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Risk assessment of herbal preparations containing *Huperzia serrata*

RIVM letter report 2024-0028
J. A. de Heer | L. de Wit-Bos

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Synopsis

Risk assessment of herbal preparations containing *Huperzia serrata*

Herbal preparations (food supplements) containing extracts of the herb *Huperzia serrata* are sold in the Netherlands. These herbal preparations are mainly available online. According to the manufacturers, *Huperzia serrata* can support memory and improve concentration.

Herbal preparations containing this herb turn out to be harmful to human health. RIVM advises consumers not to use herbal preparations with *Huperzia serrata*, especially during pregnancy. The number of people using such products is not known.

People can experience acute undesirable effects on the cholinergic system. Examples include increased salivation, muscles weakness, abdominal cramps, diarrhoea, blurred vision, lacrimation and paralysis. There are also indications that *Huperzia serrata* is harmful to the unborn child during pregnancy. These health effects can already occur when people take the advised dose.

The effects are caused by constituents of *Huperzia serrrata*, of which huperzine A is most studied. People using herbal preparations with *Huperzia serrata* extract ingest enough huperzine A to experience the harmful effects. No information is available on other constituents in *Huperzia serrata*, apart from an indication that eight compounds, other than huperzine A, may also inhibit the enzyme acetylcholinesterase. It is therefore possible that these eight compounds can enhance the effect of huperzine A.

Keywords: food supplements, huperzine A, *Huperzia serrata*, safety

Publiekssamenvatting

Risicobeoordeling van kruidenpreparaten met *Huperzia serrata*

In Nederland worden kruidenpreparaten (voedingssupplementen) met extracten van het kruid *Huperzia serrata* verkocht. Deze kruidenpreparaten zijn vooral online verkrijgbaar. Volgens fabrikanten kan *Huperzia serrata* het geheugen ondersteunen en de concentratie verbeteren.

Kruidenpreparaten met dit kruid blijken schadelijk te zijn voor de gezondheid. Het RIVM adviseert consumenten daarom geen kruidenpreparaten met *Huperzia serrata* te gebruiken, vooral niet tijdens de zwangerschap. Het is niet bekend hoeveel mensen dit product gebruiken.

Mensen kunnen last krijgen van wat we noemen acute ongewenste effecten op het cholinergische systeem. Voorbeelden daarvan zijn meer speekselproductie, zwakke spieren, buikkrampen, diarree, wazig zicht, tranende ogen en verlamming. Er zijn ook aanwijzingen dat *Huperzia serrata* schadelijk is voor het ongeboren kind tijdens de zwangerschap. Deze gezondheidseffecten kunnen al ontstaan als mensen de aanbevolen hoeveelheid gebruiken.

De effecten worden veroorzaakt door de stoffen in het kruid; huperzine A is daarvan de meest onderzochte stof. Mensen die kruidenpreparaten met *Huperzia serrata* gebruiken, krijgen genoeg huperzine A binnen om schadelijke effecten te kunnen ervaren. Over andere stoffen in *Huperzia serrata* is geen informatie bekend, behalve een aanwijzing dat acht stoffen, net als huperzine A, de werking van het enzym acetylcholinesterase remmen. Daarom is het mogelijk dat deze acht stoffen het effect van huperzine A kunnen versterken.

Kernwoorden: voedingssupplementen, huperzine A, *Huperzia serrata*, veiligheid

Contents

Summary

Introduction

In December 2020, the Minister for Medical Care and Sport of the Ministry of Health, Welfare and Sport (VWS) announced actions that would be taken to better regulate food supplements and herbal preparations in the Netherlands, thereby facilitating enforcement. One of those actions is to expand the list included in the Herbal Preparations Decree of the Dutch Commodities Act[1] with substances/botanicals that are either forbidden or restricted (i.e. subject to a maximum level) in food supplements or herbal preparations (Van Ark, 2020). In order to determine whether a substance or botanical needs to be included in this list, a risk assessment is warranted. The selection of substances and botanicals chosen for risk assessment was based on the prerequisite that the substances/botanicals are sold on the Dutch market and (widely) used and there are indications for possible health risks, e.g. Rapid Alert System for Food and Feed (RASFF) reports, from enforcement institutes. The current risk assessment is about herbal preparations containing *Huperzia serrata* (Thunb.) Trevis[2] extract.

H. serrata is also known as Chinese clubmoss and in Traditional Chinese Medicine as Qian Ceng Ta (Ma et al., 2007). Most herbal preparations containing *H. serrata* specify the huperzine A content of the product. Huperzine A is one of the active constituents and the only well-studied alkaloid in *H. serrata*.

Currently, there are no specific restrictions for the use of *H. serrata* extract in herbal preparations included in the Herbal Preparations Decree of the Dutch Commodities Act. In addition there are no European legislations for the herb *H. serrata*.

Previous evaluations

H. serrata (aerial parts) is mentioned in EFSA's compendium of botanicals, due to the presence of huperzine A and huperzine B (EFSA, 2012a).

In 2001, RIVM conducted a limited risk assessment of food supplements containing huperzine A (RIVM, 2001). Huperzine A showed inhibitory acetylcholinesterase (AChE) activity. The critical endpoints were cholinergic toxicity and embryotoxicity. In this assessment, the calculated margin of safety (MOS) for food supplements containing huperzine A at the expected intake was 13 and 33 for embryotoxicity and cholinergic toxicity, respectively. As this is far below the minimal MOS of 500[3] it was recommended to set maximum use levels (RIVM, 2001).

In 2016, DTU assessed the risk of exposure to a specific food supplement containing 2 mg huperzine A (DTU, 2016). DTU concluded

[1] https://wetten.overheid.nl/BWBR0012174/2020-07-01. Accessed December 2023.
[2] Further revered to as *H. serrata*.
[3] Minimal MOS of 500 takes into account an uncertainty factor for intra- and interspecies differences (factor of 10 each) and an additional uncertainty factor for the insuffisient quality of the data (factor 5).

that this dose poses a health risk based on the inhibition of AChE (DTU, 2016). It was not assessed if lower exposures also pose a health risk.

Products on the Dutch market

Several herbal preparations containing *H. serrata* extract were found on the Dutch market. Some herbal preparations only contain *H. serrata* extract and others contain a combination of various other ingredients, including other (extracts of) herbs. Herbal preparations containing *H. serrata* extract are marketed for two different target populations: the general population and sport participants (pre-workout supplements). The herbal preparations for the general population are marketed as supporting normal functioning of the brain, concentration, focus and memory or enhancing memory. The pre-workout supplements are marketed to boost energy and focus. They are available as capsules containing 0.05 to 0.2 mg huperzine A or as powder containing 10 mg *H. serrata* per scoop. The recommended use ranges from 1 to 4 capsules or one scoop.

Exposure

For herbal preparations containing *H. serrata* extract available on the Dutch market, the estimated exposure to huperzine A ranged from 0.05 to 0.8 mg per day (i.e. 0.7–11.4 µg/kg bw per day for an individual weighing 70 kg), based on the recommended use of the products and the reported dose of huperzine A.

Biological data

- Huperzine A is absorbed relatively fast and distributed throughout the whole body. Plasma half-life is found to be around 5 – 12 hours. Huperzine A can also pass the blood-brain barrier and the placenta. Part of huperzine A is excreted unchanged in urine, while the rest is metabolised.
- Huperzine A inhibited the enzyme AChE (Laganière et al., 1991) and bound to the N-methyl-D-aspartate receptor as an inhibitor (Wang et al., 1999).
- Acute oral exposure to huperzine A led to (adverse) effects on the cholinergic system, liver and intestine motility in mice and rats (Zhang et al., 2013; Ma et al., 2003b; Schmidt & van der Staay, 1998). A Lowest Observed Adverse Effect Level (LOAEL) of 0.3 mg huperzine A per kg bw was derived for acute cholinergic adverse effects in rats.
- After subchronic exposure to huperzine A, adverse effects were observed in the brain, intestine and heart of different animal species. Also sperm cell growth was reduced in some animal species (Yu et al., 1993).
- Huperzine A was not genotoxic according to the available studies. However, the studies did not comply with the relevant internationally approved test guidelines for genotoxicity assays (Tu & Wu, 1990; Yu et al., 1993). Therefore, it is not possible to adequately evaluate the genotoxicity of huperzine A.
- No chronic toxicity or carcinogenicity studies were identified.
- Studies on reproductive and developmental toxicity indicated that huperzine A was embryotoxic in rabbits and mice, although these

studies did not comply with the relevant internationally approved test guidelines (Yu et al., 1993).
- The National Poisoning Information Centre (NVIC) received two notifications about adverse effects in patients who took an overdose of herbal preparations containing only *H. serrata*. Effects included nausea, tremors, dysarthria, diarrhoea and blurred vision (NVIC personal communication).
- A case report described the adverse cholinergic effects seen in two patients after consumption of tea with *Lycopodium selago*, a plant from the same family and genus as *H. serrata* which also contains huperzine A.
- Several clinical trials with huperzine A were identified. The observed adverse effects – including diarrhoea, vomiting and dizziness – were mild and not statistically significantly different between the control and experimental groups in the trials (Xu et al., 2012; Zhang et al., 2002; Xu et al., 1995; Zhang et al., 1991). One trial mentioned that adverse effect were not related to the treatment (Rafii et al., 2011).

The website natural medicines reported two possible pharmacokinetic interactions of huperzine A, i.e. with scopolamine, an anticholinergic drug, and cholinergic drugs[4].

No safe use level

As a first step in the risk assessment, it was investigated whether the presumption of safety can be applied to *Huperzia serrata*. Botanical preparations for which an adequate body of knowledge exists, can benefit from a presumption of safety without any need for further testing (EFSA, 2009; EFSA, 2014). The presumption of safety cannot be applied to *H. serrata* and more information was needed to assess its safety.

It was not possible to establish a health-based guidance value (HBGV) for *H. serrata* extract, nor for huperzine A which is one of the active constituents of *H. serrata*.
An Acute Reference Dose (ARfD) could not be derived as the genotoxicity of huperzine A could not be adequately evaluated.
An Acceptable Daily Intake (ADI) could also not be established as the toxicological dataset for huperzine A was insufficient. No reproductive toxicity studies, nor oral toxicity studies with a duration longer than 30 days were identified and genotoxicity could not be adequately evaluated. Furthermore, the were unresolved concerns regarding developmental toxicity. As no HBGV could be established, no safe use level for herbal preparations containing *H. serrata* extract or huperzine A could be determined.

In different animal species, acute cholinergic overstimulation was observed after exposure to huperzine A. In rats, a LOAEL of 0.3 mg/kg bw huperzine A was identified, which was used in the risk assessment to assess the acute effects of huperzine A.

[4] https://naturalmedicines.therapeuticresearch.com/databases/food,-herbs-supplements/professional.aspx?productid=764.

Risk assessment

For the risk assessment, a Margin of Exposure (MOE) approach was applied using the LOAEL of 0.3 mg huperzine A per kg bw. A minimal MOE was considered to be 1500 and included an overall uncertainty factor of 100 for intra- and interspecies variation, a factor 5 for low quality and incompleteness of the data and a factor 3 for LOAEL to NOAEL extrapolation.

For herbal preparations containing *H. serrata* extract, the estimated exposure to huperzine A ranged from 0.7 to 11.4 µg/kg bw for an individual weighing 70 kg.

