidzein (purity > 98%) from ICN Pharmaceutical Inc.	**Inconclusive** Cytotoxicity after 48 h: slight cytotoxicity - daidzein did not cause changes in cell morphology, even at 100 µM. At a concentration of 100 µM, at 12 h post treatment, only a marginal increase of MN, leading to a doubling of the control value, was observed. Both CREST-negative and CREST-positive MN were observed. 4-Nitroquinoline-N-oxide (0.5 µM, 24 h) induced 4-fold increase in MN frequency.	**3** Study not according to OECD TG and GLP. Only 1 concentration with no cytotoxic effect was tested, no SD values reported, only 6 h of exposure, no S9-mix used, no data on historical controls)	**Low**	Kulling SE and Metzler M. Induction of Micronuclei, DNA Strand Breaks and HPRT Mutations in Cultured Chinese Hamster V79 Cells by the Phytoestrogen Coumoestrol. Food and Chemical Toxicology **1997**, 35: 605-613
2	Micronucleus test L5178Y tk+/− mouse lymphoma cells (clone 3.7.2c)	0, 25, 50, 100 µM (the solubility limit) Exposure for 5h plus 20h incubation, without S9-mix. Staining with acridine orange.	Daidzein (Sigma Chemie GmbH)	**Inconclusive** Daidzein did not induce increased frequency of micronuclei up to 100 µM	**2** Study not according to OECD TG and GLP. Only 5 h of exposure without S9-	**Limited**	Schmitt E, Metzler M, Jonas R, Dekant W, Stopper H. Genotoxic activity of four metabolites of the soy

	Test system/ Test object	Exposure conditions (concentration/duration /metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		N=3 MMC was used and genistein (30 µM) was used as a positive control in experiments testing metabolites.			mix was used.		isoflavone daidzein. Mutation Research **2003**, 542: 43–48
3	Chromosomal aberrations SHE cell cultures were established from 13-day-gestation fetuses	Cells in 75 cm^2 flasks were treated with daidzein (50, 100, 200 µM) for 24 hr. Three hours before harvest, colcemid was administered and metaphase chromosomes prepared. For determination of chromosome aberrations 100 metaphases were scored per experimental group.	Daidzein from Indofine Chemical Company, Inc. (Somerville, NJ), purity >98% (HPLC and GC/MS) after trimethylsilylation	**Negative** Cytotoxicity: relative growth of SHE cells treated with daidzein at 100 or 200 µM for 48 h showed almost 50% and 100% inhibition, respectively, comparing to the control.	**2** Only 100 metaphases were scored which is not in line with the current OECD TG 473. No routine positive control substance was used, however, genistein and coumestrol used in the same study induced a high % of aberrant metaphases.	**Limited**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320

	Test system/ Test object	Exposure conditions (concentration/duration /metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
					No historical control values provided.		
4	Micronucleus test V79 cells	Daidzein: 0, 25, 50, 75, 100 µM Exposure for 18 h, without S9-mix. Staining with acridine orange. N=2 Positive controls: methylmethanesulfonate (50 µg/mL) and vincristine (10 nM) CREST analysis as the 'standard' MN assay 3 slides per concentration, and 1000 cells per slide were scored.	Daidzein from Biomol Feinchemikalien GmbH	**Weakly positive** A shallow increase of the number of the MN with increasing concentrations of daidzein. Starting from 50 µM, there was a significant increase in MN formation. From 75 µM daidzein, the MN frequency was doubled comparing to the control. Partly CREST(+) and CREST(−) micronuclei.	**2** Study not according to OECD TG and GLP; no historical control data were provided to assess validity of the weakly positive result.	**Limited**	Di Virgilio AL, Iwami K, Wätjen W, Kahl R, Degen GH. Genotoxicity of the isoflavones genistein, daidzein and equol in V79 cells. Toxicology Letters **2004**, 151: 151–162
5	Micronucleus test HTC cells from a Rattus norvegicus hepatoma from the Cell Bank of Rio de Janeiro (RJCB—Brazil)	Cells treated with daidzein at 10 µM for 26 h with fresh medium containing cytochalasin B at a final concentration of 3.0 µg/mL and the chemicals. For anti-genotoxicity, the cells were treated with	Daidzein (CAS no. 486-66-8) from Acros Organics	**Inconclusive** Cytotoxicity: at concentrations of 50 and 100 µM, a marked inhibition of proliferation occurred. Daidzein (0.1, 1, 10 µM) did not show protective effects against	**3** Study not according to OECD TG and GLP. Only one concentration was used	**Low**	Lepri SR, Luiz RC, Zanelatto LC, Gonzalves da Silva PB, Sartori D, Ribeiro LR, Mantovaniet MS. Chemoprotecti

