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SCIENTIFIC OPINION

Saponins in *Madhuca Longifolia* L. as undesirable substances in animal feed[1]

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2005-221)

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PANEL MEMBERS

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SUMMARY

Saponins are a diverse group of low molecular-weight secondary plant metabolites that are widely distributed in the plant kingdom. The chemical structure of saponins consists of an aglycone of either steroidal or a triterpenoid nature and one or more sugar chains (glycosides). Saponins can form stable foam in aqueous solutions, hence the name "saponin" from the Latin word for soap (*sapo*). Traditionally, they have been used as detergents, piscicides and molluscicides in addition to industrial applications as foaming and surface active agents.

Madhuca longifolia and other *Madhuca* species are large evergreen or semi-evergreen trees with a dense spreading crown extensively cultivated in warm climates for their oil-containing seeds. The present opinion deals with saponins in *Madhuca longifolia* and other *Madhuca* species as potentially undesirable compounds in feed. Possible occurrence of saponins and also cyanogenic glycosides in "unhusked beech mast" from *Fagus silvatica* with regard to its listing as an undesirable substance in animal feed was also included in the request. However, since it does not contain saponins or cyanogenic glycosides in significant amounts and its reported toxicity in cattle and horses can most likely be attributed to its high content of oxalates, beech mast is not further discussed in this opinion.

[1] For citation purposes: Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Saponins in *Madhuca Longifolia* L. as undesirable substances in animal feed. *The EFSA Journal* (2009) 979, 1-36.

In food and feed, saponins can have an "anti-nutritional" effect and cause toxic effects, but have also been claimed to cause beneficial health effects. Many saponins have a general action on lipid membranes and cause haemolysis *in vitro* or when injected intra-venously. In general, saponins, as glycosides, have low oral bioavailability, but may be hydrolysed in the intestinal tract and cause systemic toxicity dependent on the structure and absorption of the aglycone. No individual saponin isolated from any of the *Madhuca spp*. has been tested in any *in vivo* toxicity assay. Toxicity studies and observations of toxic effects in feeding studies have been reported using crude total saponins or defatted seed meal from various *Madhuca* species. The oral LD_{50} in mice of crude *Madhuca* saponins (exact botanical source not given) was about 1.0 g/kg body weight. In mice and rats *Madhuca* saponins caused local gastro-intestinal toxicity as well as liver and kidney toxicity. At lower doses, *Madhuca* saponins can cause feed refusal and starvation with reduced body weight gain and increased mortality. The Panel confirmed that although Mahua oil (oil from *Madhuca longifolia*) caused bilateral testicular atrophy with degenerative changes in the seminiferous tubules in rats; saponins are the substances mainly responsible for the toxicity of *Madhuca longifolia* in animal feed. No studies on mutagenicity, genotoxicity and carcinogenicity of saponins from *Madhuca* species have been identified. Studies on other saponins do not indicate a genotoxic or carcinogenic potential. Because of the limited data available, no health-based guidance value (ADI, TDI) can be established for *Madhuca* saponins..

Results from studies on *Madhuca* seed cakes, which contain saponins, on ruminants indicate that they are more tolerant to *Madhuca* saponins than monogastric animals and can tolerate inclusion levels of up to a maximum of 20% of the total diet. Toxicity studies of *Madhuca* seeds on monogastric target animals are scarce. *Madhuca* seed cake in chick mash at approximately 12% level was lethal. No studies have been conducted on horses, pigs, rabbits or dogs. Except for piscicidal effect of *Madhuca* saponins by water exposure in guppy fish, no toxicity studies after dietary exposure were identified in fish.

Data on occurrence of *Madhuca* as a botanical impurity in feed are not available. Because of its limited value as feed for livestock *Madhuca* is not imported into the EU either as whole seeds or as meal. In producing countries, *Madhuca* cake is used mainly as a fertiliser and to a limited extent as feed because of its protein content. Data for the carry-over and residues of *Madhuca* saponins are not available. *Madhuca* products are not consumed by humans as part of the diet and human dietary exposure to *Madhuca* saponin through the consumption of animal products is very unlikely as the only potential source would be imported food of animal origin from animals fed *Madhuca.* The CONTAM Panel concludes that human dietary exposure to *Madhuca* saponins in the EU can be considered as negligible.

Key words: Saponins, *Madhuca*, unhusked beech mast, *Madhuca* seed cake, Mahua oil, toxicity, exposure, carry-over, animal health, human health.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

1. General background

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed[2] replaces since 1 August 2003 Council Directive 1999/29/EC of 22 April 1999 on the undesirable substances and products in animal nutrition[3].

The main modifications can be summarised as follows

- extension of the scope of the Directive to include the possibility of establishing maximum limits for undesirable substances in feed additives.
- deletion of the existing possibility to dilute contaminated feed materials instead of decontamination or destruction (introduction of the principle of non-dilution).
- deletion of the possibility for derogation of the maximum limits for particular local reasons.
- introduction the possibility of the establishment of an action threshold triggering an investigation to identify the source of contamination ("early warning system") and to take measures to reduce or eliminate the contamination ("pro-active approach").

In particular the introduction of the principle of non-dilution is an important and far- reaching measure. In order to protect public and animal health, it is important that the overall contamination of the food and feed chain is reduced to a level as low as reasonably achievable providing a high level of public and animal health protection. The deletion of the possibility of dilution is a powerful means to stimulate all operators throughout the chain to apply the necessary prevention measures to avoid contamination as much as possible. The prohibition of dilution accompanied with the necessary control measures will effectively contribute to safer feed.

During the discussions in view of the adoption of Directive 2002/32/EC the Commission made the commitment to review the provisions laid down in Annex I on the basis of updated scientific risk assessments and taking into account the prohibition of any dilution of contaminated non-complying products intended for animal feed. The Commission has therefore requested the Scientific Committee on Animal Nutrition (SCAN) in March 2001 to provide these updated scientific risk assessments in order to enable the Commission to finalise this review as soon as possible (Question 121 on undesirable substances in feed).

The opinion on undesirable substances in feed, adopted by SCAN on 20 February 2003 and updated on 25 April 2003 provides a comprehensive overview on the possible risks for animal and public health as the consequence of the presence of undesirable substances in animal feed.

[2] OJ L140, 30.5.2002, p. 10
[3] OJ L 115, 4.5.1999, p. 32

It was nevertheless acknowledged by SCAN itself and by the Standing Committee on the Food Chain and Animal Health that for several undesirable substances additional detailed risk assessments are necessary to enable a complete review of the provisions in the Annex.

2. Specific background

Madhuca longifolia (Mahua, Mowarah, Bassia, Madhuca and many others) is a large evergreen or semi-evergreen tree with a dense spreading crown extensively cultivated in warm climates for its oil-containing seeds. The distinction made in the Annex to the Directive 2002/32/EC between *Madhuca longifolia* and *Madhuca indica* is no longer supported and the species are considered synonymous.

The seed oil, which is a common ingredient of hydrogenated fat in India, contains oleic (46.3%) and linoleic (17.9%) acids as the major unsaturated fatty acids and the saturated fatty acids palmitic (17.8%) and stearic (14.0%) acids. Defatted seed meal contains 29.4% protein and 98 g/kg saponins which are toxic at this level. Detoxification can be done by a heat treatment but the digestibility decreases significantly through the heat treatment. The levels of saponins can also be reduced by treatment with isopropanol. Detoxified seed meal appears to be a good source of protein for food and feed[4].

SCAN[5] indicated the toxic effect of unhusked beech mast *(Fagus silvatica)* may be due to the presence of saponins (saponic glycosides). Horses are said to be particularly sensitive although most reported cases seem to have been involved cattle. Another source however refers to hydrocyanic acid as toxic substance in beech mast[6]

SCAN concluded[7] that the ease of microscopic detection of botanical contaminants is inversely related to the degree of processing, particularly contamination, of feedingstuffs. It would be advantageous if the physical detection of the presence of a potentially contaminant could be supported or replaced by a quantitative chemical analysis of the specific compound (s) presumed responsible for their toxicity and maximum limits set accordingly based on a risk assessment of the toxic compound.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

In accordance with Article 29 (1) a of Regulation (EC) No 178/2002 the European Commission asks the European Food Safety Authority to provide a scientific opinion on the presence of saponins from Madhuca longifolia in animal feed.

This scientific opinion should

- confirm that saponins are the substances responsible for the toxicity of Madhuca longifolia in animal feed.

[4] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, adopted on 20 February 2003, updated on 25 April 2003, point 9.2.7. *Madhuca longifolia* (http://europa.eu.int/comm/food/fs/sc/scan/out126_bis_en.pdf)

[5] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.2.9. *Fagus silvatica* – unhusked beech mast.

[6] Fact Sheets Undesirable Substances and Products, Product Board Animal Feed, The Netherlands, http://www.pdv.nl/lmbinaries/beuk.pdf

[7] Opinion of the Scientific Committee on Animal Nutrition on Undesirable Substances in Feed, point 9.5. Conclusion and point 9.6 Recommendations.