MOEs were calculated using the LOAEL and the estimated exposure and ranged from 26 to 429. The calculated MOEs for acute exposure to huperzine A via herbal preparations containing *H. serrata* extract were insufficient (well below 1500). Therefore, it can be concluded that the current use of herbal preparations containing *H. serrata* and huperzine A may lead to acute cholinergic adverse effects.

It is important to consider that this risk assessment is solely based on toxicological data for huperzine A, one of the constituents of *H. serrata*. However, many more constituents, including eight other alkaloids with AChE inhibitory activity, are present in *H. serrata*. Therefore, cholinergic effects of *H. serrata* extract might be stronger than calculated in this risk assessment. To be able to include the other constituents of *H. serrata* in the risk assessment, concentration and toxicological data are needed of all individual constituents, which are currently not available.

Conclusions and recommendations

Use of herbal preparations containing *H. serrata* extract and huperzine A that are currently available on the Dutch market may lead to acute cholinergic adverse effects, including increased salivation, muscular weakness, cramps, lacrimation, diarrhoea, paralysis and blurred vision. In addition, there are data indicating that huperzine A is embryotoxic.

It is noted that only the acute effects of huperzine A could be evaluated in this risk assessment. Data on repeated exposure were not sufficient to evaluate the risk of prolonged exposure to huperzine A.

Based on the possible acute adverse effects of huperzine A, RIVM advises consumers to not use herbal preparations containing *H. serrata* and/or huperzine A, especially not during pregnancy.

1 Introduction

1.1 Background

In December 2020, the Minister for Medical Care and Sport of the Ministry of Health, Welfare and Sport (VWS) announced the actions that would be taken to better regulate food supplements and herbal preparations in the Netherlands, thereby facilitating enforcement. One of those actions is to expand the list included in the Herbal Preparations Decree of the Dutch Commodities Act[5] with substances/botanicals that are either forbidden or restricted (i.e. subject to a maximum level) in food supplements or herbal preparations (Van Ark, 2020). In order to determine whether a substance or botanical needs to be included in this list, a risk assessment is warranted. The selection of substances and botanicals chosen for risk assessment was based on the prerequisite that the substances/botanicals were sold on the Dutch market and (widely) used and there were indications for possible health risks, e.g. Rapid Alert System for Food and Feed (RASFF) reports, from enforcement institutes. The current assessment is about herbal preparations containing *Huperzia serrata* (Thunb.) Trevis[6] extract.

1.2 Information on existing assessments

In 2001, a limited risk assessment of food supplements containing huperzine A was conducted by RIVM (RIVM, 2001). Huperzine A showed inhibitory acetylcholinesterase (AChE) activity, and the critical endpoints observed in animal studies were cholinergic toxicity and embryotoxicity. The authors derived a Lowest Observed Adverse Effect Level (LOAEL) of 0.3 mg huperzine A per kg bw, based on acute cholinergic adverse effects in rats seen at this dose. A factor 3 was used to extrapolate from LOAEL to NOAEL. In addition, a No Observed Adverse Effect Level (NOAEL) of 0.04 mg huperzine A per kg bw was derived, based on embryotoxicity in rabbits seen at a higher dose. The (estimated) NOAELs were compared to the estimated exposure using the margin of safety (MOS) approach. The minimal MOS was 500, taking into account intra- and interspecies differences (factor of 10 each) and the insufficient quality of the data (factor 5). The calculated MOS for food supplements containing huperzine A at the expected intake was 13 and 33 for embryotoxicity and cholinergic toxicity, respectively. In that risk assessment it was recommended to set maximum levels, since the MOS is too small at recommended use levels (RIVM, 2001).

In 2016, the Danish food safety authority assessed the risk of exposure to a specific food supplement containing 2 mg huperzine A (DTU, 2016). Also in this risk assessment the inhibitory AChE activity of huperzine A was found as the critical effect. The authors mentioned that the recommended daily intake of 2 mg was five to ten times higher than doses at which adverse effects were observed in clinical trials. The Danish food safety authority concluded that a dose of 2 mg huperzine A per day poses a health risk, based on the inhibition of AChE (DTU,

[5] https://wetten.overheid.nl/BWBR0012174/2020-07-01. Accessed December 2023.
[6] Further revered to as *H. serrata*.

2016). Other effects of huperzine A or the effect of lower exposure were not accessed.

H. serrata is described in the European Food Safety Authority (EFSA) Compendium of Botanicals, indicating that there could be a health concern when *H. serrata* (aerial parts) is consumed. However, the Compendium only mentions the *H. serrata* constituents huperzine A and huperzine B and no specific adverse effect was ascribed (EFSA, 2012a).

1.3 Information on existing legislations

Currently, there are no specific restrictions for the use of *H. serrata* extract in herbal preparations included in the Herbal Preparations Decree of the Dutch Commodities Act. In addition there are no European legislations for the herb *H. serrata*.

2 Methodology

The risk assessment for herbal preparations containing *H. serrata* extract was conducted using the template for the safety assessment of plant food supplements as a basis (De Wit et al., 2019).

A quick search was performed to identify the most relevant constituents of *H. serrata*. No indications of adverse effects were found for flavonoids, diterpenoids, triterpenoids, catechin, quercetin, chlorogenic acid and ferulic acid. For nine alkaloids indications of adverse effects were found, but only one – huperzine A – was well studied. In addition, the huperzine A content of herbal preparations is regularly specified.

A search strategy was therefore developed to collect all available information on *H. serrata* (no specific plant part) and the constituent huperzine A. The literature databases PubMed, Scopus and Embase were searched on 6 December 2023. The database Toxcenter was also searched. The search terms used were chosen to find information about the herb *H. serrata* and its constituent huperzine A on toxicokinetics, toxicity or adverse outcomes and to include in vitro data, animal data and human data. An overview of the search terms can be found in Appendix 1. Fifty-four articles were found in Pubmed, Scopus and Embase. Furthermore, 104 articles were found in Toxcenter. In total, 129 unique articles were found.

The relevance of the articles was determined based on the title and abstract. Articles only about the fungi living on *H. serrata* or organophosphate poisoning were excluded. Moreover, articles solely focussing on the effectiveness and mechanisms of huperzine A in, for example, Alzheimer's disease and not on the adverse effects were excluded. In all relevant articles, references and citations were used to find additional articles, which is also called reverse and forward snowballing (Wohling, 2014).

Moreover, grey literature[7] was searched for information on *H. serrata*. The search included evaluations of EFSA, European Medicines Agency (EMA) and national health institutes of England, Norway, Denmark, Germany, United States, Canada, New Zealand, Australia and France. One risk assessment from the DTU in Denmark was found. PubChem and ChemID plus were used to collect information on the chemical structure of *H. serrata* constituents. Furthermore, the Compendium of botanicals of EFSA and the United States Department of Agriculture (USDA) plant database were searched for information on *H. serrata*. Several other sources including the European Pharmacopoeia, Hagers Handbuch der Pharmazeutischen Praxis, European Scientific Cooperative on Phytotherapy (ESCOP) monographs, World Health Organisation (WHO) monographs and the Commission E monographs were consulted regarding *H. serrata* and huperzine A. However, no information was available.

[7] Grey literature refers to research that is either unpublished or has been published in a non-commercial form. Examples include reports from governmental institutes or EFSA's Compendium of Botanicals.

Furthermore, different web shops were checked to collect information on the different herbal preparations containing *H. serrata* extract available on the Dutch market.

Lastly, the National Poisoning Information Centre (NVIC) and the Netherlands pharmacovigilance centre Lareb were contacted.

3 Description of the product

3.1 Identity and nature of the source material

The plant *Huperzia serrata (Thunb.) Trevis,* also called Chinese clubmoss or toothed clubmoss, belongs to the family *Lycopodiaceae* (EFSA, 2012a; USDA). It has to be noted that according to Ching's taxonomic system, the family of *H. serrata* is *Huperziaceae* (Ma et al., 2006). This taxonomic system is also used in many countries, including China (Ma et al., 2006). Other synonyms of *H. serrata* are shown in Table 1 below. *H. serrata* grows slowly and reaches its maximum height of 5 to 15 centimetres, 15 to 20 years after seed germination (Ma et al., 2006). The small stems have green needle like leaves.

H. serrata is found worldwide. However, it grows more abundantly in Central America, Oceania and in eastern and southern parts of Asia (Ma et al., 2006; Zhang & Zhang, 2004), as its habitat is specific and the plant prefers moist and acid soils and shade (Ma et al., 2006).

Table 1 General information of Huperzia serrata.

Scientific (Latin) name	Family: *Lycopodiaceae/ Huperziaceae*[8] Species: *Huperzia serrata (Thunb.) Trevis*
Synonyms	*Lycopodium serratum* *Urostachys serratus*
Common names	Toothed clubmoss Chinese clubmoss Qian Ceng Ta
Part used	Entire plant
Geographical origin	Distributed globally. More abundantly present in Central America, Oceania and eastern and southern parts of Asia
Growth and harvesting conditions	Prefers moist and acid soil and shade.

(Sources: Peng et al., 2020; Ma et al., 2007; Ma et al., 2006; Zhang & Zhang, 2004; USDA, z.d.)

Originally, *H. serrata* is known as Qian Ceng Ta in Traditional Chinese Medicine (Ma et al., 2007). Usage of the plant was first mentioned in 739 CE (Ma et al., 2007). The plant was subscribed for a variety of symptoms and/or diseases, including tense muscles and rheumatism (Ma et al., 2007). More recently, Qian Ceng Ta is also subscribed for myasthenia gravis in China (Jiangsu New Medical College, 1985). The current medicine for myasthenia gravis include acetylcholinesterase inhibitors (Colović et al., 2013).

[8] Different taxonomic systems are used worldwide. In Ching's taxonomic system *Huperzia serrata's* family is *Huperziaceae*, whereas Europe works with a different system which indicates *Lycopodiaceae as the family*.

3.2 Manufacturing process

H. serrata extract is used in herbal preparations for one of its constituents, huperzine A. For the production of herbal preparations containing *H. serrata* extract, the entire plant of *H. serrata* can be used as raw material. *H. serrata* extract is used in herbal preparations as a single ingredient or in combination with other compounds (Table 4). Some herbal preparations containing *H. serrata* extract, specify the huperzine A content. Information on manufacturing methods used for making herbal preparations containing huperzine A from *H. serrata* extract is currently not available. Since *H. serrata* is scarce and the huperzine A yield is relatively low, alternative methods to manufacture synthetic huperzine A are developed (Ferreira et al., 2016). Ding et al. (2014) managed to synthesize huperzine A and huperzine B from (R)-pulegone in 10 to 13 steps with a yield of 17 and 10%, respectively. However, natural huperzine A is in its [-] eutomer[9] and this synthetic huperzine A is a mixture of [-]huperzine A and [+]huperzine A (± ratio unknown), which is three times less potent compared to the natural variant (Ferreira et al., 2016). Therefore, studies explored methods to only synthesise [-]huperzine A. White et al. (2013) found a way to synthesise only [-]huperzine A from (S)-4-hydroxycyclohex-2-enone in 17 steps. To what extent these synthetic manufacturing processes are used for herbal preparations containing huperzine A is not known.