	Test system/ Test object	Exposure conditions (concentration/duration /metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		daidzein at 0.1, 1 or 10 µM along with either doxorubicine at 0.2 µM or 2-aminoanthrace at 13 µM. 1000 binucleated cells were scored on coded slides in three experimental repetitions (1,000 cells per treatment). Cytotoxicity with MTT reduction: 0.1, 1, 10, 50, and 100 µM for 24 h.		doxorubicine or 2-aminoanthracene mutagenicity.	(10 µM) for testing the mutagenic effect, which did not induce a noticeable cytotoxicity. No S9-mix was used.		ve activity of the isoflavones, genistein and daidzein on mutagenicity induced by direct and indirect mutagens in cultured HTC cells. Cytotechnology **2013**, 65: 213–222
6	micronucleus test on cultured human lymphocytes	Micronucleus test in the presence and absence of metabolic activation Aroclor 1254 - induced rat livers, Treatment 4h with and without S9 mix 4 h, concentrations 125, 62.5, 31.25, 15.63 µg/mL; 24 treatment without S9-mix 125 – 62.5, 31.25, 15.63, 7.81 µg/mL. Positive control with S9-mix cyclophosphamide 10 µg/mL; without S9-mix mitomicyn C 0.15 (4h treatment), and for 24h treatment mitomicyn C	Daidzein (batch TFS20211018)	**Negative** no genotoxic potential of daidzein *in vitro* Increases in MN frequency in the cells after 24h exposure, however these were not concentration dependent, were not significant (less then 2-fold comparing to the control cultures values) and were within the range of historical vehicle control values. Therefore, the slight increases were considered by the SCCS not to be biologically meaningful.	**1** in compliance with OECD Guideline 487 (2016)	**High**	Study report Number FSR-IPL 220304, INSTITUT PASTEUR DE LILLE, Genetic Toxicology Laboratory, 2022

	Test system/ Test object	Exposure conditions (concentration/duration /metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		0.075 µg/mL and griseofulvin 10 µg/mL 2000 cells per sample were scored.					

Table B3: *In vitro* mammalian cell gene mutation assays

Test system/ Test object	Exposure conditions (concentration/ duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
Hypoxanthine guanine phosphoribosyltransferase (HPRT) assay Chinese hamster V79 lung fibroblasts	The cells were seeded into a 250 mL cell culture flask and incubated for 24 hr. Then the medium was removed and the cells were incubated with daidzein at 100 µM for 3 hr in FCS-free DMEM. 6-day expression period Mutation frequency represented by the number of 6-TG-resistant mutants per 10^6 viable cells.	Daidzein (purity > 98%) from ICN Pharmaceutical Inc.	**Inconclusive** 100 µM daidzein did not cause a significant effect. NQO at 0.5 µAM led to a ~8-fold increase in number of mutants.	**3** Study not according to OECD TG and GLP. Only 1 concentration with no cytotoxic effect was tested, only 3 h of exposure, no S9-mix used, no data on historical controls were provided.	**Low**	Kulling SE and Metzler M. Induction of Micronuclei, DNA Strand Breaks and HPRT Mutations in Cultured Chinese Hamster V79 Cells by the Phytoestrogen Coumoestrol. Food and Chemical Toxicology **1997**, 35: 605-613
Gene mutations at the Na^+/K^+ ATPase or *Hprt* loci	Cells treated with daidzein at 50, 100 or 200 µM or B[a]P for 48 hr. After cells were trypsinized, a part of the cell	Daidzein from Indofine Chemical Company, Inc. (Somerville, NJ), purity >98% (HPLC GC/MS after trimethylsilylation)	**Equivocal** Mutation frequencies were slightly increased at the Na^+/K^+ ATPase locus in cells treated at 50 µM and significantly increased at the hprt locus in cells treated at 200 µM.	**2** Study not according to OECD TG and GLP. Not clear how the MF was calculated	**Limited**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and