- identify the saponins from *Madhuca longifolia* which are relevant for their impact on animal health or their impact on public health through a possible carry-over into food of animal origin.
- assess if unhusked beech mast has been listed as undesirable substance in animal feed because of the presence of saponins or of the presence of hydrocyanic acid or of both with indication of their relative importance to the overall toxicity of unhusked beech mast.
- identify botanical impurities other than *Madhuca longifoli*a and unhusked beech mast which could possibly contribute significantly to the presence of saponins in animal feed. The relative importance of all identified botanical impurities should be determined.
- determine the toxic daily exposure levels of the saponins (as a group or for relevant individual saponins for the different animal species of relevance (difference in sensitivity between animal species) above which
- signs of toxicity can be observed (impact on animal health)
- the level of transfer/carry over of these undesirable substances from the feed to the products of animal origin results in unacceptable levels of these undesirable substances or possibly their toxic metabolites in the products of animal origin in view of providing a high level of public health protection.

- identify feed materials which could be considered as sources of contamination by these undesirable substances (saponins or botanical impurities sources of saponins) and the characterisation, insofar as possible, of the distribution of levels of contamination.
- assess the contribution of the different identified feed materials as sources of contamination by these undesirable substances.
  - to the overall exposure of the different relevant animal species to these undesirable substances,
  - to the impact on animal health,
  - insofar relevant, to the contamination of food of animal origin (the impact on public health), taking into account dietary variations and carry over rates.
- identify possible gaps in the available data which need to be filled in order to complete the evaluation.

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ASSESSMENT

1. Introduction

The present opinion deals with saponins in *Madhuca longifoli*a and other *Madhuca* species as undesirable compounds in feed. The possible occurrence of saponins and/or cyanogenic glycosides in "unhusked beech mast" from *Fagus silvatica* is also discussed with regard to its listing as an undesirable substance in animal feed. In addition, as part of the terms of reference, the occurrence and possible contribution of saponins in other plants used as food/feed and potential sources of botanical impurities are briefly discussed.

Saponins are low molecular weight secondary plant constituents containing either a tetracyclic steroidal or a pentacyclic triterpenoid aglycone and one or more sugar chains, which can form a stable foam (as can soap) in aqueous solutions, hence the name "saponin" from the Latin word for soap (*sapo*) (Vincken *et al.*, 2007). A broader definition, which is not used in this opinion, would include also the steroidal alkaloid glycosides found in potatoes (Friedman, 2006). The closely related, very bitter tasting and highly toxic cucurbitacins, mostly found in plants belonging to cucurbitaceaous plants, are not saponins (Gry *et al.*, 2006) nor are the non-glycosylated microbial sterol surrogates of pentacyclic triterpenoid origin - the hopanoids (Ourisson *et al.*, 1987). The saponins are widely distributed in the plant kingdom and constitute a diverse group of compounds, varying in structure, physicochemical properties and biological effects. Traditionally, saponins have been extensively used as detergents, as piscicides (fish poison) and molluscicides, in addition to their industrial applications as foaming and surface active agents. If present in food or feed, saponins can have "anti-nutritional" effects and may even cause toxic effects (Price *et al.* 1987), whereas some saponins may have beneficial health effects (Shi *et al.*, 2004; Güclü-Üstündag and Mazza, 2007; Isanga and Cuo-Nong, 2008).

2. Saponin containing plants

2.1. Saponin containing plants of major importance

Saponins have been reported to be present in more than 100 plant families (Sparg *et al.*, 2004; Güclü-Üstündag and Mazza, 2007) and in a few marine sources, such as in most star fish and sea cucumber species, and even in a few fish that secrete saponins as shark repellents (Sparg *et al.*, 2004; Williams and Gong, 2004).

Saponins can be classified into the two groups: pentacyclic triterpenoid saponins and steroidal saponins (see further under "Chemistry"). The steroidal saponins are mainly found in monocotyledons (such as in the families Agavaceae, Dioscoreaceae and Liliaceae), while triterpenoid saponins mostly are present in dicotyledons (Fabaceae, Araliaceae and Caryophyllaceae) (Sparg *et al.*, 2004). According to the structure of the carbon skeleton of the aglycone, saponins are sometimes further classified into 12 main classes, namely the: dammaranes, tirucallanes, lupanes, hopanes, oleananes, 23-nor oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids (Vincken *et al.*, 2007).

Besides saponins in *Madhuca* species (genus *Madhuca* Hamilton ex Gmelin), which is assessed in this opinion, significant concentrations of saponins are found in some food and feed plants[8] (Table 1) such as alfalfa (*Medicago sativa* L.), soybean (*Glycine max*), quinoa (*Chenopodium quinoa* Willd.), balanites (*Balanites aegyptiaca* L.).

Studies have been pursued to reduce the amount of bitter saponins, especially in soybeans which are intensively cultivated (Masakazu and Kazumi, 2004).

Table 1. **Saponin content of selected plant materials (modified from Güclu-Üstündag and Mazza, 2007)**

Source	Saponin content (g/kg)[1]	References
Madhuca saponin	> 200	Hegnauer, 1973
Soybean (f.w. basis)[2]	2.2 - 5.83	Ireland *et al.* 1986, MacDonald *et al.*, 2005
Chickpea ("d.w." basis) [3]	2.3	Price *et al.*, 1986
Green pea ("d.w." basis)[3]	1.8- (42)[4]	Price *et al.*, 1986
Quillaja bark (d.w. basis)[5]	90-100	San Martin and Briones, 1999
Yucca (*schidigigera*) (d.w. basis)	100	Oleszek *et al.,* 2001
Fenugreek	40-60	Sauvaire *et al.*, 2000
Alfalfa (whole leaf; d.w. basis)	1.4-17.1	Livingston *et al.* 1984, Price *et al.*, 1987
Licorice Root (d.w. basis)[6]	222-323	Spinks and Fenwick, 1990
American ginseng (*Panax quinquefolium* L.)		
Young leaves (d.w. basis)	14.2-26.4	Li *et al.,* 1996
Mature leaves (d.w. basis)	41.4-55.8	Li *et al.,* 1996
Roots (4 years old)(d.w. basis)	24.4-38.8	Li *et al.,* 1996
Oat (d.w. basis)	1-1.3	Price *et al.*, 1987
Horse chest nut (d.w. basis)	30-60	Price *et al.*, 1987
Sugar beet leaves (d.w. basis)	58	Price *et al.*, 1987
Quinoa (d.w. basis)	1.4-23	Fenwick *et al.,* 1991, Ridout *et al.,* 1991

[1]fresh weight (f.w.). dry weight (d.w.)
[2] For mature seeds f.w. and d.w. nearly identical (Shimoyamada *et al.*, 1991)
[3]Dry weight defatted sample.
[4]Gravimetric method.
[5]Fresh bark given as around 50 g/kg in saponin content (San Martin and Briones, 1999).
[6]Information is not given on d.w. versus f.w., but since bought as commercial confectionary (Spinks and Fenwick, 1990) it is probably d.w.

2.1.1. Madhuca

Madhuca Hamilton ex Gmelin is a genus of tropical plants growing from India to New Guinea, and most species inhabit evergreen or deciduous forests at low altitude. In addition to the 84 species listed by Van Royen, (1960), a few additional ones have been recently described from Thailand (Chantaranothai, 1998). The genus *Madhuca* belongs to the family of Sapotaceae, which includes more than 800 tree species many of which are used in the production of latex (e.g. Guttapercha). Saponins in this family are triterpenoids and they also contain other secondary metabolites, including tannins, and in some species also alkaloids and cyanogens. In *Madhuca*, the triterpenoids mostly occur in the seeds ("mowrin"), up to a

[8] Feed in this respect often means extracted seed material (extracted through pressing and/or extraction with solvents). The remaining material typically contains the starch, fibers and proteins together with minerals and hydrophilic secondary constituents (such as saponins). The extracted material may e.g. be termed cake, extraction cake, or defatted seed meal. Non extracted but comminuted material may be termed seed meal. In the present opinion materials are referred to as they are named in the articles cited.

concentration of around 100 g/kg as for example in *M. butyracea* (Shanmugasundaram and Venkataraman, 1985) and *M. longifolia* (Singh and Singh, 1991; Jakhmola *et al.*, 1987). The seeds are rich in oil, and several species are used for the production of seed oil. The oil content of mature seeds of *M. longifolia* (L) MacBr. (syn. *Bassia longifolia* Koenig) collected in Sri Lanka varied from 480 g/kg to 570 g/kg, with an average of 510 g/kg (Senaratne *et al.*, 1982). Oil is also extracted from *M. indica* J.F. Gmel. (syn. *M. latifolia* Macbr., *Bassia latifolia* Roxb.), *M. butyraceae* Macbr. (syn. *Aisandra butyracea* (Roxb.) Baehni) (Council of Scientific and Industrial Research CSIR, 1986; Jakhmola *et al.*, 1987), and a few other species. The seed oil is mainly used for non-food purposes, such as the manufacturing of laundry soap (CSIR, 1986) and biodiesel. The oils (often termed "Mahua oil/butter" or for *M. butyracea* "Phulwara butter") have also been tested as potential cocoa butter extenders (Reddy and Prabhakar, 1989; Lipp and Anklam, 1998; Ghadge and Raheman, 2005).

The press cake, after oil extraction which contains > 200 g/kg saponins (measured as "raw saponin") (Hegnauer, 1973), is unsuitable as feed and has traditionally been used as fertiliser.