3.3 Chemical composition

Many phytochemical constituents are present in *H. serrata* (Ma et al., 2007). These phytochemical constituents can be divided in several classes including phenolic constituents, flavonoids, diterpenoids, triterpenoids and alkaloids (Table 2) (Jaswinder et al., 2016; Yang et al., 2009). Some alkaloids have inhibitory AChE activity and are considered the active ingredients (Wu et al., 2011; Ma et al., 2007). For example, these include huperzine A, huperzine B, N-methyl-huperzine B, huperzine Y2, huperzine Y3, huperserine E, carinatumine A, carinatumine B and huperzinine (Jiang et al., 2019; Liu et al., 2017; Jiang et al., 2014; Wu et al., 2011; Ma et al., 2007).

Of all the alkaloids with inhibitory AChE activity, huperzine A is most potent (Wu et al., 2011). Bai (2007) reported variation in huperzine A concentration in *H. serrata* ranging from 0.0047 to 0.025% w/w in dried herb. One of the variables is the season (Ma et al., 2005). The measured huperzine A concentration was 100 µg/g in October, whereas a lower concentration of 64 to 70 µg/g was measured in late winter (Ma et al., 2005). Moreover, the concentration of Huperzine A in *H. serrata* also varies per tissue. Ma et al. (2005) reported a huperzine A concentration of 117 µg/g in the leaves, 38 µg/g in the sporangia, 78 µg/g in the stem and 25 µg/g in the roots. The relatively high huperzine A concentration in leaves is in line with the study of Wu & Gu (2006), where the huperzine A content in leaves was 0.0175% and at least twiceas high as the concentration in the roots (0.0019%) and stem (0.0071%). Since the whole plant can be used for herbal preparations

[9] The more pharmacologically active form of the enantiomers of an active substance. Enantiomers are different stereoisomers of a compound. Stereoisomers have the same molecular groups, but are not identical as they are a mirror image of each other.

Table 2 Examples of phytochemical constituents of Huperzia serrata

Phytochemical constituents

Phenolic constituents

- catechin
- quercetin
- chlorogenic acid
- ferulic acid

Flavanoids

- 5-7-2`4`-tetrahydroxy5`methoxyflavone
- 5-7-4`-trihydroxy3`methoxyflavone
- 5-7-4`-trihydroxy3`5`dimethoxyflavone apigenin
- 5,5'-dihydroxy-2',4'-dimethoxyflavone-7-O-β-D-(6"-O-Z-p-coumaroyl)-glucopyranoside

Triterpenoids

- 21α-hydroxyserrat-14-en-3β-yl propanedioic acidmonoester
- serrat-14-en-3α,21β-diol
- 21β-hydroxyserrat-14-en3α-ol
- 3β,21β-dihydroxyserrat14-en-29-ol
- 14β,15β-epoxy-3β-hydroxyserratan-21α-ol

Diterpenoids

- 3β-hydroxysandaracopimaric acid
- 12, 16-epoxy-11,14-dihydroxy-8,11,13-abietatrien-7-one

Alkaloids

- huperzine A
- huperzine B
- N-methyl-huperzine B
- huperzine Y2
- huperzine Y3
- huperserine E
- carinatumine A
- carinatumine B
- huperzinine

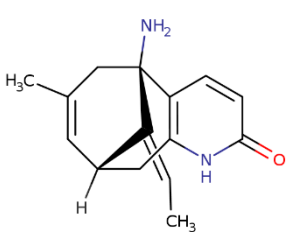
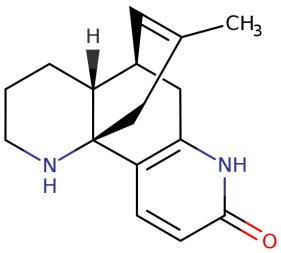
(Sources: Wu et al., 2020; Jiang et al., 2019; Liu et al., 2017; Jaswinder et al., 2016; Jiang et al., 2014; Yang et al., 2010; Yang et al., 2009; Ma et al., 2007; Yuan, et al., 2004; Zhou et al., 2004; Yuan & Zhao, 2003; Zhou et al., 2003a; Zhou et al., 2003b; Tan et al., 2002a, 2002b; Tan et al., 2002c)

containing *H. serrata* extract, the variation per tissue is less relevant for the risk assessment. Overall, Wu & Gu (2006) found a huperzine A content of 0.0118% when the entire plant was measured.

Another variable for huperzine A concentration in *H. serrata* is the specific location of the plant (Ma et al., 2005). Ma et al. (2005) measured the huperzine A concentration in 21 plants collected from different regions in China during the period May to August. The concentration varied from 46 µg/g in Guangdong to 133 µg/g in Yuannan (Ma et al., 2005). The average huperzine A concentration of all 21 *H. serrata* plants was 80 µg/g (Ma et al., 2005). Zhang et al. (2009) found a huperzine A concentration of 202 µg/g in *H. serrata*. It has to be noted that the origin of the plant and period of harvesting is unknown.

Interestingly, also the concentration of a second alkaloid, huperzine B, was measured. The average concentration of huperzine B was 113 µg/g, which was substantially lower than average concentration of huperzine A measured in this study (Zhang et al., 2009). No other studies reported huperzine B concentrations in *H. serrata*. However, in related species the concentration of huperzine B is also lower than the concentration of huperzine A in almost all cases (Lim et al., 2010; Goodger et al., 2008).

Table 3 Two of the active ingredients of Huperzia serrata (source: ChemID Plus & PubChem)

Active ingredient	Huperzine A	Huperzine B
Chemical structure		
Systematic name	5,9-Methanocycloocta(b)pyridin-2(1H)-one, 5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-, (5R,9R,11E)-	Lycodin-1(18H)-one, 8,15-didehydro-
CAS No.	102518-79-6	103548-82-9
Molecular formula	$C_{15}H_{18}N_2O$	$C_{16}H_{20}N_2O$
Molecular weight	242.32	256.34

For the other seven alkaloids with known inhibitory AChE activity (N-methyl-huperzine B, huperzine Y2, huperzine Y3, huperserine E, carinatumine A, carinatumine B and huperzinine), no information on concentrations in *H. serrata* is available. Therefore, Table 3 contains only information on huperzine A and B. Regarding huperzine B, besides the concentration and inhibitory AChE activity, no other information is available.

3.4 Selected compound(s) for risk assessment

The whole plant of *H. serrata* can be used in herbal preparations, so the risk assessment does not focus on a specific plant part. *H. serrata* contains many phytochemical constituents. A quick search was performed to identify the most relevant consitutents of *H. serrata*. No indications of adverse effects were found for flavonoids, diterpenoids, triterpenoids, catechin, quercetin, chlorogenic acid and ferulic acid. For nine alkaloids indications of adverse effects were found, but only one, huperzine A, was well studied. In addition, the huperzine A content of herbal preparations is regularly specified. Therefore, this risk assessment focussed on huperzine A, besides *H. serrata*.

3.5 Stability

No information is available on the stability of *H. serrata.* However, one of the active ingredients, huperzine A, is very stable in its natural form (Zangara, 2003). Long term incubation at 24ºC with butyrylcholinesterase (BuChE), AChE, 0.1 N hydrochloric acid or sodium hydroxide did not cause structural changes (Ashani et al., 1992). Moreover, huperzine A was also stable after long term storage at -20ºC in blood plasma (Li et al., 2008).

3.6 Use and use levels

Several herbal preparations containing *H. serrata* extract were found on the Dutch market and the recommended intake varies strongly between the herbal preparations (table 4, based on an internet search on Dutch websites, December 2023). Herbal preparations containing *H. serrata* extract are marketed for two different target populations, the general population and sports participants.

Herbal preparations marketed to support brain function
The herbal peparations for the general population are marketed to support brain function including normal functioning, concentration, focus and memory or enhancing memory. The recommended use varies between one to four capsules a day depending on the product. On some webpages warnings are listed, such as: this supplement is not intended for pregnant or breastfeeding women, this supplement can cause stomach problems, this supplement should not be used when having heart problems, discuss usage of this supplement with a doctor when there is a health problem, when medication is used or when AChE inhibitors are used.

Pre-workout supplements
In addition, pre-workout supplements containing *H. serrata extract* are on the market. Those can contain multiple ingredients and are designed to be used before workouts. *H. serrata* is intended to boost energy and focus. One capsule or one scoop (of the product specific measuring spoon; approximately 8 to 10g per scoop) of product is recommended before a workout. On the webpages selling the pre-workout supplements no warnings are listed.

Table 4 Examples of herbal preparations containing Huperzia serrata extract available on the Dutch market

Ingredients	**Recommended use per day***	**Dose of *H. serrata* per unit**	**Total recommended daily dose* of *H. serrata***	**Dose of huperzine A per unit**	**Total recommended daily dose* of huperzine A**	**Warnings**
Huperzia serrata extract	1-4 capsules	Not stated	Not stated	0.2 mg	0.2 – 0.8 mg	Yes[10]
Huperzia serrata extract	1 capsule	Not stated	Not stated	0.05 mg	0.05 mg	Yes[11]
Huperzia serrata extract; *Ginkgo biloba* extract	1-2 capsules	15 mg	30 mg	0.15 mg	0.15 - 0.3 mg	No
Huperzia serrata; *Ginkgo biloba*; *Piper nigrum*; *Ziniber officinale*; *Vinca minor*; *Mucuna pruriens*; …	2 capsules	0.5 mg	1 mg	Not stated	Not stated	Yes[12]
Huperzia serrata extract	1 capsule	25 mg	25 mg	0.25 mg	0.25 mg	Yes[13]
Huperzia serrata extract	1-2 capsules	Not stated	Not stated	0.2 mg	0.2 - 0.4 mg	Yes[14]
Huperzia serrata extract	2 capsules	5 mg	10 mg	0.05 mg	0.1 mg	Yes[15]
Huperzia serrata extract	1 capsule	20 mg	20 mg	0.2 mg	0.2 mg	No
Huperzia serrata; ginkgo extract; *Mucuna pruriens* extract; PurCaf; Active TC caffeine; Lion's mane; vitamin B6; citicoline; L-tyrosine;…	1 scoop	10 mg	10 mg	Not stated	Not stated	No

*For product 8 & 9 is the dose per workout

[10] Not suitable during pregnancy or breastfeeding. Do not use when having heart problems. Consult with a physician when AChE inhibitors are used. This product can cause stomach problems.
[11] Not suitable during pregnancy or breastfeeding. First consult usage with a physician when having heart or lung problems.
[12] This product contains an ingredient that may affect blood sugar. Check with your physician before using this product if you are using medication, including anti-coagulants (blood thinners), or have any medical conditions, including heart disease or high/low blood pressure. Do not use if you may become pregnant, are pregnant or nursing. Do not exceed recommended daily intake. Not intended for use by persons under 18. Keep out of reach of children. Store in a cool, dry place. (State of California Prop 65) This product contains a chemical known to the State of California to cause birth defects or other reproductive harm.
[13] Not suitable during pregnancy or breastfeeding. First consult usage with a physician when having a medical condition or when using medication.
[14] Not suitable during pregnancy or breastfeeding. Do not use when having heart problems. This product can cause stomach problems.
[15] Consult with a physician when pregnant, breastfeeding or having health problems

4 Exposure: extent and duration

4.1 Exposure from herbal preparations use

Based on the recommended use levels and the reported dose of huperzine A mentioned in Table 4, the exposure to huperzine A can be estimated. For pre-workout supplements, it is assumed that they are used daily.The recommended use for herbal preparations containing *H. serrata* extract varies from one to four capsules a day, resulting in an exposure of 0.05 to 0.8 mg huperzine A. For an individual with a bodyweight of 70 kg this equals an exposure to huperzine A of 0.7 to 11.4 µg/kg bw per day. For two herbal preparations (Table 4), the dose of huperzine A is not reported. These products are therefore not included in the range calculated above.