Test system/ Test object	Exposure conditions (concentration/ duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
SHE cell cultures were established from 13-day-gestation fetuses	suspension was assayed for morphological transformation, and the remaining cells were subcultured for mutation experiments where gene mutations were measured in the same cells by induction of cells resistant to Oua or TG. Expression time: 4 days; plus 7 days for colony formation			in both tests, no data were provided on negative historical controls. B[a]P, a known indirect mutagen was apparently used without S9-mix.		mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320

Table B4: *In vitro* DNA damage (e.g. Comet assay, DNA elution)

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
1	DNA breaks analysed by the alkaline filter elution technique (Kohn *et al.*, 1981). V79 cells	3 x 10^6 V79 cells per vial plated in 650 mL cell culture flasks were grown for 48 h and then exposed to daidzein for 6 h. Eluted fractions from filters were analysed for DNA by the Hoechst 33258 fluorometric method.	Daidzein (purity > 98%) from ICN Pharmaceutical Inc.	**Negative** When the cells were treated with 50 and 100 µM daidzein for 1, 12 and 24 hr and subsequently analysed, no indication of strand breaks was obtained.	**3** No detailed data on results were provided.	**Low** The method is not among the methods recommended for regulatory purposes.	Kulling SE and Metzler M. Induction of Micronuclei, DNA Strand Breaks and HPRT Mutations in Cultured Chinese Hamster V79 Cells by the Phytoestrogen Coumoestrol. Food and Chemical Toxicology **1997**, 35: 605-613
2	Comet assay on human sperm cells and isolated peripheral blood leukocytes (1 male and 1 female donor)	Daidzein concentrations: 0, 1, 10, 25, 50, 100, 200, or 400 µM Lymphocytes treated for 0.5 h: 2 separate studies Sperm cells treated for 1h: 3 separate studies COMET 3.0 (Kinetic Imaging Ltd., Liverpool, UK) was used to	Daidzein from Sigma Chemical Co. (Gillingham, Dorset, UK)	**Positive** In lymphocytes only concentration of 100 µM induced a statistically significant increase in tail moment. Sperm cells, significant effect from 10 µM was observed.	**2** No effect of hydrogen peroxide on percentage head DNA in human sperm was shown. Relatively short treatment	**Limited**	Anderson D, Dobrzynska MM, and Basaran N. Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the comet assay. Teratogenesis, Carcinogenesis, and

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		measure comet parameters.			times were used. Tail moment parameter was used for lymphocytes.		Mutagenesis, **1997**, 17:29–43
3	Comet assay on human sperm cells Samples were from fertile and infertile donors: two were fresh samples and the other four were received deep frozen (in total 6 donors).	Daidzein concentrations: 0, 10, 50, 100 µM Exposure time of sperm cells not clear.	No data provided	**Positive** All 6 donors showed similar results. There were no differences between fertile and infertile donors in the extent of DNA damage, and daidzein produced a response in human semen (significant effect from 10 µM).	**2** The description of methodology lacks basic details. No data provided on positive control substances.	**Limited**	Anderson D, Dobrzynska MM, Yu TW, Gandini L, Cordelli E, and Spano M. DNA integrity in human sperm. Teratogenesis, Carcinogenesis, and Mutagenesis, **1997**, 17:97–102.
4	Comet assay Isolated lymphocytes from a healthy donor. Semen sample from another healthy donor.	Comet assay: lymphocytes treated for 30 min at 37°C, semen treated for 1 hr at 37°C to daidzein at 250 µM. Viability checked by Trypan blue exclusion. Analysis with Komet 4.0; Kinetic Imaging, Liverpool, U.K.). Tail moment values for	Daidzein (CAS #486-66-8) from Sigma-Aldrich	**Positive** Daidzein , as well as H_2O_2, induced significant increases in tail moments in the lymphocytes and significant decreases in % head DNA in human sperm. Cytotoxicity: the viability of sperm and lymphocytes exceeded 80% in all Comet assays.	**2** Only single relatively short exposure times and only one relatively high concentratio	**Limited**	Cemeli E, Schmid TE, and Anderson D. Modulation by flavonoids of DNA damage induced by estrogen-like compounds. Environmental and Molecular Mutagenesis