A reduction of the saponin content of *Madhuca* seed products has been achieved by processing, and seed cake/flour experiments have been performed in order to make them acceptable as animal feed ingredients (Jakhmola *et al.*, 1987; Singh and Singh, 1991; Shanmugasundaram and Venkataraman, 1985, 1989; Saxena *et al.*, 2002). Resulting products have also been tested in feeding experiments on e.g. bull calves (Katiyar *et al.*, 1991), but are generally used only at a local scale.

2.1.2. Alfalfa

Alfalfa (*Medicago sativa* L.), also known as lucerne, Spanish trefoil, Chilian clover, Brazilian clover, French clover, medic, and purple medic (Coburn, 1904), contains saponins of the triterpenoid type (Sen *et al.*, 1998; Pecetti *et al.*, 2006). Alfalfa is a legume native to Iran and is widely grown throughout the world as forage for cattle, most often harvested as hay. Alfalfa has the highest feeding value of all common hay crops, being used less frequently as pasture. It is widely used as a feed for many livestock, but there are reports of toxicity when fed to particularly sensitive species, e.g. poultry, even when saponin concentrations are relatively low (Pecetti *et al.,* 2006), indicating that other substances may be responsible for such toxicity.

2.1.3. Soybean

Soybean (*Glycine max* L.) is native to Eastern Asia. The plant contains saponins of the triterpenoid type (Güclü-Üdtündag and Mazza, 2007; Csaky and Fekete, 2004) and is an important global crop, grown for oil and protein content; soybean meal, a major ingredient in livestock diets and in non-ruminant diets, may account for up to 20-25% of the ration (depending on species and levels of production). The bulk of the crop is harvested as seeds and solvent-extracted for vegetable oil, and the defatted soy meal is used for animal feed. Most of the characterised soy-saponins are derived from one of three different triterpenoid aglycones termed soyasapogenol A, B and E, with a total saponin content ranging between 2 and 5 g/kg (Güclü-Üdtündag and Mazza, 2007). The content of individual saponins vary within the different plant organs and between soybean variety as reviewed by Shi *et al.* (2004), who give examples including 11 plant parts and 6 soybean varieties. Concentrations in whole soybean seeds are typically about 5 g/kg by weight (Ireland *et al.*, 1986), while Knudsen *et al*. (2006) reported that total level of saponins in 15 samples of commercial defatted soybean meal ranged from 5.1−7.0 g/kg (4.8−6.8 μmol/g). The breeding programmes

have apparently not changed the content of saponins in soya beans substantially (MacDonald *et al*., 2005).

Soy beans contain a number of toxic/antinutritional substances, such as lectins (phytohemaglutinins) and different types of proteinase inhibitors and phytoestrogens. However, there are no reports of saponin toxicity associated with feeding livestock with current soybean rations, and Birk (1969) reported that soybean saponins were harmless to poultry, even in approximately a three-fold concentration than that found in a 50% soybean supplemented diet.

2.1.4. Quinoa

Quinoa (*Chenopodium quinoa* Willd.), originating from the Andean region of South America where it has been grown for food production for over 6000 years, is grown as a crop primarily for its edible seeds which are used to make flour, soup, breakfast cereal, and alcohol. It is a pseudo-cereal that grows best in well-drained soils and requires a relatively long growing season. Its leaves are also eaten as a leaf vegetable, much like amaranth, but the commercial availability of quinoa greens is restricted to local markets. Leaf protein from quinoa has been examined as a potential source of feed protein (Carlson *et al.*, 1984). Seeds of *Chenopodium* spp. used for human consumption come from *C. quinoa* (quinoa), *C. pallidicaule* (canihua) and *C. berlandieri ssp. nuttaliae* (Safford) Wilson and Heiser (huauzontle) (Heiser and Nelson, 1974). The seeds contain bitter pentacyclic triterpenoid saponins of the aglycones oleanoic acid, hederagenin, phytolaccagenic acid, and possibly other sapogenols (Ridout *et al*., 1991; Gee *et al*., 1993; Madl *et al.*, 2006). The levels of total saponin contents reported for different cultivars vary between 1.5-23 g/kg (Güclü-Üdtündag and Mazza, 2007). The compounds occur in the outer layers (seed coat; seed hulls), making these parts of the seed essentially unpalatable. It is not grown widely in the European Union (EU), and there are no reports of the use of quinoa as a feed for livestock. Most quinoa is sold commercially as health food in North America, after removal of the seed coat.

2.1.5. Sugar beet

Sugar beet (*Beta vulgaris* L.) also contains saponins of the triterpenoid type (Murakami *et al.*, 1999; Sparg *et al.*, 2004; Güclü-Üdtündag and Mazza, 2007). Even for this well-established industrial crop, new saponins are continuously isolated and structures elucidated (Brezhneva *et al.*, 2001). The presence of saponins, particularly in the leaves (up to 6 g/kg total saponin content) (Price *et al*., 1987), restricts their use as feed for non-ruminants (Draycott and Christenson, 2003). Root pulp remaining after the extraction of the sugar may still contain some saponins (3 g/kg reported in the surface layer of the root), particularly in the tail (Silin, 1964, cited by Birk, 1969). Cases of saponin poisoning have been reported in livestock grazing the tops of the sugar beet plants (Cooper and Johnson, 1984). Sugar beet tops may be fed to livestock, usually sheep, once the roots have been harvested and at some point they may have accounted for a significant (15-20%) proportion of animals daily dry matter intake, but the practice of grazing beet tops is less common now. This is largely for logistic reasons, since the beet tops are now usually ploughed back into the soil as a green manure.

2.2. Beech mast

The leaves of *Fagus silvatica* contain triterpenoid saponins with oleanoic acid as the aglycone; 5 g of 28-(-β-**D**-glucopyranosyloxy)-28-oxoolean – 12-en-3β-yl 3-*O*-(β-**D**-glucopyranosyl)-β-**D**-glucopyranosiduronic acid together with 2.5 g of gensenoside Ro [28-(-

β-**D**-glucopyranosyloxy)-28-oxoolean – 12-en-3β-yl 2-*O*-(β-**D**-glucopyranosyl)-β-**D**-glucopyranosiduronic acid were isolated from 21 kg of fresh young leaves (Romussi *et al.*, 1987). In the wood/bark of the plant the free triterpenoids β-amyrin and betulin and β-amyrin as the acetate are present (Pisova and Soucek, 1973). However, Krauze and Dziedzianowicz (1959) did not find saponins in the seeds of *Fagus silvatica* L. No information in support of the occurrence of significant concentrations of saponins in the fruits has been identified in the literature, including the comprehensive reviews of Hegnauer (1966, 1989). Krauze and Dziedzianowicz (1959) investigated extracts of seeds by the following methods: ability to form foam, reactions with a number of chemical reagents to detect the occurrence of saponins, haemolytic effect on blood from cattle, and toxicity to two aquatic organisms, tadpoles of edible frog (*Rana esculenta*) and guppy (*Lebistes reticulatus*). The overall conclusion is that the seeds did not contain any saponins. Whilst Krauze and Dziedzianowicz investigated seeds, there is no information on the content of saponins of whole unhusked beech mast or on the husk of beech mast.

No information has been found in support of the occurrence of cyanogenic glycosides or other cyanogenic compounds in any part of *Fagus sylvatica* L, including unhusked beech mast, neither in the comprehensive reviews of Hegnauer (1966, 1989) nor in other scientific databases (EFSA, 2007).

Up to the beginning of the 20th century, beech mast was of considerable importance for pig production in many European countries. Pigs were driven to the woods to feed on the fruit, and a few feeding trials have been performed (Broendegaard, 1979). Understandably, the beech mast also makes up a considerable part of the diet of wild boar (*S. scrofa*) (Groot-Bruinderink, 1977). Likewise deer - such as the sika deer - forage on beech mast (Obrtel *et al.* 1985) as do red deer, roe deer, and fallow deer (Anke *et al.,* 1980).

Although the above mentioned animal species seem to tolerate beech mast well, evidence exists that this botanical material may be toxic to horses and cattle (Wilkens and Cranwell, 1990; Hayes and Turner, 1990; Volker, 1950). Beckmann and Manz (1959) suggested that the toxicity is due to thiaminase. Another hypothesis is that the beech mast toxicity is due to oxalic acid as the symptoms observed were compatible with those of oxalate toxicity (Hayes and Turner, 1990). This interpretation is supported by the finding that the beech mast contains 2.41% non-water soluble and 0.54% water soluble oxalates (Krauze and Dziedzianowicz, 1959). In the leaves of *F. silvatica*, quinate, malate and oxalate are the dominating anions (Gabriel and Kesselmeier, 1999).