4.2 Possibility of additional/combined human exposure

H. serrata (dried or wet) extract is only available on the Dutch market as an ingredient for herbal preparations. Moreover, related plant species which also contain huperzine A are not used in herbal preparations. Therefore, it is not likely that the exposure is above the estimated exposure described in 4.1. However, it should be mentioned that individuals possibly take both the regular herbal preparations containing *H. serrata* extract and the pre-workout supplements as they are marketed for different purposes. Especially since some regular herbal preparations containing *H. serrata* extract are not only marketed to improve memory, but are also claiming to improve concentration and focus.

4.3 Information on historical use of the ingredient

In China, *H. serrata* is known as Qian Ceng Ta and has been used for centuries as Traditional Chinese Medicine(Ma et al., 2007). However, it is not known what the exposure to huperzine A is when Qian Ceng Ta is used. No information on *H. serrata* is available in sources as the European Pharmacopoeia, Hagers Handbuch der Pharmazeutischen Praxis or monographs of ESCOP, the WHO and Commission E.

5 Biological data

5.1 Toxicokinetics

5.1.1 Absorption

Animal data

Toxicokinetics after a single dose

The toxicokinetics after a single dose of huperzine A in different animal species were studied in five studies. A full overview of the toxicokinetic parameters can be found in Appendix B, the results after oral administration of huperzine A will be briefly described below.

The toxicokinetic parameters of huperzine A were first studied in rats by Wang et al. (1988). In this study, rats (n=3) were exposed to a single dose of ^{3}H-radiolabelled huperzine A (13.9 MBq) via intravenous and intragastric administration. After intragastric administration, blood was collected at seven time points between 3 and 240 minutes. The highest maximum concentration (C_{max}) was 98569 dpm/ml and the time to reach the maximum concentration (T_{max}) was 21 minutes. The average plasma half-life ($t_{1/2}$) was 203 minutes. As the dose is not converted to mg/kg bw, the relevance of this study is low. Huperzine A had a high bioavailability of about 97% (Wang et al., 1988).

Yue et al. (2007) used Sprague-Dawley rats to study the toxicokinetic parameters of huperzine A after intranasal, intravenous and intragastric administration. The dose level used for intragastric administration was 0.5 mg/kg bw. After administration, blood plasma and cerebrospinal fluid (CSF) samples were collected at ten different time points between 5 to 360 minutes. The C_{max} of huperzine A in plasma was 72 ng/ml and was reached after 51 minutes. The $t_{1/2}$ was 2,5 hours. It was concluded that the data for both blood plasma and CSF fitted a two-compartment model (Yue et al., 2007).

Wang & Chen (2009) studied the toxicokinetic parameters of 0.5 mg/kg bw huperzine A in Sprague-Dawley rats after intranasal or intravenous administration in blood plasma and CSF. The results are presented in Appendix B.

Chu et al. (2006) studied the toxicokinetic parameters of huperzine A after oral or intravenous administration in beagle dogs (n=5). For studying oral administration, the dogs received 0.1 mg (9.5 µg/kg bw on average) of huperzine A orally in the form of tablets. Blood samples were collected before and at 13 time points, ranging from 15 minutes to 24 hours post-dosing. The C_{max} of huperzine A was 3 ng/ml and was reached after 75 minutes. The $t_{1/2}$ was approximately 6 hours. The oral bioavailability was 94% and comparable to the number found in rats. It was concluded that the data of oral administration of huperzine A fitted best to a one-compartment open model (Chu et al., 2006).

Ye et al. (2008) studied the toxicokinetic parameters of 0.5 mg huperzine A after oral administration in beagle dogs (n=6). Blood samples were collected before and at 12 time points between 0 and 24

hours post-dosing. The C_{max} of huperzine A was 10 ng/ml and was reached after 3 hours. The $t_{1/2}$ was approximately 6 hours. (Ye et al., 2008).

Toxicokinetics after repeated dosing
The toxicokinetics after repeated dosing of huperzine A in dogs was investigated in two studies. A full overview of the toxicokinetic parameters can be found in Appendix B, the results after oral administration of huperzine A will be described below.

Ye et al. (2008) studied the effect of daily oral exposure to 0.5 mg (27 µg/kg bw on average) huperzine A for 5 consecutive days in dogs (n=6). Blood samples were collected at 3 and 24 hours after the first four doses and at 12 time points between 0 and 24 hours after the last dose. The results obtained for the C_{max} and the T_{max} were similar to the results found in the single administration study mentioned above (Ye et al., 2008). The elimination half-life was not determined. Wang et al. (2004) studied the effect of daily intermuscular injection of 10 mg/kg huperzine A for 15 days in dogs. The results are presented in Appendix B.

Human data
The toxicokinetics after a single dose of huperzine A in humans were studied in five studies. A full overview of the toxicokinetic parameters can be found in Table 5. The studies will be briefly described below.

In the study of Qian et al. (1995), six human volunteers ingested a tablet containing 0.99 mg huperzine A. Before and at 11 time points between 15 minutes and 10 hours post-dosing blood samples were collected. The C_{max} of 8.4 ng/ml was reached after 80 minutes. Furthermore, the $t_{1/2}$ was approximately 5 hours. The toxicokinetic parameters fitted a one-compartment open model with first-order absorption (Qian et al., 1995).

In the study of Li et al. (2007), 12 human volunteers ingested a tablet containing 0.4 mg of huperzine A. Subsequently, blood was collected at 15 time points ranging from 0 to 24 hours post-dosing. Five to ten minutes post-dosing, traces of huperzine A were present in the blood. The C_{max} of huperzine A in blood, 2.6 ng/ml, was reached after approximately 1 hour. Huperzine A showed a $t_{1/2}$ of approximately 12 hours in plasma. It was concluded that the results fitted a two compartment open model (Li et al. 2007).

In the study of Li et al. (2008), 18 human volunteers ingested a tablet containing 0.2 mg of huperzine A. After administration blood was collected at 13 time points ranging from 0 to 24 hours. The C_{max} of huperzine A in the blood, 2.5 ng/ml, was reached after 1.3 hours. Furthermore, the $t_{1/2}$ was approximately 6 hours (Li et al., 2008).

In the study of Zou et al. (2009), 20 human volunteers ingested a tablet containing 0.1 mg of huperzine A. Blood was collected before and at 12 time points ranging from 15 minutes to 24 hours post-dosing. The C_{max} of 1.0 ng/ml was reached after 1.2 hours. Moreover, the $t_{1/2}$ was approximately 6 hours (Zou et al., 2009).

In the study of Wu et al. (2017), 23 human volunteers received 0.2 mg of huperzine A in capsules or tablets from three different companies. Blood was collected at 13 time points ranging from 0 to 72 hours post-dosing. All the parameters were similar for the three different tablets containing 0.2 mg huperzine A. The C_{max} was around 1.5 ng/ml. Furthermore, the T_{max} was around 0.8 hour and the $t_{1/2}$ approximately 12 hours (Wu et al., 2017).

Table 5 Toxicokinetic parameters of huperzine A exposure in humans after a single oral dose

Dose (mg)	**C_{max} (ng/ml)**	**T_{max} (h)**	**AUC_{0-t} (ng*h/ml)**	**$t_{1/2}$ (h)**	**Ref**
0.1	1.0±0.2	1.2±0.3	7.8±1.9	5.8±0.3	1
0.2	2.5±0.5	1.3±0.4	16.4±3.4	5.9±0.8	2
0.2	1.4±0.5	0.8±0.7	15.3±3.4	12.1±2.1	3
0.2	1.5±0.6	0.7±0.5	15.7±3.6	12.3±1.7	3
0.2	1.6±0.5	0.9±1.0	17.6±3.8	12.1±2.9	3
0.4	2.6±0.4	1.0±0.1	1986.96 (µg/L*min) [16]	11.9±2.2	4
0.99	8.4±0.9	1.3±0.2	4.1±1.2	4.8±1.1	5

(maximum concentration C_{max}; time to reach the maximum concentration T_{max}; area under the curve AUC; plasma half-life $t_{1/2}$; reference Ref; 1. Zou et al., 2009; 2. Li et al., 2008; 3. Wu et al., 2017; 4. Li et al., 2007; 5. Qian et al., 1995)

5.1.2 Distribution

Wang et al. (1988) studied the specific distribution of huperzine A by administering radioactive ^{3}H-huperzine A intravenously in mice. The highest radiolabel concentrations were found in the kidneys, liver, lung, spleen and heart. Interestingly, when ^{3}H-huperzine A was intravenously administered to 14 days pregnant mice, it was present in the foetus after 15 minutes, which means huperzine A is able to cross the placenta. Furthermore, radiolabel was also present in the brain (Wang et al., 1988). Another study used radioactive ^{3}H-huperzine A to track the presence of radiolabel in the brain of mice (Tang et al., 1989). Radiolabel was present in all brain regions 60 minutes after intravenous injection. The highest concentrations of radiolabel were found in the hippocampus, the frontoparietal cortex, the striatal cortex, the nucleus accumbens, the anterior lobe of the pituitary, hippocampus and the basal area of the fourth ventricle at both 15 minutes and 3 hours post-dosing. The tissue concentration significantly decreased after 180 minutes post-dosing (Tang et al., 1989).

Yue et al. (2007) studied the toxicokinetics in male Sprague–Dawley rats. After 167 or 500 µg/kg of huperzine A administration via various routes, there was a fast onset of adverse effects, such as sialorrhea and muscle trepidation. According to the authors this showed that huperzine A has the ability to easily pass membranes. The presence of huperzine A in the CSF was established within 7 minutes after an intravenous injection of 167 µg/kg huperzine A (Yue et al., 2007). The equilibrium dialysis method showed a binding rate between radioactive huperzine A and plasma protein of 17% (Wang et al., 1988).

[16] Equal to 119217.6±164.6 ng*h/ml

5.1.3 *Metabolism*

In vitro data

Ma et al. (2003a) studied the effect of cytochrome P450 (CYP) antibodies on the huperzine A metabolism in rat liver microsomes. The liver microsomes were exposed to 200 nM of huperzine A. Both CYP1A2 and CYP3A1/2 antibodies reduced the huperzine A metabolism by 76 and 18%, respectively. Only minor inhibitory effects were observed for CYP2C11 and CYP2E1 antibodies. These results indicate a large contribution of CYP1A2 in the huperzine A metabolism and a smaller role for CYP3A1/2 (Ma et al., 2003a).

Lin et al. (2016) studied the metabolism of huperzine A in human hepatocytes. A concentration of 10 ng/ml of huperzine A was not metabolised after 90 minutes.