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		lymphocytes and % head DNA for sperm were determined. Because of the high levels of background damage in sperm (about 15–30%), % head DNA was used for statistical analysis as an indicator of induced strand breaks.			n of daidzein was used. High sperm % head DNA in control was observed.		**2004**, 44: 420 – 426
5	Comet assay ± formamido-pyrimidine DNA glycosylase (FPG) HT29 human colon carcinoma cell line	Comet assay: cells were exposed to the solvent control or daidzein at 1, 10, 50 µM in serum-free medium for either 1 or 24 h (details in Pelka *et al.* DNA damaging properties of single walled carbon nanotubes in human colon carcinoma cells. Nanotoxicology 2011, 7, 2–20). UV irradiation used as a positive control. Cell viability determined by trypan blue was	Daidzein (purity >99%) from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany)	**Negative -/+Fpg** Cytotoxicity: no cytotoxic potential up to 100 µM after 24 h.	**1**	**High**	Baechler SA, Schroeter A, Walker J, Aichinger G and Marko D. Oxidative metabolism enhances the cytotoxic and genotoxic properties of the soy isoflavone daidzein. Mol. Nutr. Food Res. **2014**, 58: 1269–1281

	Test system/ Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
		>80% throughout the comet experiment. Cytotoxicity assessed 24 h, by LDH release and WST-1 reduction					
6	Comet assay MIA PaCa-2 human pancreatic carcinoma cells and HT-29 human colon cancer cells	Comet assay: cells incubated with the IC50 (200 µM) concentration of daidzein for 48 h. At least 50 randomly selected comets on each triplicate slide were captured, comet tail length was measured using Comet Assay IV Version 4.3.2 Cytotoxicity: XTT reduction test: 25 µM - 1 mM after 24 h and 48 h of exposure.	Daidzein (purity >99%) from LC Laboratories (USA).	**Equivocal** In **MIA PaCa-2** cells, DNA damage results were all significantly higher than those in the control cells. However, while DNA tail length, tail intensity, and DNA tail moment in **HT-29** cells were all higher, only DNA-TI and DNA-TM were statistically higher ($p<0.01$). Cytotoxicity: higher concentrations of daidzein exhibited significant cytotoxic effects on both cell lines treated for 24 h and 48 h. IC50 concentration of daidzein was determined as 200 µM in both cell lines for 48 h.	**2** Only one concentration inducing IC50 was used (it is not clear if the DNA damage effect was dependent on developing cytotoxicity). Only one relatively long exposure time was used. No positive control substance was used.	**Low**	Gundogdu G, Dodurga Y, Cetin M, Secme M and Cicek B. The cytotoxic and genotoxic effects of daidzein on MIA PaCa-2 human pancreatic carcinoma cells and HT-29 human colon cancer cells. Drug and Chemical Toxicology, **2018**, DOI: 10.1080/01480545.2018.1527849

Table B5: Other *in vitro* assays

Test system/Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
Cell transformation assay (CTA) SHE cell cultures were established from 13-day-gestation fetuses	Cells treated with daidzein at 50, 100 or 200 µM or B[a]P at 4 µM for 48 hr. Thereafter, cells were trypsinized, a part of the cell suspension was assayed for morphological transformation, and the remaining cells were subcultured for mutation experiments. For transformation, 2,000 cells were replated on 100 mm dishes (20 dishes for each group) and incubated for 7 days for colony formation.	Daidzein from Indofine Chemical Company, Inc. (Somerville, NJ), and were of purity >98% (HPLC GC/MS after trimethylsilylation)	**Positive** The frequency of morphological transformation of cells treated with daidzein at 200 µM was higher than that induced by B[a]P at 4 µM used as a positive control.	**1** No data on historical control values were provided.	**High**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320