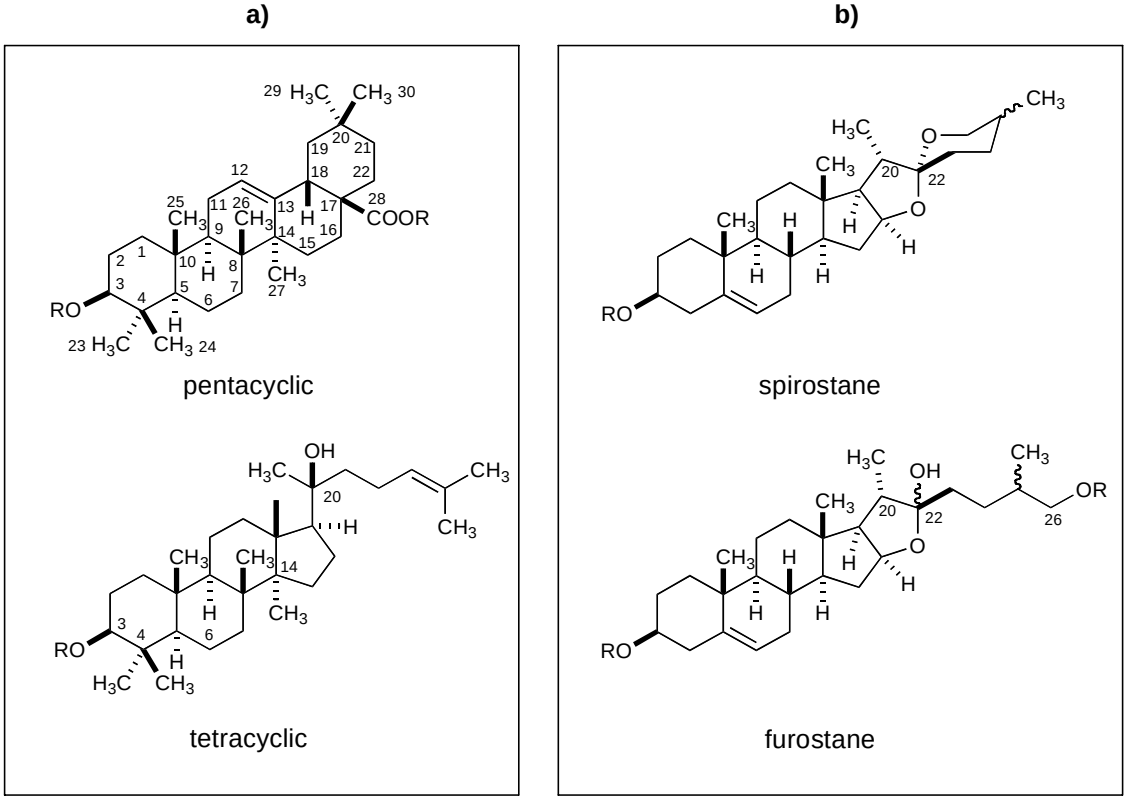
In conclusion, although there is some evidence that beechnuts and beech mast can be toxic to cattle and horses when consumed in larger quantities, the toxic syndrome is likely to be related to the presence of oxalates. Since beech mast does not contain any significant concentrations of saponins (or cyanogenic glycosides), it is not further discussed in this opinion.

3. Chemistry of saponins

Saponins are glycosides that, depending on the structure of the genin (aglycone), may belong either to the class of triterpenoid saponins or to steroidal saponins (Güclü-Üstündag and Mazza, 2007) (Figure 1a and 1b). The aglycone part of triterpenoid saponins can either be a pentacyclic triterpenoid or a tetracyclic triterpenoid, both containing 30 carbon atoms.

Figure 1. **Main classes of saponins: a) triterpenoid saponins, which can have either a pentacyclic or a tetracyclic triterpenoid as the aglycone; b) steroidal saponins, which are divided into spirostane and furostane types (in the furostane type, a double bond between C-20 and C-22 is often present as a result of water elimination). R indicates typical position of attachment of carbohydrate residue(s).**

Steroids are modified triterpenoids with a tetracyclic structure containing 27 carbon atoms. Steroids present in photosynthetic organisms are derived from cycloartenol, a cyclic triterpenoid (Rees *et al.*, 1968). The first step in this biosynthetic pathway of steroids is thus the oxidation of squalene via squalene 2,3-epoxide to form cycloartenol (Siegler, 1998a). Further transformations lead to opening of the cyclopropane ring of cycloartenol and loss of both methyl groups from C4 as well as of the methyl group from C14. Steroidal saponins typically contain either a spirostane or a furostane skeleton (Figure 1 b) (Sparg *et al.*, 2004;



Siegler, 1998 b). The aglycone may either have an A/B ring *trans* or an A/B ring *cis* relationship (annelation/annulation), but very often a double bond between C5 and C6 is present, as shown in Figure 1b. If the double bond is absent, the *trans* annulation is the most common, the *cis* annulation being known especially from the structurally related and clinically useful cardiac glycosides (Siegler, 1998b).

Whether steroidal or triterpenoid, saponins may be mono, bi- or tridesmodic. Monodesmodic saponins have a single sugar chain, normally attached at C-3. Bidesmodic saponins have two sugar chains, often with one attached through an ether linkage at C-3 and the other either attached through an ester linkage at C-28 or through an ether linkage at C-20 (pentacyclic and tertacyclic triterpene saponins, respectively), or through an ether linkage at C-26 (furostane saponins) (Güclü-Üstündag and Mazza, 2007). During the last decade some results have come

up to suggest that some triterpenoid saponins genuinely may occur as pyronyl-derivatives (chromosaponins) (Tsurumi *et al.*, 1992; Kudou *et al.*, 1994).

Most saponin-containing plants contain a complex mixture of various saponins. For example, soybeans contain saponins of three types, soyasaponins A, B and E, each type being categorized according to the number, linkage and kind of sugar moieties bound to the soyasapogenol (aglycone structure) (Güclü-Üstündag and Mazza, 2007). Similarly, at least 29 saponins based on a total of no less than 12 aglycones have been isolated and identified from the important feed plant alfalfa (*Medicago sativa* L.; Fabaceae) (Sen *et al.*, 1998).

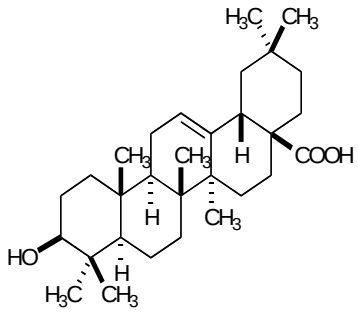
The total saponin content in plant tissues generally considered as saponin-bearing can vary considerably, from between 1.5-23 g/kg in seed crops such as quinoa and soybean, 100 g/kg in *Madhuca* seeds, up to 100-300 g/kg in quillaja bark and licorice root, respectively (Güclü-Üstündag and Mazza, 2007).

A few saponins are of economic importance as starting material for the semi-synthesis of other chemicals or as additives in different products. These include steroidal saponins such as dioscin from *Dioscorea* spp. and hecogenin from species of *Agave,* which are used as starting materials for industrial steroid hormone synthesis (Hardman, 1975; Bruneton, 1995). Furthermore, the pentacyclic triterpene saponin glycyrrhizinic acid found in the roots and rhizomes of *Glycyrrhiza glabra* (the liquorice plant, family Fabaceae) and present in liquorice confectionery is used as flavouring agent (FAO/WHO, 2006a) due to its characteristic aroma. Mixtures of up to 60 different pentacyclic triterpenoid saponins occurring in commercial extracts of the inner bark or wood of the pruned stems and branches of the *Quillaja saponaria* tree (family Rosaceae) are used as food additives, e.g. as foaming agents in soft drinks (FAO/WHO, 2002, 2004, 2006b).

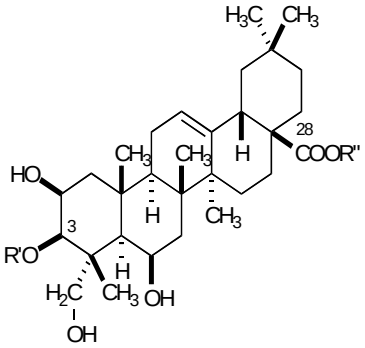
3.1. Saponins in *Madhuca*

Species of *Madhuca* contain pentacyclic triterpenoid saponins based on an oxygenated oleanolic acid skeleton (see Figure 2, Table 1). Thus, known *Madhuca* saponins contain protobassic acid (2β,6β,28-trihydroxyoleanolic acid), 16α-hydroxyprotobassic acid (2β,6β,16α,28-tetrahydroxyoleanoic acid), or their 2-oxo derivatives as aglycones, with sugar residues attached in most case to both C-3 and C-28 to form bidesmidic saponins (Li *et al.*, 1994; Jakhmola *et al*., 1987; Yoshikawa *et al*., 2000). New saponins are continuously being isolated and their structures determined (Lalitha *et al.*, 1987; Misra *et al.*, 1991; Nigam *et al*., 1992; Li *et al*., 1994; Yoshikawa *et al.*, 2000). However, structural details proposed for some of the isolated saponins are not reliable, and it is therefore difficult to conclude about the exact number of known *Madhuca* saponins. Structures shown in Table 2 should therefore be regarded as representative examples rather than as a comprehensive list.