Animal data

Garcia et al. (2004) compared blood extracts obtained from rats exposed to huperzine A via intramuscular injection to unexposed rats. This resulted in the identification of a phase I epoxide metabolite of huperzine A, namely 13,14-epoxy Hup-A. Other minor peaks found by HPLC were not identified. Also, possible metabolites in the urine were not identified. Therefore, it is unknown if 13,14-epoxy Hup-A is the end-product for excretion (Garcia et al., 2004).

5.1.4 *Excretion*

Animal data

The excretion of huperzine A was studied in mice (Wang et al., 1988). After intravenous administration, 73% of the dose of huperzine A was excreted via the kidneys within 24 hours. A part of the huperzine A was metabolized in more water-soluble molecules before being excreted (Wang et al., 1988).

Human data

The excretion of huperzine A is also studied in humans (Lin et al., 2016). Huperzine A was measured in urine of 14 elderly participants for 48 hours after oral exposure to 0.1 mg huperzine A. After 48 hours, 35% of the huperzine A was found unchanged in the urine. The authors concluded that the percentage of unchanged huperzine A excreted in the urine is probably larger, since the experiment only collected urine for 48 hours (Lin et al., 2016). Sheng et al. (2016) found a discrepancy between some toxicokinetic parameters of huperzine A in younger and older people. Using a PBK model it was observed that the age of the participant influenced the clearance of huperzine A; the clearance was lower in older people (Sheng et al., 2016). Also the Area Under the Curve (AUC) increased by 75% in elderly (Sheng et al., 2016). However, 48 hours is 4 to 12 times as long as the $t_{1/2}$ for huperzine A. It is therefore considered likely that a large part of huperzine A is metabolised to polar (phase II) metabolites, which in turn are excreted via the urine.

5.1.5 *Summary*

Overall, huperzine A is well absorbed in the intestine, as animal studies showed a bioavailability of almost 100% after oral exposure. The bioavailability was not studied in humans, but it is expected that results

would be similar.
In humans, huperzine A was absorbed relatively fast, with the maximum concentration reached around one hour after dosing, which was faster than observed in animals. For the $t_{1/2}$, some human studies showed values around 5 hours and others around 12 hours. The time profiles of the differrent studies were compared to see if different phases could be distinguished, which might explain the discrepancy in the results. This was not the case, since the time profiles had linear axes, which made it not possible to distinguish two phases. So the large differences for $t_{1/2}$ could not be explained.
It has to be noted that the human toxicokinetic studies are conducted in young and healthy people.This means that the above mentioned studies might not be representive for the older population. Since some herbal preparations are marketed to improve brain function they likely attract an older age group, which might not be represented by the toxicokinetic parameters presented in this chapter.
Animal studies indicated that huperzine A was distributed throughout the whole body and can pass the blood-brain barrier as well as the placenta.
The metabolism and excretion of huperzine A in humans is relatively unknown. It was shown that huperzine A can be excreted unchanged.
No information on toxicokinetics was available for *H. serrata*.

5.2 Mode of action

5.2.1 Acetylcholinesterase inhibition

AChE is mainly present in the brain at cholinergic synapses and in muscles at neuromuscular junctions (Colović et al., 2013). The enzyme has an important function in the breakdown of the neurotransmitter acetylcholine, since it catalyzes the hydrolysis of acetylcholine into choline and acetic acid (Colović et al., 2013). AChE inhibitors can stop the breakdown of acetylcholine by inhibiting AChE activity (Colović et al., 2013). As a result, acetylcholine accumulates (Colović et al., 2013). Excessive accumulation can result in cholinergic crisis, since receptors are overstimulated (Adeyinka & Kondamudi, 2021). Adverse effects associated with acetylcholine accumulation include increased salivation, muscular weakness, cramps, lacrimation, diarrhoea, paralysis and blurred vision (Adeyinka & Kondamudi, 2021).

H. serrata contains several alkaloids which can inhibit AChE (See section 3.3). The effect of huperzine A on AChE inhibition was studied in vitro (Zhao & Tang, 2002; RIVM, 2001; Cheng et al., 1996; Ashani et al., 1992). Ashani et al. (1992) studied the inhibitory effect of huperzine A on fetal bovine serum (FBS) AChE and purified human AChE (rHuAChE brain cDNA). At a concentration of 0.41 µM huperzine A, both human and FBS AChE activity was stronlgy inhibited within 15 minutes post-dosing. The percentage of remaining AChE activity was approximately 15% for FBS AChE and 10% regarding human AChE. Moreover, titration of FBS AChE by huperzine A revealed a dose-response curve. The reduction of the first 50% of AChE activity was linear, followed by a non-linear part (Ashani et al., 1992).

Another in vitro study reports half-maximal inhibitory concentration (IC_{50}) values for AChE inhibition by huperzine A. The lowest IC_{50} value

was 19 ng/ml found in human erythrocyte membrane, followed by 20 ng/ml in rat cortex, 21 ng/ml in rat erythrocyte membrane and 22 ng/ml in bovine erythrocyte membrane (As cited by RIVM, 2001). Cheng et al. (1996) found huperzine A was a selective for AChE inhibition and not BuChE inhibition, which also hydrolyses choline (Darvesh et al., 2003).

Zhao & Tang et al. (2002) studied the AChE activity in rat brain after huperzine A exposure for the two forms of AChE: the tetrameric G4 form and monomeric G1 form. Both forms of AChE are inhibited by huperzine A, although it depends on the brain region which form is inhibited more potently (Zhao & Tang, 2002).

The in vivo studies studying the AChE inhibitory activity of huperzine A will be further discussed in chapter on toxicological data (5.3.6). For the other alkaloids with known AChE inhibitory activity (chapter 3.3) no studies were available.

5.2.2 N-methyl-D-aspartate receptor antagonist

N-methyl-D-aspartate (NMDA) receptors are important for neuronal gene expression, neuronal signalling, neuronal plasticity and neuronal survival (Sucher et al., 1996). They consist of four subunits, based on availability and affinity of specific subunits (Ulbrich & Isacoff, 2008). Overstimulation of the NMDA receptor can results in neuronal death (Sucher et al., 1996). On the other hand, when normal synaptic signalling is inhibited, psychomimetic adverse effects can occur (Vyklicky et al., 2014).

Wang et al. (1999) and Zhang & Hu (2001) showed that huperzine A can inhibit NMDA receptors in vitro. The effect of huperzine A was reversible (Wang et al., 1999). Several studies focussed on finding the binding site of huperzine A on NMDA receptors and a few potential binding sites were identified (Gordon et al., 2001; Zhang & Hu, 2001). As no toxicological studies focussed on the effect of NMDA receptor inhibition, the toxicological relevance of this mode of action is unknown.

5.2.3 Other modes of action

Since huperzine A might be an effective drug for Alzheimer's disease, many studies looked into the effects of huperzine A on the brain (Mao et al., 2016; Tang et al., 2005a 2005b; Wang et al., 2001). Besides its role as an AChE inhibitor and NMDA antagonist, many positive effects were found. Wang et al. (2001) observed improved expression of apoptosis-related genes and protection agains hydrogen peroxide induced apoptosis after pre-incubation with huperzine A in rat cells in vitro. Moreover, other studies demonstrated an increased excretion and expression of neuronal growth factor in primary astrocytes of rat cells in vitro (Tang et al., 2005a; 2005b). The same studies also showed that huperzine A induced neurite outgrowth (Tang et al., 2005a; 2005b). Lastly, Mao et al. (2016) found reduced formation of reactive oxygen species and a protective effect on cellular damage after huperzine A exposure in vitro in hippocampal cells from mice.

5.3 Toxicological data

This chapter gives an overview of all relevant toxicity studies found in the literature search. No toxicity data were identified for *H. serrata*. Therefore, toxicity studies for huperzine A were reviewed in this risk assessment. Studies exposing animals to huperzine A via other routes of exposure than oral exposure, will only be briefly described unless specified otherwise.

5.3.1 Acute toxicity

LD_{50}

A study reported the lethal dose for 50% of the tested animals (LD_{50}) for different routes of administration in mice and rats (Yu et al., 1993). The LD_{50} after intragastric injection was 5.2 mg/kg bw and 25.9 mg/kg bw for mice and rats, respectively (Yu et al., 1993).

Oral administration

Zhang et al. (2013) exposed mice (n=10) to 0 (vehicle, no loperamide), 0 (only loperamide, control for intestinal mobility), 0.05, 0.1 or 0.2 mg/kg bw of huperzine A via intragastric administration in order to measure the effect of huperzine A on the intestine. An hour post-dosing, the mice received 4 mg/kg loperamide orally, followed by 0.2 ml of a charcoal meal 30 minutes later. The intestinal motility was measured by measuring the covered distance of the charcoal meal after 15 minutes. A significant larger distance was seen in animals exposed to the highest dose of huperzine A, indicating increased gastrointestinal motility. (Zhang et al., 2013).

Ma et al. (2003b) studied the effect of 0 (distilled water), 0.1 or 2 mg/kg bw of huperzine A in the diet in female and male Sprague-Dawley rats. Each experimental group consisted of 3 male and 3 female rats. Twenty-four hours after dosing, blood samples were collected to conduct a biochemical assay on several parameters like aspartate aminotransferase (AST), bilirubin, alkaline phosphatase, alanine aminotransferase (ALT), albumin and total protein. Furthermore, the liver was collected for a histological examination, calculation of liver coefficients and the isolation of hepatocytes for cytotoxicity testing. The dose of 0.1 mg/kg bw huperzine A did not induce significant changes in liver coefficients or any of the measured parameters. In female rats, 2 mg huperzine A per kg bw resulted in a significant increase in liver coefficients ($p<0.05$) 24 hours post-dosing; it was not reported which liver coefficients were increased. Further, those changes were not observed in male rats for the same dose. In the serum parameters AST (227% of control; $P<0.05$) and ALT (274% of control; $P<0.01$) a significant increase was observed in rats 24 hours after exposure to 2 mg/kg bw. Serum parameters like bilirubin, albumin, total protein and alkaline phosphatase did not change significantly. It was not specified if the same results were observed for both genders or if the parameters was not studied separately for the genders. Additionally, the histological examination of the liver 24 hours after dosing did not show any huperzine A-induced changes. The authors concluded that the change in liver coefficients and serum parameters was a result of AChE inhibition (Ma et al., 2003b).

In the study of Schmidt & van der Staay (1998), male Wistar rats (n=6) were exposed to a single dose of 0, 0.3, 1 or 3 mg/kg bw of synthetic (±, ratio unknown) huperzine A via oral gavage. The rats were observed for physiological and behavioural symptoms of cholinergic overstimulation immediately after administration and every 15 minutes thereafter over a period of 3 hours. The symptoms included tremors, paw beating, vocalization, tonic and clonic seizures, Straub tail, prone and lateral positions, limb abduction, arched back, sedation, excitation, diarrhoea, piloerection, salivation, lacrimation, exophthalmos and ptosis. The adverse effects showed a sharp increase with increasing dose. At the lowest dose, tremors and increased salivation were observed and the symptoms worsened with increasing dose[17]. In higher dose groups animals took a prone position and one animal experienced diarrhoea in the highest dose group. All adverse effects were reversible within 135 minutes (Schmidt & van der Staay, 1998). Based on this study, RIVM (2001) previously established a LOAEL for acute cholinergic overstimulation of 0.3 mg/kg bw based on adverse effects at that dose. It has to be noted that a racemic mixture of huperzine A (ratio unknown) was used in this study. An in vitro study showed that natural huperzine A (extracted from *H. serrata*) is three times more potent compared to the racemic mixture (Ferreira et al., 2016).