Test system/Test object	Exposure conditions (concentration/duration/metabolic activation	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
DNA adducts by ^{32}P-postlabeling SHE cell cultures were established from 13-day-gestation fetuses	Cells in 75 cm^2 flasks were treated with daidzein (50, 100, 200 µM) for 24 hr.	Daidzein from Indofine Chemical Company, Inc. (Somerville, NJ), and was of purity >98% (HPLC and GC/MS)	**Positive** DNA adducts shown as an extra spot on the chromatogram not seen in the control cells were detected in the cells treated with daidzein at the concentrations that induced morphological transformations. Treatment with daidzein produced a single DNA adduct with the intensities in a concentration-dependent manner. Cytotoxicity: relative growth of SHE cells treated with daidzein at 100, 200 µM for 48 h showed almost 50% and 100% inhibition, respectively comparing to control.	**1**	**High**	Tsutsui T, Tamura Y, Yagi E, Someya H, Hori I, Metzler M and Barrett JC. Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer **2003**, 105: 312–320

Table B6: *In vivo* mammalian gene mutation studies

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
Gene mutations in female (3-wk-old) Big Blue transgenic rats lacI Mutagenesis Assay in **uterine** cells	Group I (INT): sets of N=5 rats/group for mutagenesis study were fed diets containing 250 ppm or 1,000 ppm daidzein, until termination of the experiments. Group II (OVX): at the same time, sets of rats were gavaged with 250 ppm or 1,000 ppm daidzein and at 9 wk of age, were bilaterally ovariectomised. The uteri were harvested for lacI Mutagenesis Assay at 23 wk of age in both Groups. Proliferating Cell Nuclear Antigen/apoptosis analysis in uterine tissues and histopathological analysis were performed.	Daidzein (lot 1-FSS-31–1) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	**Negative** Dietary daidzein (250 or 1000 ppm; 0.250 or 1 g/kg fodder) resulted in variable changes in lacI mutation frequency in both OVX and INT rats, however, the responses were not statistically significant.	**1**	**High**	Aidoo A, Bishop ME, Shelton SD, Lyn-Cook LE, Chen T and Manjanatha MG. Effects of Daidzein, Genistein, and 17β-Estradiol on 7,12-Dimethylbenz[a]anthracene-Induced Mutagenicity and Uterine Dysplasia in Ovariectomized Rats. Nutrition and Cancer **2005**, 53, 1: 82-90

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
	Based on consumption calculations, a dose of 1,000 ppm daidzein represented ~20 mg of daily dose per rat; (estimated daily dose of 20–50 mg/day consumed per person in Asian populations).					
Gene mutations in female (3-wk-old) Big Blue transgenic rats lacI Mutagenesis Assay in **mammary gland** cells	Group I (INT): N=5 rats for mutagenesis study per group were fed diets containing 250 ppm or 1,000 ppm daidzein, until termination of the experiments. Group II (OVX): at the same time, sets of rats were gavaged with 250 ppm or 1,000 ppm daidzein and at 9 wk of age, were bilaterally ovariectomised. The mammary glands were harvested for lacI Mutagenesis Assay at 23 wk of age in both Groups. Proliferating Cell Nuclear Antigen/apoptosis analysis in mammary	Daidzein (lot 1-FSS-31–1) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	**Negative** Daidzein intake in diet (0.250 or 1 g/kg fodder) generally produced a modest increase in the lacI mutation frequency in both OVX and INT Big Blue rats, but none of the responses was significantly higher than the control.	**1**	**High**	Manjanatha MG, Shelton S, Bishop ME, Lyn-Cook LE and Aidoo S. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz[a]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic rats. Carcinogenesis **2006**, 27, 12: pp.2555–2564