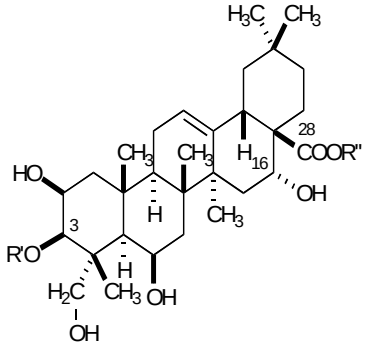
Figure 2. **Structure of oleanolic acid and of derived aglycones present in Madhuca saponins; R' and R'' indicate positions of sugar chains.**



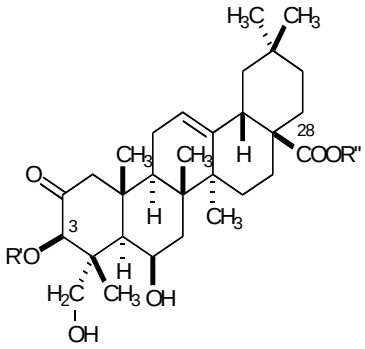
oleanolic acid



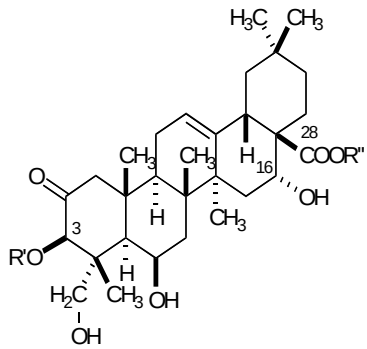
R' = R'' = H: protobassic acid



R' = R'' = H: 16α-hydroxyprotobassic acid



R' = R'' = H: 6β,28-dihydroxy-2-oxooleanolic acid



R' = R'' = H: 6β,16α,28-dihydroxy-2-oxooleanolic acid

Table 2. **Names and structures of representative saponins isolated from seeds of different species of *Madhuca*.**

Species of *Madhuca*	Saponin name	Structure	Reference
M. longifolia	Madlongiside A	6*β*,23-dihydroxy-2-oxooleanoic acid 28-*O*-α-L-arabinopyranoside	Yoshikawa *et al.* (2000)
M. longifolia	Madlongiside B	3-O-*β*-D-glucopyranosyl 6*β*,23-dihydroxy-2-oxooleanoic acid 28-*O*-α-L-arabinopyranoside	Yoshikawa *et al.* (2000)
M. longifolia	Madlongiside C	Protobassic acid acid 28-*O*-α-L-arabinopyranoside	Yoshikawa *et al.* (2000)
M. longifolia	Madlongiside D	Protobassic acid 28-O-*α*-L-rhamnopyranosyl(1-2)-*O*-α-L-arabinopyranoside	Yoshikawa *et al.* (2000)
M. longifolia M. butyracea	Mi-saponin A	3-O-*β*-D-glucopyranosyl protobassic acid 28-O-*β*-D-rhamnopyranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-arabinopyranoside	Yoshikawa *et al.* (2000); Kitagawa *et al.*(1975); Nigam *et al.* (1992)
M. butyracea	Butyroside A	3-O-*β*-D-glucopyranosyl protobassic acid 28-O-*β*-D-apiofuranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-rhamnopyranosyl(1-2)-*α*-L-arabinopyranoside	Nigam *et al.*(1992)
M. butyracea	Butyroside B	3-O-*β*-D-glucopyranosyl 16α-hydroxyprotobassic acid 28-O-*β*-D-apiofuranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-rhamnopyranosyl(1-2)-*α*-L-arabinopyranoside	Nigam *et al.*(1992)
M. butyracea	Butyroside C	3-O-*β*-D-glucuronopyranosyl protobassic acid 28-O-*β*-D-rhamnopyranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-rhamnopyranosyl(1-2)-*α*-L-arabinopyranoside	Li *et al.* (1994)
M. butyracea	Butyroside D	3-O-*β*-D-glucuronopyranosyl 16α-hydroxyprotobassic acid 28-O-*β*-D-apiofuranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-rhamnopyranosyl(1-2)-*α*-L-arabinopyranoside	Li *et al.* (1994)
M. butyracea	16α-hydroxy Mi-saponin A	3-O-*β*-D-glucopyranosyl 16α-hydroxyprotobassic acid 28-O-*β*-D-rhamnopyranosyl(1-3)-*β*-D-xylopyranosyl(1-4)-*α*-L-arabinopyranoside	Nigam *et al.* (1992)

From the bark of M. indica (now considered identical with M. longifolia ; ref "Specific background") two additional compounds (protobassic acid glycosides) were isolated (Pawar and Bhutani, 2004). Misra *et al.* (1991) claim on the basis of mild acidic hydrolysis followed by isolation of released aglycones, that protobassic acid is the **major** aglycone (in terms of yield) found in saponins from M. butyracea seed.

Apart from a claim that the protobassic acid is the dominating aglycone of *M. butyracea* saponins (see above), the relative concentrations of the different saponins present in *Madhuca* species are not available from the literature.

4. General toxicology and hazard assessment for humans

Due to the great structural diversity within the saponins group, a large variation is seen in their biological activities, both with regard to nature and potency of effects. Besides the general action of many saponins on membranes, a number of these compounds, depending on the structure of their aglycones, also cause specific systemic toxicity, following hydrolysis and absorption in the gastrointestinal tract. Usually, the glycosides have low oral bioavailability and toxicity, but when given intravenously many saponins often show strong toxicity and cause haemolysis (rupture of the erythrocyte membrane). The general membrane disrupting properties includes pore formation by an unknown mechanism (Johnson *et al.,* 1986; Francis *et al.,* 2002; Güclü-Üstündag and Mazza, 2007).

Toxicity of saponins in *Madhuca*

None of the single saponins isolated from any of the *Madhuca spp.* (whether isolated from seeds or from e.g. bark material) have been tested in any *in vivo* toxicity assay. However, toxicity studies and observations of toxic effects in feeding studies have been reported using crude extracts of total saponins or defatted seed meal from various *Madhuca* species.

The oral lethal dose (LD_{50}) in mice of crude *Madhuca* saponins (botanical source not given) was about 1.0 g/kg b.w. The saponins caused destruction and sloughing of the superficial layers of the intestinal mucous membranes followed by intense inflammation and some degree of absorption into circulation through damaged hyperaemic tissues (AICPR, 1980 cited by Jakhmola *et al.* 1987).

Liver and kidney sections of rats fed *Madhuca* crude protein with unknown amounts of saponins showed cytoplasmic vacuolation (AICPR, 1980 cited by Jakhmola *et al.*, 1987). In rats fed *M. latifolia* meal containing 50-60 g/kg of saponin at an inclusion rate of 10-12% in the feed (corresponding to 5-7.2 g saponin/kg feed), acute inflammation of the intestine and death within a month were observed (Mulky, 1976 cited by Shanmugasundaram and Venkataraman, 1985).

Male weanling (21 days old at start of experiment) Wistar rats were fed defatted *M. latifolia* seedmeal with a total saponin content of 104 g/kg at a 10% protein level (corresponding to 416g of *Madhuca* meal or 43.3 g of saponins /kg feed) for 12 weeks. Haematological data showed an increase in neutrophilic cells to 40 % in comparison with around 20% for all other treatment groups (Shanmugasundaram and Venkataraman, 1985).

In another rat study, defatted meal from seeds of *M. latifolia* containing 163 g/kg of protein, 7 g/kg of fat, 12 g/kg of tannins, and 71 g/kg of saponin, was included in the feed. The study was divided into two sub studies, the first using weaning Wistar rats weighing 40-50 g and the second using adult rats of the same strain weighing about 120 g (Cherian *et al.,* 1996). In the first experiment groups of weaning rats (n=12, 6 males and 6 females) were fed seed meal for 4 weeks at inclusion rates of 0, 10, 20, 30 or 40% of the basic rat feed (corresponding to 0, 7.1, 14.2, 21.3 or 28.4 g saponins/kg feed). In the second experimental groups of adult Wistar rats (n=6, 3 males and 3 females) were for a period of 32 days fed diets containing 10, 15 or 20% of seed meal (corresponding to 7.1, 10.6 or 14.2 g saponins/kg feed per day). All saponin treatment groups showed a marked reduction in feed intake. Young animals given the lowest inclusion rate (10% of the diet) consumed 2.5 g saponins/kg feed per day (corresponding to a daily saponin intake of about 0.44 g/kg b.w.) and the mortality was of 50 % after two weeks. In comparison, the control group ingested 7.5 g saponins/kg feed per day. In adult rats at the lowest dose, feed intake and absorbed nutrients were just enough for survival but the supply was not adequate to support growth. In the young animals which were more vulnerable than adults, the toxic action of mowrah seed meal with saponins became apparent in the absorptive cells of the intestinal mucosa, especially those near the tips of the villi. The concomitant intestinal inflammation caused an increased mucoid secretion from the goblet cells. At higher inclusion rates in the diet (20-40%), the damage extended to deeper layers of the intestine. Toxicity was also seen in the epithelium of the renal tubules (Cherian *et al*., 1996).

In vitro studies

Full haemolysis of red blood cells *in vitro* has been shown at 7 μM or more with a number of triterpenoid saponins (Voutquenne *et al*., 2002).

Analysis of 59 different triterpenoid saponins has shown that such haemolytic activity is highly dependent on the overall saponin structure including the number of sugar units, the sugar linkage(s), the substitutes on the sugar unit(s) and the nature of the aglycone. However, no simple conclusion on structure activity relationship could be drawn (Voutquenne *et al.*, 2002). The four *Madhuca* saponins madlongiside A-D were tested for cytotoxicity against a human gastric signet ring carcinoma cell line, KATO-III, but none of them showed any activity (concentrations not given) (Yoshikawa *et al.*, 2000).