Little et al. (2008) described results of several studies, but the underlying data were not published. One study investigated which dose levels of huperzine A would be tolerated by male and female rats. The meaning of tolerated was not specified in Little et al. (2008). The maximum tolerated dose in male rats was a single oral dose of 6 mg/kg bw. Interestingly, in females only 3 mg/kg bw was tolerated which is half of the dose tolerated by male rats. In another study, the effect of huperzine A in dogs was studied. At an oral dose of 10 mg/kg bw, severe adverse effects like death, convulsions, tremors and emesis were observed[18]. The adverse effects were less severe at a lower dose of 0.5 mg/kg bw huperzine A. However, adverse effects like licking, tremors, salivation and decreased activity were still present. It is unknown if more dose levels were tested (unpublished data found in Little et al., 2008).

Other routes of administration
In the study of Boudinot et al. (2005) breathing activity was measured in mice exposed to 1, 2, 3 or 6 mg/kg bw huperzine A via a single subcutaneous injection. The dose 1 mg/kg bw did not induce significant changes. At 2 or 3 mg/kg bw, breathing frequency decreased and tidal volume increased[19]. All changes were reversible within 90 minutes. The dose of 6 mg/kg bw was lethal for all three animals within 20 minutes. In AChE deficient mice, the same dose of huperzine A did not result in respiratory changes or death indicating AChE activity was probably responsible for the adverse effects (Boudinot et al., 2005).

In the study of Pohanka et al. (2012) guinea pigs were exposed to 5, 25, 125, 625 µg/kg bw huperzine A via a single injection in the pelvic limb. In the guinea pigs exposed to 625 µg/kg bw, seizures were observed from 30 minutes to 5 hours post-dosing. Also, stress markers

[17] It was not specified in the study whether the observed effect was statistically significant from the control
[18] It was not specified in the study whether the observed effect was statistically significant from the control
[19] It was not specified in the study whether the observed effect was statistically significant from the control.

in several organs and antioxidant markers significantly changed at the three highest doses. The lowest dose did not induce any changes (Pohanka et al., 2012).

In a patent, Yu et al. (1993) describes results of several studies, but the underlying data were not published. In one study, the heart and blood pressure was measured in unconscious dogs exposed to 0.306 or 1 mg/kg bw huperzine A via a single intravenious injection. Overall, no changes were observed (Yu et al., 1993). In a study in rabbits, the animals were exposed to huperzine A (0.5 – 2 mg/kg bw) via a single intramuscular or intravenous injection. In the first 3 to 4 hours post-dosing, several adverse effects were observed including induced bronchial secretion, twitching, drooling, tearing and fecal and urinary incontinence[20]. The highest dose, administered intravenously, was lethal for one rabbit (Yu et al., 1993).

In the study of Hamilton et al. (2017) behavioural changes were measured in monkeys exposed to 5, 10, 20, 40 µg/kg bw huperzine A via a single intramuscular injection. Overall, no behavioural toxicity was observed (Hamilton et al., 2017).

Summary acute toxicity
Overall, five studies researched acute toxicity after oral exposure. For two studies, only the main finding was available as the original study was unpublished. These unpublished studies cannot be used in the risk assessment, as the available information is too limited. In the study of Zhang et al. (2013), higher gastrointestinal motility was observed; the toxicological relevance of this effect is unknown. In the study of Ma et al. (2003), effects on the liver were observed. The data were not suitable for deriving a NOAEL. A LOAEL of 0.3 mg/kg bw for adverse cholinergic effects (the lowest dose tested) was identified from the study of Schmidt & van der Staay (1998).

5.3.2 Short-term and subchronic toxicity

Oral administration
Zhang et al. (2013) exposed rats (n=10) to 0 (vehicle), 0 (loperamide), 0.05, 0.1 or 0.2 mg/kg bw of huperzine A daily for seven or 28 consecutive days by intragastric administration. An hour after the last dose, the mice received 4 mg/kg loperamide orally (control for intestinal mobility), followed by 0.2 ml of a charcoal meal 30 minutes later. The intestinal motility was measured by measuring the covered distance of the charcoal meal after 15 minutes. No change in intestinal propulsion rates were observed at all dose levels for both the 7 and 28 days experiment, in contrast to the single dose experiment (Zhang et al., 2013).

Little et al. (2008) described results of several studies, but the underlying data were not published. In a study in rats, the animals were orally exposed to huperzine A daily for 30 consecutive days. The highest dose showing no adverse effects was 1 and 3 mg/kg bw for female and male rats, respectively. It is unknown which other dose levels were tested or which parameters were measured. It has to be noted that although no adverse effects were seen, a reduced food consumption was

[20] It was not specified in the study whether the observed effect was statistically significant from the control

observed. These maximum dose levels found were considered as the NOAEL in male and female rats. In another study, dogs were orally exposed to huperzine A daily for 30 consecutive days. It was not reported which dose levels were given. Lacrimation in female dogs was observed at 0.1 mg/kg bw/d. No other effects were observed at this dose (unpublished data found in Little et al., 2008).

Other routes of exposure
In a patent, Yu et al. (1993) describes results of several studies, but the underlying data were not published. In the first study, rats were exposed daily to 0.5 or 1.5 mg/kg bw huperzine A via intraperitoneal injection for 90 days or to 1.5 or 3 mg/kg bw huperzine A via intragastric administration for 180 days. Several tests were conducted, including blood test and microscopic evaluation. Not all results were given separately for the different routes of administration, so it is unclear whether the results were seen for both routes of administration. In the high-dose groups a few rats died between 30 to 150 days after exposure. However, no effects were observed after tests in the remaining rats, apart from a small increase in ALT values in both groups receiving 1.5 mg/kg bw of huperzine A. Microscopic evaluation of the organs revealed inflamed areas on the heart muscle. Also myocardial cell denaturation atrophy was observed. In the brain, cerebral spongiocyte growth was noticed. Furthermore, a few rats showed inhibition of sperm cell growth and interstitial growth[21]. All other organs seemed unaffected by huperzine A (Yu et al., 1993).

In a second study, rabbits were exposed daily to huperzine A via intramuscular (0.6 mg/kg bw; 180 days) or intravenous administration (0.3 or 0.6 mg/kg bw; 90 days). Intramuscular injection of 0.6 mg/kg bw huperzine A was lethal for 3 out of the 5 rabbits between 66 and 136 days post-dosing. However, none of the rabbits showed visible clinical signs before death. On the other hand, microscopic examination revealed interstitial growth and myocardial cell denaturization atrophy in the heart and cerebral spongiocyte growth in the cerebral cortex (both doses)[22] (Yu et al., 1993).

In the third study, dogs were exposed daily to 0.3 or 0.6 mg/kg bw huperzine A via intramuscular injection for 180 days. At 0.6 mg/kg bw, the dogs experienced twitching of the muscles, which decreased some time post-dosing. No other abnormalities were observed, apart from fat infiltration in the heart and cerebral spongiocyte growth (both dose groups)[23] found during microscopic evaluation (Yu et al., 1993).

In the last study, rats were exposed daily to 0.3 mg/kg bw huperzine A via intraperitoneal injection for 51 days. Overall, no deviations were found in urea nitrogen, creatinine, zinc turbidity, number of platelets, number of red and white blood cells and percentage haemoglobin (Yu et al., 1993).

Liver toxicity was not found in rabbits or dogs after an experiment of six months with huperzine A. No other information was available about this experiment (unpublished data in Tang et al., 1994).

[21] It was not specified in the study whether the observed effect was statistically significant from the control
[22] It was not specified in the study whether the observed effect was statistically significant from the control
[23] It was not specified in the study whether the observed effect was statistically significant from the control

Summary short-term and subchronic toxicity
Overall, four studies investigated short-term and subchronic toxicity after oral exposure. For three studies, only the main finding was available as the original study was unpublished. The unpublished studies cannot be used in the risk assessment, as the available information is too limited. In contrast to the acute exposure, Zhang et al. (2013) did not observe effects on gastrointestinal motility in short-term exposure.

In addition to the oral toxicity studies, several toxicity studies with other exposure routes were described. Overall those studies showed adverse effects on heart, intestine and brain in rats, rabbit and dogs. In rats, a few animals also showed reduced sperm cell growth. However, also for these studies the underlying data were not available.

5.3.3 Genotoxicity

In vitro
A limited bacterial reverse mutation assay, also called Ames test, was conducted with huperzine A in two *Salmonella typhimurium* strains TA98 and TA100. In total, four different concentrations were tested: 1, 10, 100 and 1000 µg/container. As a positive control 1500 µg/container of cyclophosphamide was used. Both compounds were tested with the metabolic activation system (S9). The positive control cyclophosphamide showed a significant increase ($P<0.05$) in reversed mutation colony numbers (566) in the TA100 strain compared to the negative control (150). All concentrations of huperzine A had a reverse mutation colony number below the negative control (85-117) in the TA100 strain ($p>0.05$). Regarding the TA98 strain no information on the positive control was available. The reverse mutation colony numbers for all huperzine A concentrations were slightly above the negative control, but the difference was not significant and did not increase with the dose. This study did not comply to the OECD standards for the bacterial reverse mutation assay (OECD, 2020). Some of the deviations from the protocol were the usage of only two bacterial strains compared to five and only test results with S9 mix were available. Moreover, only four concentrations were tested instead of five and the positive control was only shown for one of the bacterial strains (Yu et al., 1993).

Tu & Wu (1990) studied the mutagenicity of huperzine A in *S. typhimurium* strains. Four different strains were used, TA97, TA98, TA100 and TA102 to test five different concentrations of huperzine A: 1, 10, 100, 1000 and 2000 µg per plate. Every test was conducted with and without S9. Several positive controls were used including 9-Aminoacridine (TA97, -S9), p-Nitroquinoline (TA98, -S9), Methyl methanesulfonate (TA100, -S9), Mitomycin (TA102, -S9), 1,8-dihydroxyanthraquinone (TA102, +S9), 2-Aminofluorene (TA97, TA98, TA100, +S9). The number of reversed mutation colonies at all the concentrations of huperzine A tested were similar to negative control. The number of reversed mutation colonies of the positive controls were four to 14-fold higher compared to values found in huperzine A exposed strains. Since no increase in reverse mutation colony numbers was observed for all of the tested concentrations of huperzine A, this suggested that huperzine A is not mutagenic in *S. typhimurium* (Tu & Wu, 1990). This study did not fully comply to the OECD standards for the bacterial reverse mutation assay, since only four and not five

bacterial strains were used (OECD, 2020). Thereby, no information on the agar, culture medium or incubation procedure was provided.

In vivo
Furthermore, Tu & Wu (1990) performed a micronucleus test. Mice were exposed to 0, 0.1, 0.3 or 1 mg/kg bw via intraperitoneal injection. The highest dose used was approximately half of the LD_{50}. The bone marrow cells of the mice were studied. No significant difference was observed between the treatment and control group (Tu & Wu, 1990). Not enough information about the experiment is known to conclude if it complies to the OECD standards (OECD, 2016).

Summary genotoxicity
Huperzine A was not genotoxic according to the available studies. However, the studies did not comply with the relevant internationally approved test guidelines for genotoxicity assays (Tu & Wu, 1990; Yu et al., 1993). Therefore, it is not possible to adequately evaluate the genotoxicity of huperzine A.