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
	gland tissues and histopathological analysis were performed.					
Gene mutations in female (3-wk-old) Big Blue transgenic rats ***Hprt gene*** mutagenesis assay in lymphocytes isolated from the spleen and cultured for 2 days	Group I (INT): N=5 rats for mutagenesis study per group were fed diets containing 250 ppm or 1,000 ppm daidzein, until termination of the experiments. Group II (OVX): at the same time, sets of rats were gavaged with 250 ppm or 1,000 ppm daidzein and at 9 wk of age, were bilaterally ovariectomised. The lymphocytes were harvested for *Hprt* Mutagenesis Assay at 23 wk of age in both Groups.	Daidzein (lot 1-FSS-31–1) from Toronto Research Chemicals (Toronto, Canada). Purity as determined by nuclear magnetic resonance, desorption electron ionization, gas chromatography/mass spectrometry, and high-performance liquid chromatography–ultraviolet (detector) analyses was >99%.	**Negative** Daidzein intake in diet (0.250 or 1 g/kg diet) generally produced a modest increase in the *Hprt* mutant frequency in both OVX and INT Big Blue rats, but none of the responses was significantly higher than the control.	**1**	**High**	Manjanatha MG, Shelton S, Bishop ME, Lyn-Cook LE and Aidoo S. Dietary effects of soy isoflavones daidzein and genistein on 7,12-dimethylbenz[a]anthracene-induced mammary mutagenesis and carcinogenesis in ovariectomized Big Blue transgenic rats. Carcinogenesis **2006**, 27, 12: pp.2555–2564

Table B7: *In vivo* Comet assay

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance including source/manufacturer, CAS number, purity of the test material	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
Comet assay with FpG modification on mice stomach mucosa cells Effects of co-administration of daidzein with $NaNO_2$ was also studied	Daidzein dissolved in saline was orally administered at dose of 1 mg/kg bw (mice N=5 per group). FPG-modified comet assay 3h after administration of daidzein. Fifty cells were examined per mouse (250 cells per group). Tail moment of DNA was measured using Comet Analyzer Youworks BioImaging Software. Measurement of nuclear 8-oxodG after coadministration of Daidzein (1 mg/kg) and NaNO2 (10 mg/kg)	Daidzein from LKT Laboratories Inc. (St. Paul, MN, USA).	**Inconclusive**	**3** Only one dose of daidzein was used, only 3 h exposure time used, no positive control substance used, apparently 50 cells per mouse scored, tail moment used for scoring	**Low**	Toyoizumi T, Sekiguchi H, Takabayashi F, Deguchi Y, Masuda S, Kinae N. Induction effect of coadministration of soybean isoflavones and sodium nitrite on DNA damage in mouse stomach. Food and Chemical Toxicology **2010**, 48: 2585–2591

Table B8: Other *in vivo* assays

Test system/Test object	Exposure conditions (concentration/duration)	Information on the characteristics of the test substance	Result as evaluated by SCCS	Reliability/ Comments	Relevance of the result as evaluated by SCCS	Authors_year
Sister chromatid exchange test in bone marrow Female ICR mice	Groups of 5 mice were pretreated i.p. with DMSO (solvent control) or daidzein at 3 doses: 10 or 20 mg/kg daily for 6 days with the last dose given just before 5-bromodeoxyuridine (BrdU) treatment, or 50 mg/kg at 12 h intervals for 3 days with the 5th dose given just before BrdU treatment (6x50 mg/kg bw).	Daidzein from Indofine Chemical Company, Inc. (Somerville, NJ).	**Weakly positive** At 6x50 mg/kg daidzein significantly increased SCE by 22%.	**2** No historical control values provided, what hampers evaluation of the results.	**Limited** SCE test is not recommended for regulatory purposes. Daidzein was administered i.p.	Giri and Lu, 1995