4.1. Mutagenicity, genotoxicity and carcinogenicity

No studies on mutagenicity, genotoxicity or carcinogenicity of saponins from *Madhuca* species have been identified. Mutagenicity studies for other saponins (medicagenic acid, medicagenic acid 3-0-glucopyranoside from alfalfa roots; soya saponin from soya) did not show any mutagenicity in the Ames/Salmonella test (Czeczot *et al.*,1993; 1994). Carcinogenicity studies in rodents were not available for saponins. Information available for other triterpenoid saponins does not indicate a risk for such effects (FAO/WHO, 2006a; Wina *et al.,* 2005; Francis *et al.*, 2002).

4.2. Saponins in other plants

Because of their use in some foods and food related products, two different extracts of quillaia bark both containing triterpenoid saponins were assessed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2002, 2004, 2006a), as was the triterpenoid saponin glycyrrhizinic acid (FAO/WHO, 2006b) from the licorice root (*Glycyrrhiza glabra*).

Quillaia extracts: Two different products have been assessed:

- "unpurified extract" *Quillaia* extract (type 1), which is obtained by aqueous extraction of the milled inner bark or of the wood of pruned stems and branches of *Q.saponaria* Molina (family Rosaceae), followed by clarification and purification. The product contains triterpenoid saponins consisting predominantly of four different glycosides of quillaic acid with a saponin content of 200-260 g/kg), and

- "semi-purified extract" *Quillaia* extract (type 2), which is obtained by subjecting the aqueous extract to several clarification and purification steps: ultra-filtration or affinity chromatography, resulting in a saponin content of 750–800 g/kg).

For both products JECFA noted that little was known about the non-saponin fraction, which for both extracts contains polyphenols, tannins, salts such as calcium oxalate, carbohydrates and fat (FAO/WHO, 2004). The toxicity of *quillaia* extract (type 1) was low *in vivo* in comparison with parenteral administration. In mice, the acute oral LD_{50} was 1600 mg/kg b.w. Oral long-term studies in mice and rats mainly showed reduced feed intake, while reduced relative organ weight at the highest intakes and generally no histopathological effects attributable to treatments were found in the studies with no-observed-adverse-effect levels (NOAELs) of 0.5 and 1.0 % extract in the diets in mice and rats, respectively. JECFA established in 1985 an acceptable daily intake (ADI) of 0-5 mg/kg b.w. for *quillaia* extract (type 1) (FAO/WHO, 1986, 2002, 2004, 2006a). With regard to *Quillaia* extract (type 2),

JECFA concluded that the limited information available prevented the committee from establishing an ADI (FAO/ WHO, 2004).

Glycyrrhizinic acid has been isolated from the roots and stolons of licorice (*Glycyrrhiza glabra)*. The most significant adverse effect of glycyrrhizinic acid can be ascribed to the biological activity of its aglycone, the glycyrrhetic acid, which is formed by hydrolysis of glycyrrhizinic acid in the gut. Glycyrrhetic acid mainly inhibits type-2 11β-HSD in mineralocorticoid tissues and increases the cortisol concentrations resulting in retention of sodium and water, a syndrome of apparent mineralocorticoid excess. Based on information from effects in humans, JECFA concluded that an intake of 100 mg/day would be unlikely to cause adverse effects in the majority of adults (FAO/WHO, 2006b; Stoermer *et al.,* 1993; Schambelan, 1994; Kerlan *et al*., 1994; Haberer *et al*., 1984).

4.3. Saponins as adjuvants

Concerns have been raised about the possibility that saponins in food or feed may promote oral sensitisation to allergens through their action on the cell membrane in the gastrointestinal tract, resulting in enhanced uptake of the potential allergens (Johnson *et al.,* 1989). Indeed, saponins have been shown to act as oral adjuvants (Dalsgaard *et al.,* 1990). This was first shown for rabies vaccine in mice (Maharaj *et al.,* 1986), and has further been explored by adding cholesterol and phospholipids to the matrix in order to reduce the toxicity of the final vaccine (Skene and Sutton, 2006). Structure activity relationships have been studied with respect to the "immunostimulating" effects of saponins, giving rise to a very complex picture, as was also seen in the structure-activity studies on haemolytic effect and cytotoxicity, and no simple conclusion could be drawn (Press *et al.*, 2000; Oda *et al.*, 2000). Indeed, enhanced permeability of biological membranes such as frog skin (Blankemeyer *et al.*, 1997), and transmucosal passage of proteins as a result of contact with steroidal glycoalkaloids and with saponins, have been shown *in vivo* (Gee *et al.,* 1997). Ongoing research has very recently confirmed these effects, as stimulation of the immune response to ovalbumin in mice was seen with the use of *Anemone raddeana* saponins as an adjuvant (Sun *et al.*, 2008).

4.4. Toxicity of oil from *Madhuca* species

Two studies in rats fed *Madhuca* seed oil have been published. Whilst weanling albino rats fed alkaline-refined edible grade mahua oil (from *Madhuca latifolia*) at an inclusion rate of 10% in the diet for fourteen weeks responded as the control rats, a subsequent reproductive study showed poor reproductive performance in the second generation. Histological studies showed bilateral testicular atrophy with degenerative changes in the seminiferous tubules. The effect was apparently reversible (Rukmini, 1990). Similar effects were observed in Wistar rats fed mahua oil of unknown quality mixed into other edible oils such as red palm oil (Manorama *et al.,* 1993*).* These effects may be of importance if whole seeds or seedmeal with significant oil residues are used in feed.

4.5. Possible beneficial effects of saponins

Saponins from a variety of sources have also been shown to have a range of biological activities and potential health benefits such as hypocholesterolemic, anti-coagulant, anti-carcinogenic, hepato-protective, hypoglycemic, immunomodulatory, neuroprotective, anti-inflammatory anti-oxidant activity, inhibition of dental caries, and platelet aggregation (Rao and Gurfinkel, 2000; Güçlü-Üstündağ and Mazza, 2007.) They might also be used in the

treatment of hypercalciuria and have also been found to significantly affect growth, feed intake and reproduction in animals. Saponins have also been observed to kill protozoans and molluscs, and act as antifungal and antiviral agents. To date there are very few human data and most of the data have been obtained in *in vitro* cell systems or in animal studies. The reader is referred to recent reviews (Rao and Gurfinkel, 2000; Francis *et al*., 2002; Shi *et al*., 2004).

5. Method of analysis

No validated method for the determination of saponins in feeding stuff, including *Madhuca*, is found in the EU Directive 71/250/EEC and no validated method from the Association of Official Analytical Chemists (AOAC) exists.

Two common and non-specific methods of screening for the occurrence of significant concentrations of steroidal and/or triterpenoid saponins in a plant rely on (1) their general physico-chemical characteristics, e.g. formation of stable foam (Hansen *et al.*, 2003), or (2) their haemolytic action *in vitro* (Lemmiche *et al*., 1995). Preferably, chemical analysis should be performed for total or individual saponins as discussed below.

5.1. Extraction

Due to the ability of certain saponins to facilitate the formation of foam/emulsions, care must be taken during extraction and pre-analytical extract purification steps to avoid this. Saponins are traditionally extracted into water/ethanol mixtures, after which the ethanol is removed by evaporation and the saponins extracted from the water phase into 1-butanol (Madl *et al.*, 2006; Brimer *et al.*, 2007). During the last decade there have been considerable efforts to improve this methodology, mainly on the extraction of ginseng saponins and glycyrrhizic acid, and also escin from horse chestnut. These extraction studies were recently reviewed by Güclü-Üdtündag and Mazza (2007). Especially the use of supercritical CO_2 extraction in combination with modifiers such as methanol, ethanol or aqueous methanol has proven successful (Güclü-Üdtündag and Mazza, 2007).

Due to the presence of a lipid-soluble aglycone and water soluble sugar chain(s) in their structure, saponins are surface active compounds. In aqueous solutions they form micelles as concentration reaches a critical level. Thereby they have solubilisation properties for other compounds (Güclü-Üstündag and Mazza, 2007). However, due to the inherent relatively low solubility of many saponins, both in water and in a number of more lipophilic solvents, it may be difficult to keep them in solution for analysis, as the addition of other compounds (even other saponins) may enhance the solubility of the saponin(s) in question for analysis (Güclü-Üstündag and Mazza, 2007). Due to these problems, when doing analysis on saponins, one should be observant on whether the compounds are dissolved.

5.2. Chemical determination

High performance liquid chromatography (HPLC) is the method of choice for the separation of saponins (Oleszek, 2002; Oleszek and Bialy, 2006). Both normal phase and reverse phase columns have been used. However, reverse phase HPLC, mostly by the use of C_{18} columns and gradient elution, seems to be the preferred method. As most of the saponins do not possess chromophoric groups, either spectrophotometric detection with pre-column derivatisation with e.g. benzoyl chloride has been used (Oleszek, 2002), or detection with electrospray ionization mass spectrometry is possible (Fan *et al*, 2006; Madl *et al.*, 2006).