5.3.4 Chronic toxicity and carcinogenicity
No chronic toxicity or carcinogenicity studies were identified.

5.3.5 Reproduction and developmental toxicity
No studies on reproduction and developmental toxicity after oral exposure were identified for hupezine A. However, two studies with intraperitoneal or intramuscular administration were identified and described in more detail below.

In a patent, Yu et al. (1993) describes results of two studies, but the underlying data were not published. In the first study, pregnant mice were exposed to huperzine A at gestational day (GD) 6 to 15 via intraperitoneal injections. Five different dose levels were administered, namely 0.019 (n=9), 0.038 (n=12), 0.08 (n=9), 0.19 (n=15) and 0.38 (n=10) mg/kg bw. The control group of 16 mice received an intraperitoneal injection with distilled water. Several aspects were measured including body weight and length of the foetus, number of resorbed foetuses and number of stillbirths (no further information available). At dose levels 0.19 and 0.38 mg/kg bw the number of absorbed foetuses was significantly higher compared to the control group ($p<0.01$). Also, the amount of stillbirths was significantly increased after a dose of 0.38 mg/kg bw. A single injection on GD10 had similar effects as daily injections from GD6 to 15. The embryos showed no physical abnormalities for all tested dose levels (Yu et al., 1993). Based on this study, RIVM (2001) previously established a NOAEL for huperzine A of 0.08 mg/kg bw.

In a second study, rabbits were exposed to 0.02 (n=2), 0.04 (n=3), 0.08 (n=6) or 0.2 (n=3) mg/kg bw of huperzine A via intramuscular injections at GD 7 to 18. The control group of four rabbits was exposed to distilled water. The same aspects were measured as in the mice experiment (no further information available). A significant difference ($p<0.05$) with the control group was only observed for number of stillbirths after administration of 0.08 mg/kg bw. No physical or internal abnormalities were observed in the rabbit foetuses (Yu et al., 1993).

Based on this study, RIVM (2001) previously established a NOAEL for huperzine A of 0.04 mg/kg bw.

For both studies only the results were described. Based on this information it could be determined that the developmental toxicity studies were not conducted according to the OECD standard, since the group sizes were under 16 pregnant animals, the compound was not orally administered, the study length was too short and the foetuses were not examined following the protocol (OECD, 2018). Literature was searched to further examine if AChE inhibition could be the mode of action for developmental toxicity found for huperzine A, but no strong evidence was found. An overview of developmental toxicity found for AChE inhibitors was available (Tsiaoussis et al., 2018). Many different effects were found in this study including fetal death, for the AChE inhibitors methyl parathion and Dimecron, the same effect as observed for huperzine A (Tsiaoussis et al., 2018; Sahu & Ghatak, 2002). As fetal death is observed for multiple AChE inhibitors, there is a possibility that it is a result of AChE inhibition, but no strong evidence supports this theory. Therefore, the mode of action for developmental toxicity for huperzine A remains unknown.

5.3.6 AChE inhibition

The effect of huperzine A on AChE inhibition was studied in vivo in different animals (Hamilton et al., 2017; Boudinot et al., 2005; RIVM, 2001; Cheng & Tang, 1998; Tang et al., 1994; Laganière et al., 1991; Tang et al., 1989).

Tang et al. (1989) studied the inhibition of AChE in Sprague-Dawley rats. In the first experiment, 2 mg/kg bw huperzine A via intramuscular injection resulted in a peak AChE inhibition of 39% after 30 minutes in red blood cells. In the brain, the peak AChE inhibition of 42% was observed 60 minutes post-dosing. However, in the brain the percentage of AChE activity inhibition was already above 40% after 30 minutes and did not lower below 40% until 4 hours post-dosing. After 6 hours still 32% of AChE activity was inhibited in the brain. On the other hand, in red blood cells the AChE activity was already restored to approximately 90% after 6 hours.

In another experiment the effect of huperzine A (0.1 - 2.0 mg/kg bw) via intraperitoneal injection on AChE activity was studied in several brain regions in Sprague-Dawley rats. The maximum dose of 2 mg/kg bw of huperzine A resulted in a decrease of AChE activity of at least 30% in all brain regions, except the striatum. The inhibition was especially high in the frontal cortex, where AChE activity was already decreased with 40% at a dose of 0.25 mg/kg bw. A concentration of 0.1 mg/kg bw of huperzine A did not decrease AChE activity for more than 20% in all brain regions (Tang et al., 1989).

Boudinot et al. (2005) studied the inhibition of brain AChE in mice after a subcutaneous injection of 1 mg/kg bw of huperzine A. The AChE activity in the brain decreased by 42% compared to the control group.

Laganière et al. (1991) studied the inhibition of AChE in male Sprague-Dawley rats after an intraperitoneally injection of 0.1 or 0.5 mg/kg bw of

huperzine A. Forty-five minutes after administration, 0.1 mg/kg bw of huperzine A did only slightly, but not significantly inhibit AChE activity in any of the brain regions. A dose of 0.5 mg/kg bw significantly decreased (15-30%) AChE activity in all measured brain regions: the hippocampus ($p<0.01$), striatum ($p<0.001$) and septum ($p<0.005$). No significant effects were observed 90 minutes post-dosing. The effect of multiple doses of huperzine A was similar to the results seen in the single dose experiment (Laganière et al., 1991).

Tang et al. (1994) studied the effect of huperzine A (0.1 - 2.0 mg/kg bw) via intraperitoneal injection on AChE inhibition in brain of Sprague-Dawley rats. In the hippocampus, cortex and hypothalamus the AChE activity was significantly reduced ($P<0.05$ or $P<0.01$) from 0.25 mg/kg bw of huperzine A onwards compared to the control group. In the striatum this was the case from the dose of 0.5 mg/kg bw onwards (Tang et al., 1994).

Cheng & Tang (1998) studied the effect of huperzine A on AChE activity in the brain of male Sprague-Dawley rats after oral administration. The brain regions of interest were the cortex, hippocampus, hypothalamus and striatum. Four to 12 rats per group received single doses of 0.242 (1 µmol), 0.484 (2 µmol) or 0.969 (4 µmol) mg/kg bw of huperzine A in saline or a saline control. After 30 minutes the animals were killed, brain regions were homogenized and incubated with a reaction mixture. In the ChE assay, the colour production was measured at 440 nm using a spectrophotometer. The AChE activity was calculated. In both the hypothalamus and striatum only the dose of 4 µmol/kg bw of huperzine A significantly decreased AChE activity by 20% ($p<0.05$) and 18% ($p<0.01$), respectively. Regarding the cortex and hippocampus the lowest dose of 1 µmol/kg bw of huperzine A did not significantly alter the AChE activity. However in the cortex both the 2 and 4 µmol/ kg bw of huperzine A significantly decreased ($p<0.01$) the AChE activity by 17 and 28%, respectively. Regarding the hippocampus, the AChE activity was significantly ($p<0.01$) reduced after both 2 and 4 µmol/kg bw of huperzine A, with 12 and 20%, respectively.

The data from this study were chosen for BMD modelling, since the animals were dosed orally and control values for AChE activity in the brain parts were available. Table 6 shows the raw data of AChE inhibition, extracted from the article of Cheng & Tang (1998) and the AChE activity as calculated using the control values. The assumption was made that all groups consisted of 4 rats, since this was the lowest value from the range for group size of 4 to 12 rats given in the article. The mean AChE activity and standard deviation of every group was used as input value in PROAST[24] combined with the group size of 4. The BMR was set at 0.20. This is the critical limit of inhibition of AChE activity set by the WHO (WHO, 2015). This means that an inhibition of AChE activity of 20% or more is seen as adverse by the WHO.

Figure 1 shows the output of our PROAST analysis. The approach, fitted models and model weighing, which show the models used for this analysis, can be found in the appendix. Moreover, the BMDL and BMDU visualised in Figure 1 can be found in Table 7. The lowest BMDL was

[24] Program developed by RIVM. Version 70.1, released on 19-10-2020

found in the hypothalamus (590 µg/kg bw), followed by the striatum (595 µg/kg bw), hippocampus (616 µg/kg bw) and cortex (657 µg/kg bw). The confidence intervals are twofold of the BMDL, which is an indication for suitable data.

It has to be noted that a group size of 4 used in this model is the minimal group size possible, since the sizes used in the study ranged from 4 to 12. Therefore, the analysis was repeated with a group size of 12, to study the effect of the group size. The lowest BMDL of this analysis was 642 µg/kg bw in the hippocampus. This is higher compared to the previous analysis. The confidence interval was slightly smaller, except for the confidence interval of the hippocampus. In this risk assessment, the worst case scenario will be used, which is the model including group size four.

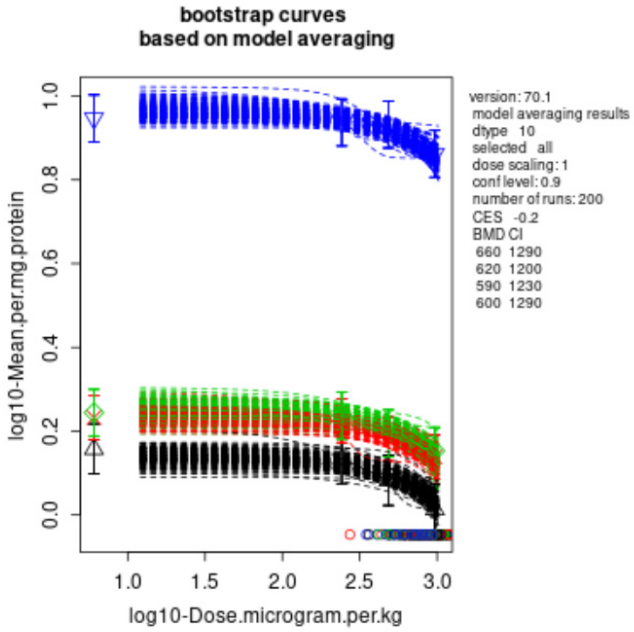


Figure 1 The bootstraps curves of the different brain parts; hypothalamus (green), cortex (black), hippocampus (red) and striatum (blue) after 200 runs in rats.

Table 6 The raw data of AChE inhibition (%) ± standard error of the mean (SEM) extracted from Cheng & Tang (1998) and the AChE activity (/mg protein) calculated using the known AChE activity in the control group in different brain parts at different dose levels administered as single doses to rats.

Dose (µg/kg bw)	**AChE**	**Cortex**	**Hippocampus**	**Hypothalamus**	**Striatum**
0	Inhibition	0	0	0	0
	Activity	1.46±0.13	1.73±0.14	1.78±0.15	9.01±0.87
242	Inhibition	6.06±5.74	2.98±2.02	2.95±2.18	3.91±2.69
	Activity	1.37±0.08	1.68±0.03	1.73±0.04	8.66±0.24
484	Inhibition	17.2±1.10	12.2±1.80	11.2±5.50	4.99±2.22
	Activity	1.21±0.02	1.52±0.03	1.58±0.10	8.56±0.19
968	Inhibition	28.7±4.70	19.8±5.90	19.9±4.50	18.6±5.10
	Activity	1.04±0.07	1.39±0.10	1.43±0.07	7.33±0.44

Table 7 The BMDL (µg/kg bw) and BMDU (µg/kg bw) determined by PROAST for the different brain regions in rats (n=4).