Table B9: In Vivo Studies investigating ED effects of Daidzein

Ref	Compound	Species	Sex	Age of animals/ model	Route of exposure	Dose	duration of exposure	+ Control	Effects observed	Conclusion from the authors	SCCS? Comments
Ajdžanović V *et al.*, 2020 PMID: 32120001	Daidzein	Wistar rat	male	16 months, orchidectomized	sc	30 mg/kg	3 weeks	estradiol	Daidzein treatment significantly increased volumes of the zona glomerulosa cell and nuclei but decreased circulating aldosterone levels. Daidzein significantly decreased both the adrenal and circulating levels of corticosterone Daidzein significantly increased the circulating level of DHEA.	Given the coherence of its effects and relative safety, daidzein could be the remedy of choice for the treatment of ageing-caused androgen deprivation and the hypothalamo-pituitary-adrenal axis hyperfunction/related metabolic issues in males.	LOEL = 30 mg/kg sc
Trifunović S *et al.*, 2018; PMID: 29338944	Daidzein	Wistar rats	male	2 years, orchidectomized	sc	30 mg/kg	3 weeks	estradiol	Daidzein decreased the volume density of CRH neurons within the paraventricular nucleus as well as CRH immunofluorescence in the ME. The total number of ACTH cells was decreased, while ACTH cell volume and the intensity of ACTH fluorescence were increased. Both ACTH and corticosterone blood levels were increased.	The results of performed experiments clearly demonstrate that volume density of CRH neurons; total number and volume of ACTH cells, as well as stress hormones levels are vulnerable to the effects of daidzein	LOEL = 30 mg/kg sc
Nestorović N *et al.*, 2018, PMID: 29569839	Daidzein	Wistar rats	male	2 months, orchidectomized	sc	30 mg/kg	3 weeks	estradiol, genistein	Daidzein decreased the cell volume of gonadotropic cells but increased their number and numerical density		LOEL= 30 mg/kg sc s

Retana Marquez, 2016, PMID: 27163522.	Daidzein	Wistar rat	male	3 months	sc	5 mg/kg	50 days	estradiol, genistein	Mount latencies in males treated with Leucaena extract, DAI and E2 were higher than in control males in some of the days of treatment [F5, 55=3.653; p = 0.0032], although there were no differences among days of treatment. Intromission latency increased in almost all groups, except in the Leucaena extract group, and it increased with days of treatment [F5, 55 = 7.801; p = 0.0001]. Ejaculation latencies increased in males from all the experimental groups, compared with control. This effect was stronger on days 40 and 50 [F5, 55 = 4.039; p = 0.0036]. The percentage of tubules with TUNEL-labeled germ cells increased in DAItreated males, compared with testes from control males. Sperm count and sperm viability and testosterone levels were decreased in DAI treated rats.	The results indicate that mesquite pod and Leucaena extracts disrupt male sexual behavior in a similar way to DAI and GEN, but less than E2	LOEL= 5 mg/kg sc

Jaric *et al.*, 2016 PMID: 29217487	Daidzein	Wistar rats	female	12 months ovarian intact	sc	35 mg/kg	4 weeks	Genistein	DAI did not change the uterine wet weight and stereological features of the main uterine compartments as well as LAC and VEGF gene expression. Absence of hyperplastic changes were illustrated by an increase in caspase-3 immunoexpression, associated with reduced PCNA expression. DAI up-regulated only the expression of ERβ, while the expression levels of ERα and PR remain unaffected. Also, DAI inhibited the activation of Akt due to down-regulation of phosphorylated and total form of Akt protein expression.	Compared to GEN, DAI did not promote events associated with the endometrial cell proliferation	NOAEL = 35 mg/kg sc
Romero *et al.*, 2019	Daidzein	Wistar rats	female	ovariectomized	sc	5 mg/kg	30 days	estradiol	**In intact females**, DAI disrupted the estrous cycle and female sexual behavior, decreased the number of follicles and corpora lutea, increased uterine and vaginal epithelium in proestrus and diestrus periods, increased uterine and vaginal relative weights during diestrus, and decreased serum progesterone during proestrus All these effects were similar to those of DAI but lower than E2-induced effects. **In OVX female**s, DAI decreased body weight, induced lordosis,	These results indicate that, in gonadally intact females, LEU (**and DAI**) can produce antiestrogenic effects in sexual behavior but estrogenic effects on uterine and vaginal weight and epithelia, without modifying serum levels of E2. In OVX females, in total absence of endogenous E2, LEU induced estrogenic effects on vaginal weight and epithelia, as	LOEL = 5mk/kg sc