As an alternative to chromatographic analysis described above, enzyme-linked immunosorbent assays (ELISA) have been applied to extracts of ginseng derived drugs in a study comparing the ELISA method with the HPLC analysis (Fukuda *et al.*, 2000). Also many other studies are available in recent literature, mostly dealing with ginsenosides as above or other saponins of medicinal interest such as saikosaponins (Zhu *et al.*, 2006).

A bottleneck for the development and use of modern validated methods for saponins, and *Madhuca* saponins in particular, is the unavailability of pure reference standards. However, for a large number of saponins essential data for the ease of compound identification - both during purification from plant sources and during analysis - such as nuclear magnetic resonance (NMR) chemical shifts -, have been collectively published (Agrawal *et al.*, 2005).

5.3. Bioassays

The haemolytic activity of saponins can be used as an alternative method to chemical analysis to detect and (semi)-quantify mixtures and single saponin compounds. However, these methods do not identify the saponin(s), but can be used to compare the total saponin content of different batches of the same type of material. Details on haemolytic actions have recently been reviewed (Oleszek, 2002).

Reviewing alfalfa saponins and the implication for their occurrence in animal feed, Sen *et al.* (1998) highlighted that there is an urgent need to improve existing methods and develop simple and specific methods for the quantification of saponins. The saponin analysis of plants used for feed or feedingstuff requires information on (1) which are the relevant saponins from a feed safety point of view, and (2) how these are identified and quantified.

6. Statutory limits for saponin containing plants in feed materials

Annex 1 to Council Directive 2002/32/EC[9] contains a list of compounds/substances that are undesirable in animal feed and their maximum levels in different feed commodities. The current EU maximum levels for certain feed materials containing saponins are given in Table 3. It should be noted that the saponin containing plant materials in table 3 refer to botanicals such as seeds and fruit of the plants.

Table 3. **EU legislation on saponin containing plant materials used as feed.**

Undesirable substances	Product intended for animal feed	Maximum content in mg/kg relative to a feedingstuff with a moisture content of 12%
Mowrah, Bassia, Madhuca — Madhuca longifolia (L.) Macbr. (= Bassia longifolia L. = Illiped malabrorum Engl.) Madhuca indica Gmelin (= Bassia latifolia Roxb.) = Illipe latifolia (Roscb.) F. Mueller)	All feedingstuffs	Seeds and fruit of the plant species listed opposite as well as their processed derivates may only be present in feedingstuffs in trace amounts not quantitatively determinable

In the control of feed materials for compliance with this directive, light microscopy is the principal means of detection used for botanical impurities. The successful identification of contaminants depends on several factors including the skill of the operator, the availability of reference material and the degree in which the sampled material has been processed.

[9] OJ L 140, 30.5.2002, p. 10–22

Comminution and heat/pressure treatments can destroy much, or all, of the anatomical/histological feature on which identification is based. Hence this method is time consuming and less accurate than chemical analysis.

7. Occurrence in feed material

No data were received after a call regarding saponin levels in *Madhuca* was launched to the EU member states.

***Madhuca*:** From a commercial point of view, the two major species of the genus *Madhuca* are *Madhuca indica* (syn. *Bassia latifolia*) and *Madhuca longifolia* (syn. *Bassia longifolia*). Madhua is the widely accepted local name for the fat and cake from both these species[10]

Following oil extraction, the remaining 'cake' may contain up to 200 g/kg raw saponins (Hegnauer, 1973). A number of processes have been examined as means of reducing the saponin content (Shahal and Sharma, 1992) in the cake. Jakhmola *et al.* (1987) reported that "simple water treatment of *Madhuca* cake to reduce the saponins content appears to improve the utilisation potential of the press cake as animal feed", but they add that further studies are required. Since then a number of investigations have studied whether the processing of seed or cake may improve acceptability of the seed products as food or feed. This has been achieved in a number of different ways, including urea-ammoniation, and soaking in cold or hot water (Katiyar *et al.*, 1991, Singh and Singh, 1991; Shanmugasundaram and Venkataraman, 1985, 1989; Saxena *et al*., 2002).

There is no information on occurrence of *Madhuca* as a botanical impurity in feed.

8. Estimated intake by farm livestock

The saponins present in *Madhuca* cake reduce its value as a feed for livestock. Because of this, and its generally low nutritional value, *Madhuca* cake is not imported into the EU. In producing countries *Madhuca* cake is commonly used as a fertiliser, but its protein can potentially contribute to the protein economy of livestock production. Consequently, attempts have been made to develop methods for reducing the saponin content of *Madhuca* cake and, as discussed above, these trials have been met with some success. As a result *Madhuca* cake is used to a limited extent in countries where the seeds are harvested and processed.

Since oil extraction of *Madhuca* is unlikely to occur where other oilseeds are being processed, cross contamination of other animal feeds imported into the EU with *Madhuca* is very unlikely. *Madhuca* cake is not, to our knowledge, fed to livestock in the EU and saponin exposure from this source cannot therefore be estimated.

9. Adverse effects on livestock

Although there are some common effects, the toxicity of saponins from different plant sources varies considerably (see section 4). As described in chapter 2, there are many saponin-containing plants that are commonly used as feed materials. An assessment of the potential adverse effects on livestock from these saponins is beyond the scope of this opinion.

[10] Source: National Oilseeds and Vegetable Oils Development Board, Ministry of Agriculture, Government of India (www.novodboard.com)

Studies investigating the toxicity of *Madhuca* saponins as individual compounds/mixtures are not available. Moreover, toxicity studies using crude extracts of the plant on monogastric target animals are scarce. Results from studies on ruminants indicate that they are more tolerant to *Madhuca* seeds than non-ruminants.

9.1. Ruminants

A number of feeding experiments have been performed in cattle where *Madhuca* press cakes (i.e from *M. latifolia or M. butyracea*) or further processed (extracted) cakes (to reduce the saponin content), were fed to ruminants. In general these feed materials were reasonably accepted and had no significant effect of body weight gain, or other toxicological effects, at inclusion levels of around 20% crude press cake in the diet (Jakhmola *et al.,* 1987). Evidently, *Madhuca* saponins affect negatively crude protein digestibility (Jakhmola *et al.,* 1987), as observed in rat studies reviewed previously in this opinion (section 1.2). The longest experiment included in the review is a 200 days trial on buffalo calves. Unprocessed *Madhuca* cake at an inclusion rate of 20% did not affect their growth rate adversely, but 30% did so. It should be noted that the literature reviewed includes studies on cattle as well as on buffaloes. A few relevant studies have been published after the review (by Jakhmola *et al.*, 1987; Tiwari *et al.,* 1996) showed a significantly improved weight gain, but a decreased protein digestibility in four 12-14 month old crossbred calves fed a standard complete feed diet containing 20% *M. latifolia* seed cake. The experiment was a cross-over study where all four first received the standard diet, and after this the diet with *Madhuca* seed meal. The *Madhuca* feeding period was 23 days. However, the latter diet had a slightly higher crude protein content of around 17 % and a higher fat content, around 1.87 %, in comparison with the standard diet with 15.5 % protein and 0.68 % fat (Tiwari *et al.,*1996).

The cellulose digestion, microbial protein synthesis, and total volatile fatty acid (TVFA) production was studied in an *in vitro* ruminal degradation set-up. With a test period of 24 h the cellulose digestion was reduced from around 25 % to about 6 % when the level of crude total seed saponin (called mowrin) was increased from 0 to 2.5 mg/ml. Microbial protein synthesis was reduced from 41 to 9.4 mg/flask, while the TVFA production was reduced from 8.9 to 1.8 meq/100 ml (Chahal and Sharma, 1991).

In conclusion, an inclusion level of *Madhuca* seed cakes up to maximum 20 % of the total diet does not seem to negatively affect ruminants.

Reported toxicity of other saponins in the feed includes different levels of gastroenteritis, diarrhoea and secondary photosensitization, following intake of plants containing different steroidal saponins, causing liver degeneration and reduced excretion of phylloerythrin (from chlorophyll degradation) in sheep (Wina *et al.*, 2005; Francis *et al.*, 2002; Kellerman *et al.,* 1994). Photosensitization does not seem to be a hazard expected from *Madhuca* saponins which are pentacyclic triterpenoids.

9.2. Horses

No studies were identified.

9.3. Pigs

No studies have been identified where *Madhuca* materials/saponins have been administered to pigs.

Toxicity of other saponins in the feed was studied using *quillaja* saponins, and reduced growth and feed utilisation in suckling piglets after the sow was fed the saponins was observed (Ilsley and Miller, 2005). A study including close to 200 weaned piglets (about 100 of each sex) concluded that quillaja saponin at a level of 750 mg/kg of diet for one week, and 300 mg/kg for the next two weeks, had no effect on the piglet growth, however, it did have a detrimental effect on the feed utilization (Ilsley *et al.*, 2005). In another study quillaja saponins at levels up to 500 mg/kg feed did not adversely affect growth performance and immune function of weanling pigs challenged with *Salmonella typhimurium* (Turner *et al.*, 2002).