									stimulated vaginal epithelium cornification, increased vaginal weight, and augmented vaginal epithelium thickness. All these effects were similar to the effects observed with E2 but lower.	well as on sexual behavior.	
Talsness et al., 2015	Daidzein	Sprague Dawley rats,	female	pregnant female	oral-gavage	0, 5, 60 mg/kg bw per day.	Gestational day		Altered estrous cyclicity: Prolonged estrous in high dose group, no change in cycle length. Altered ovary histology: reduced number of secondary and tertiary follicles at both doses, and increased number of atretic follicles at the high dose.	The morphological changes to the ovarian surface epithelium are consistent with an antiproliferative effect, while ovarian folliculogenesis was adversely affected. The effects of the high dose DZ were similar to those observed with 17-α EE.	LOEL = 5 mg/kg This study is potentially relevant but has several limitations, e.g. uncertainty about number of litters included per dose group, and the use of different control groups for each dose group in the histological evaluation (Nordic Council report, 2020)

Zhang *et al.*, 2018	Daidzein supplemented food	Sprague Dawley rats,	female	pregnant animals	diet	50 mg/kg feed	6 to 21.	Daidzein supplementation significantly increased the total litter weight and the total viable newborn weight ($p < 0.05$). **Daidzein** supplementation acutely elevated the concentrations of serum estrogen, progesterone and insulin-like growth factor-1 ($p < 0.01$) after the maternal rats' delivery. IgA and IgG were also significantly higher in the DAI than in the CON maternal rats ($p < 0.05$). Daidzein significantly increased the total antioxidant capacity (T-AOC) in maternal rats' sera and in newborns ($p < 0.05$) and elevated the concentration of superoxide dismutase (SOD) in both the maternal rats' sera and their ovaries ($p < 0.05$). Daidzein supplementation significantly elevated the expression levels of estrogen receptor β (ERβ) and NR5A2 genes in maternal rats' ovaries ($p < 0.05$) and downregulated the expression level of prolactin receptor (PRLR) in newborns ($p < 0.05$).	These results suggest that dietary daidzein supplementation improves reproductive performance and fetal development in rats, which is associated with changes in serum hormones, tissue antioxidant capacity, and expression levels of reproductive-related genes, both in maternal rats and their offspring	LOEL = 50 mg/kg oral?

Mohamad *et al.*, 2019, PMID: 30710233.	Daidzein	Sprague Dawley rats,	male (n =6 /group)	testosterone-induced prostate hyperplasia	oral	10 and 100 mg/kg	30 days	Genistein	Daidzein increased testosterone levels in testosterone-induced prostate hyperplasia by 11%. However, levels of FSH, LH, triglyceride and HDL are not affected. The zinc content increased significantly and the zinc transporter gen of ZnT4 and ZIP4 highly expressed suggesting that daidzein plays essential role in modulating prostate zinc homeostasis. Similarly, the expression of IL-6, AR and ER was significantly reduced indicating functioning in regulation of prostate growth and acts as anti-inflammatory role in preventing BPH.		LOEL = 10 mg/kg
Jeminiwa *et al.*, 2020, PMID: 32520353	Daidzein	Long-Evans rat	male	21, 35 or 75 days of age, pubertal male rat	diet	200 ppm	14 days	Genistein	Feeding of all isoflavone-containing diets decreased ($P < 0.05$) testicular T concentrations, and more so in the G + D diet group. Interestingly, Esr1 and androgen receptor protein and pituitary Fshβ with Lhβ subunit protein were increased ($P < 0.05$) by feeding of genistin and G + D diets, but not the daidzin diet. However, daidzein and genistein both caused a concentration dependent inhibition ($P < 0.05$) of T secretion by Leydig cells in vitro with IC50 of 184 ηM and 36 ηM, respectively.	Results demonstrated that altered testicular steroidogenic capacity and pituitary FSHβ and LHβ subunit expression due to soy-based diets result from specific actions by genistein and daidzein.	LOEL: 200 ppm?