9.4. Poultry

An Indian report stated that "mahua" cake in chick mash at approximately 12% in feed caused mortality of all chicks within five hours (AICPR, 1979, cited in Jakhmola *et al.*, 1987)

Quillaja saponins fed at 0.9% in feed caused reduced weight gain in chickens (Jenkins and Atwal, 1994).

9.5. Rabbits

No studies were identified.

9.6. Dogs

No studies were identified.

9.7. Fish

No toxicity studies after dietary exposure to *Madhuca* seeds have been identified. Two saponins (called A and B) from the defatted seed flour of *M. butyracea* showed a LC_{50} value for guppy fish of 11 and 14 mg/L, respectively (Lalitha *et al*., 1987). The structures of the individual saponins were not known.

Other saponins have shown toxicity to fish at concentrations as low as 1.5 mg/L (Cannon *et al*., 2004). Such toxicity has also been shown in molluscs (Brimer *et al*. 2007). However, in spite of such high toxicity in fish, the triterpenoid saponin fraction of quillaja acted as a growth promoter for farmed Nile tilapia (*Oreochromis niloticus*) when added to the diets at levels of around 100-300 mg/kg (Francis *et al.*, 2005).

10. Toxicokinetics, metabolism and tissue distribution

Saponins are metabolised by microbial hydrolysis in the gut, e.g glycyrrhizic acid, so that the aglycone of the saponin may be absorbed and the glycoside part is metabolised through common pathways (Ploeger *et al*., 2001; Stoermer *et al*. 1993). In some instances, the whole molecule has been shown to be absorbed (Brimer *et al*., 2007; Han *et al.*, 2006). Following damage to the intestinal wall, most saponins, including those of *Madhuca* saponins, may enter the systemic circulation (AICPR, 1980, cited in Jakhmola *et al*., 1987). The distribution, metabolism and excretion of the aglycone is compound-specific and has been described for saponins in licorice, ginseng, soya in rodents (Güclü-Üdtündag and Mazza, 2007).

Except for data on uptake in mice with intestinal damage (AICPR, 1980, cited in Jakhmola *et al.* 1987), there are no data available on the toxicokinetics of saponins from *Madhuca*.

11. Carry-over and residues

There are no data available on the carry over of saponins from *Madhuca* in meat, meat products, fish, milk or eggs.

12. Human dietary exposure

Madhuca products are not consumed by humans. Human dietary exposure to *Madhuca* saponins through the consumption of food of animal origin is very unlikely, since *Madhuca* meal is not used in the EU as a feed material. The only potential source of exposure would be the consumption of imported meat products, but this is very unlikely.

Human exposure to *Madhuca* saponins, if occurring, might be through its non-food use as a detergent.

Overall, human exposure to *Madhuca* saponins within the European Union can be considered as negligible.

CONCLUSIONS

Chemistry, occurrence in plants and parts used as feed materials

- Saponins are low molecular weight secondary plant metabolites containing either a triterpenoid or steroidal aglycone, and one or more sugar chains; saponins can form stable foam in aqueous solutions. Saponins are widely distributed in the plant kingdom and vary greatly in structure, physico-chemical properties and biological effects.

- Traditionally, saponins have been extensively used as detergents, piscicides and molluscicides, in addition to industrial applications as foaming and surface active agents.

- Apart from *Madhuca* species (genus *Madhuca* Hamilton ex Gmelin), saponins occur in significant concentrations in a number of feed and food plants such as alfalfa (*Medicago sativa* L.), soybean (*Glycine max* L.), quinoa (*Chenopodium quinoa* Willd.) and balanites (*Balanites aegyptiaca* L.).

- Saponins of *Madhuca* contain pentacyclic triterpenoids as aglycones; the saponin content in the seeds is about 100 g/kg. The seeds of several species of *Madhuca* are rich in oil, and the seed oil is mainly used for non-food purposes such as production of laundry soap and biodiesel. The oils have also been tested as cocoa butter extenders. The press cake contains >200 g/kg of saponins and is unsuitable as feed. Therefore, it has traditionally been used only as a fertilizer. A reduction of the saponin content in *Madhuca* seed products, especially seed cake/flour, can be achieved to make them acceptable as animal feed ingredients.

- There are no validated official methods in EU for the determination of saponins in feeding stuff, including *Madhuca*, and no validated AOAC methods exist. Pure reference standards of *Madhuca* saponins as well as other saponins are generally lacking. Since most saponins lack chromophores, liquid chromatography – mass spectrometry (LC-MS) techniques would be the analytical methods of choice. Common, but non-specific, screening methods for saponins are based on their ability to form foam or on their haemolytic activity.

- In the Terms of Reference, unhusked beech mast was also requested to be considered with respect to its possible content of saponins or cyanogenic glycosides and their relative importance for toxicity. Beech mast does not contain any significant concentrations of saponins and apparently no cyanogenic glycosides. The likely cause of toxicity to cattle and horses is their high content of oxalates. Therefore, beech mast was not further discussed in this opinion.

General toxicological effects

- In food and feed, saponins often have an "anti-nutritional" effect or cause toxic effects. Many saponins have a general action on lipid membranes and cause lysis of red blood cells *in vitro* and *in vivo* by intravenous administration. In general, the saponins have low oral bioavailability and toxicity, but they may be hydrolysed in the intestinal tract and cause systemic toxicity, depending on the structure and absorption of the aglycone. Some saponins have an adjuvant effect.

- Saponins from a variety of sources are claimed to have beneficial health effects; however, the data indicating this potential are mostly from *in vitro* studies or from animal models.

- No individual saponin isolated from any of the *Madhuca* species has been tested in *in vivo* toxicity assay. Toxicity studies and observations of toxic effects in feeding studies have been reported using crude total saponins or de-fatted seed meal from various *Madhuca* species.

- The oral LD_{50} in mice of crude *Madhuca* saponins (exact botanical source not given) was about 1.0 g/kg body weight. At high doses, in mice and rats, *Madhuca* saponins caused gastro-intestinal, liver and kidney toxicity. At lower doses *Madhuca* saponins caused feed refusal and starvation with reduced body weight gain and increased mortality.

- Although, no studies on mutagenicity, genotoxicity and carcinogenicity of saponins from *Madhuca* species have been identified, studies on other saponins do not indicate a genotoxic or carcinogenic potential.

- Mahua oil (oil from *Madhuca latifolia*) causes bilateral testicular atrophy with degenerative changes in the seminiferous tubules in rats. The Panel confirms that saponins from *Madhuca* are the substances mainly responsible for the toxicity of *Madhuca longifolia* in animal feed.

- Because of the limited data available, no health-based guidance value (ADI, TDI) can be established for *Madhuca* saponins.

Adverse effects of Madhuca saponins in target animals

- Toxicity studies on oral exposure of *Madhuca* saponins as individual compounds/mixtures are not available.

- Results from studies on *Madhuca* seed cakes on ruminants indicate that they are more tolerant to *Madhuca* seeds than monogastric animals. An inclusion level of *Madhuca* seed cake up to maximum 20% of the total diet does not seem to negatively affect ruminants.

- Toxicity studies of *Madhuca* seeds on monogastric target animals are scarce. *Madhuca* cake in chick mash at an inclusion rate of about 12% was lethal. No studies have been conducted on horses, pigs, rabbits or dogs.

- No toxicity studies after dietary exposure of *Madhuca* seeds were identified in fish. Two saponins (structure not determined) from defatted seeds of *M. butyracea* showed a LC_{50} value for guppy fish of 11 and 14 mg/L, respectively.

- Although the information about occurrence and toxicity of saponins from *Madhuca* is rather limited, further toxicological studies are currently not needed because of negligible exposure to target animals and humans within the EU.

Madhuca saponins in feed materials

- There is no information on occurrence of *Madhuca* as a botanical impurity in feed. The saponins present in *Madhuca* reduce its value as feed for livestock, and the material is not imported into the EU either as whole seeds or as the meal. In producing countries, *Madhuca* cake is used to a limited extent for its high protein content.

Fate in animals and carry-over

- There is no information on the fate and carry over of *Madhuca* saponins in animals.

Human exposure

- *Madhuca* products are not consumed by humans, therefore human dietary exposure to *Madhuca* saponin through the consumption of animal products is very unlikely and can be considered as negligible, since *Madhuca* meal is not used in the EU as a feed material.

RECOMMENDATIONS

- Analysis of plants used for feed or feeding stuffs for saponin content requires (1) information on saponins which are relevant from a feed safety point of view, and (2) availability of appropriate analytical methods. Key saponins for important feed plants should be chosen, and compound-specific analytical methods should be developed. Since pure compounds/saponin standards are generally lacking, such standards should be made available.

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ABBREVIATIONS

ADI	Acceptable daily intake
AICRP	All India Coordinated Research Project on Utilization of Agricultural By-products
AOAC	Association of Official Analytical Chemists
b.w.	Body weight
CSIR	Council of Scientific and Industrial Research
d.w.	Dry weight
ELISA	Enzyme-Linked Immunoabsorbent Assay
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
f.w.	Fresh weight
HPLC	High-performance liquid chromatography
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Lethal dose – the dose required to kill half the members of a tested animal population
NMR	Nuclear magnetic resonance
NOAEL	No-observed-adverse-effect level
TDO	Tolerable daily intake
TVFA	Total volatile fatty acid
WHO	World Health Organisation