Brain region	**BMDL**	**BMDU**
Cortex	657	1290
Hippocampus	616	1200
Hypothalamus	590	1230
Striatum	595	1290

In another experiment, the AChE activity was measured after an intracerebroventricular injection of huperzine A (0.004 – 0.016 mg) (Cheng & Tang, 1998). The lowest dose did not alter AChE activity significantly in any of the brain regions. The dose of 0.008 mg significantly reduced AChE activity by 17, 19 and 27% in the cortex, hippocampus and striatum, respectively. The highest dose, significantly reduced AChE activity by 21, 24, 11 and 41% in the cortex, hippocampus, hypothalamus and striatum, respectively (Cheng & Tang, 1998).

Another study measured the effect of huperzine A on AChE activity in rat brain after intraperitoneal injection of huperzine A (0.12 – 0.48 mg/kg bw) (RIVM, 2001). At the lowest dose, AChE activity was inhibited around 10% in the cortex, hippocampus and striatum. In the cortex, 20% inhibition was reached at the dose of 0.36 mg/kg bw. The highest concentration resulted in an AChE inhibition of 22% in both the cortex and hippocampus and 18% in the striatum (RIVM, 2001).

Hamilton et al. (2017) studied the effect of huperzine A (0.625 – 40 µg/kg bw) via intramuscular injection on AChE inhibition in red blood cells of cynomolgus macaques (monkeys). All concentrations inhibited AChE activity ranging from around 10% for the lowest dose to approximately 90% at the highest dose (Hamilton et al., 2017).

Summary AChE inhibition
Overall, there is clear evidence that huperzine A can inhibit AChE activity in vivo. BMD modelling was performed with the data from Cheng & Tang (1998), as this study showed AChE inhibition after oral administration of huperzine A. The lowest BMDL found was 590 µg/kg bw.

5.3.7 Human data

For this risk assessment, the National Poisoning Information Centre (NIVC) and the Netherlands pharmacovigilance centre Lareb were contacted to gather information on *H. serrata*. NVIC received several notifications on products containing *H. serrata*. In most cases the complaints could be attributed to another ingredient. However, in two cases, *H. serrata* was the only ingredient in the herbal preparation. In these cases, the recommended daily dose was exceeded four or ten times. The patients developed symptoms as nausea, tremors, dysarthria, diarrhoea and blurred vision (NVIC, personal communication). Lareb did not receive any notifications for *H. serrata* thusfar (Lareb, personal communication).

Case reports
No case reports of specific *H. serrata* poisoning were identified in literature. However, two cases of *Lycopodium selago* poisoning were analyzed by Felgenhauer et al. (2000). Both *H. serrata* and *L. selago* are members of the Lycopodiaceae family and the genus *Huperzia* (USDA). Since both species contain huperzine A as an active compound (Szypuła et al., 2011), these case reports were described to give an indication of possible effects of *H. serrata*. A 60-year-old female and 62-year-old male drank tea made of approximately five grams of dried *L. selago*. Approximate doses of huperzine A were not given. Within 2 hours the

female experienced extreme sweating followed by diarrhoea and vomiting. These symptoms were combined with abdominal cramps, slurred speech and lack of feeling of the mouth. Also the systolic blood pressure increased to a value of 185 mmHg. The male started with the same symptoms of sweating, diarrhoea and vomiting. However, in this case also dizziness and cramps in hands and legs were experienced. Similarly to the female, also speech was affected combined with disturbed articulation. The systolic blood pressure increased to 200 mmHg. Both patients had to be treated in the hospital, but could return home the next day. The female experienced abdominal cramps for one more day and the male felt cramps in his thumbs for two more days. Since the symptoms suggest a cholinergic reaction, the AChE inhibitory activity of *L. selago* was studied in vitro and confirmed in this study. Boiling did not reduce the AChE inhibitory activity (Felgenhauer et al., 2000). The huperzine A concentration in *L. selago* varied from 440 to 1590 µg/g dry weight depending on origin (Szypuła et al, 2011). This is higher than the concentration measured for *H. serrata*, which varied from 46 to 133 µg/g.

Clinical trials
Zhang et al. (1991) studied the effect of huperzine A on people with multi-infarcted dementia or simple memory disorder. The treatment group received a daily oral dose of either 0.03 mg huperzine A for people with simple memory disorder (n=52), or 0.05 mg huperzine A for dementia patients (n=28), for 4 weeks. The control group (52 people with simple memory disorder and 28 people with dementia) received saline. Information on the exact measurements for adverse effects and whether the adverse effects were significantly different between the two groups is not available. However some patients experienced slight dizziness for a short period of time. The patients could continue the experiment and no treatment was needed. The treatment significantly improved memory (Zhang et al., 1991).

Xu et al. (1995) performed a randomized, placebo-controlled trial on the efficiency and safety of huperzine A. In this study, people with Alzheimer's disease were orally exposed to either 0.2 mg of huperzine A (n=50) or a placebo (n=53) twice a day for 8 weeks. Every week blood pressure and heart rate were measured. Moreover, every two weeks an electrocardiogram was conducted and the treatment-emergent signs and symptoms (TESS) score was given. Thereby, ALT, alkaline phosphatase, blood urea nitrogen, creatine, hemoglobin and white blood cells were measured every month. Only a slight increase in adverse effects like diarrhoea, vomiting and nausea was observed in the treatment group. However, this difference was not significant compared to the control group. Moreover, no significant difference ($P>0.05$) was found for all the parameters measured when results after 8 weeks were compared to the pre-trail number. In 58% of the patients receiving huperzine A, the memory improved (Xu et al., 1995).

Moreover, a randomized control trial was conducted by Zhang et al. (2002). In this study, 0.4 mg huperzine A was given daily during 12 weeks to 100 patients with Alzheimer's disease and results were compared to the control group (n=102). A safety evaluation was conducted every six weeks and included evaluating medical history, vital

signs, ECG results, nerve system functioning, blood test results and urine test results. Overall, 3% of the patients in the treatment group experienced adverse effects, like insomnia and edema, nausea, anorexia. However, also adverse events were observed in the control group like bradycardia, headache and tightness in the chest area. No significant difference was observed between the groups for adverse effects, liver and kidney functioning or results of the blood tests. Huperzine A improved cognition, behaviour and mood in the patients (Zhang et al., 2002).

In the study of Rafii et al. (2011) the tolerability, safety and efficacy of huperzine A was studied in a randomized, controlled trial. Patients with Alzheimer's diseases were exposed to huperzine A or received a placebo for at least 16 weeks. Group A (n=70) received 0.1 mg huperzine A twice a day for two weeks, continued by 0.2 mg twice a day for 22 weeks. Group B (n=70) received the same amount huperzine A as group A in the first 4 weeks, continued by 0.3 mg twice a day in week 5 and 6 and 0.4 mg twice a day in the last 18 weeks. The placebo group (n=70) received a placebo until week 16, continued by 0.1 mg huperzine A twice a day during week 17 to 20 and 0.2 mg twice a day for the last 4 weeks. The tolerability and safety was measured using laboratory tests, checking of vital signs and an ECG. Vital signs were measured at each visit and an ECG was performed at week 2, 4, 8, 11, 16, 20 and 24. No significant differences in adverse effects were observed between the groups. Some severe adverse events that occurred were not considered to be related to huperzine A. Huperzine A did not improve cognition in the patients (Rafii et al., 2011).

Another randomized, double-blind placebo-controlled trial was conducted by Xu et al. (2012). Patients with vascular dementia were orally given either 0.1 mg huperzine A (n=39) or 100 mg vitamin C (n=39) daily for 12 weeks. The type and number of adverse events were monitored by performing routine clinical laboratory tests and physical examinations before the start of the experiment and after 12 weeks. Only one person exposed to huperzine A experienced adverse effects like nausea and dizziness. However, no significant changes were observed in the laboratory tests and physical examinations. Huperzine A significantly improved cognitive function in the patients (Xu et al., 2012).

Summary human data
Overall, NVIC received two notifications about adverse effects, such as nausea, tremors, dysarthria, diarrhoea and blurred vision, in patients who took an overdose of herbal preparations containing only *H. serrata*. Also one case report was available, which described the adverse cholinergic effects seen in two patients after consumption of tea with Lycopodium selago, a plant from the same family and genus as *H. serrata*, which both contain huperzine A.
In addition, several clinical trials with huperzine A showed mild adverse effects, including diarrhoea, vomiting and dizziness, but these were not statistically significantly different between the control and experimental groups in the trials. One trials mentioned that adverse effect were not related to the treatment.

5.3.8 Interactions

Natural Medicines reported two possible pharmacokinetic interactions, supported by some experimental evidence[25]. Three animal studies showed an interaction of huperzine A with the anticholinergic drug scopolamine. The effect of scopolamine was decreased by huperzine A. Moreover, a non randomized clinical trial showed an interaction of huperzine A with cholinergic drugs. In this interaction huperzine A and cholinergic drugs have additive effects, which could potentially increase side effects. Pepping (2000) reported a toxicodynamic interaction of huperzine A with cardiac medication like β-blockers, which could lead to bradyarrhythmia, as a result of an additive effect. Theoretically, interactions with CYP enzymes could result in altered concentrations of other compounds, metabolized by these CYP enzymes, or other compounds could alter the concentration of huperzine A. This could happen since it was shown that CYP1A2 and CYP3A1/2 (orthologue of human CYP3A4) are involved in the huperzine A metabolism in rats (Ma et al., 2003a). Moreover, an inducing effect of huperzine A on CYP3A4 activity was shown in humans (Zhang et al., 2014). Other compounds which affected CYP1A2 activity are for example caffeine or the medication omeprazole. These compounds can induce CYP1A2 activity and therefore might change the huperzine A metabolism (Ma et al., 2003a).

5.4 Derivation of toxicological reference value

As there were no toxicity data available for *H. serrata,* it is not possible to establish a toxicological reference value for *H. serrata.*

In addition, it was not possible to establish a health-based guidance value (HBGV) for huperzine A.
An Acute Reference Dose (ARfD) could not be derived as the genotoxicity of huperzine A could not be adequately evaluated.
An Acceptable Daily Intake (ADI) could also not be established as the toxicological dataset for huperzine A was incomplete: no reproductive toxicity studies, nor oral toxicity studies with a duration longer than 30 days were identified and genotoxicity could not be adequately evaluated. Furthermore, the were unresolved concerns regarding developmental toxicity.

As no HBGV can be established, it was considered if another reference value could be derived and used in the risk assessement.

In humans several clinical trials and a case report are available.
The clinical trials were mainly focussed on the positive effect of huperzine A on memory and brain function and reported less on adverse effects. Therefore, not all toxicological endpoints were studied in these trials. In addition, the study population was not representative for the general population, since only older and often sick individuals were included. As a consequence, the data from these trials are of limited value for establishing a reference value.
The case report only gives an indication of the severity of the adverse

[25]https://naturalmedicines.therapeuticresearch.com/databases/food,-herbs-supplements/professional.aspx?productid=764. Accessed in oktober 2021.

effects at an extreme dose, as it is about a closely related species of *H. serrata* and is not further considered.

In animals, the results of the available developmental toxicity studies suggest that huperzine A may be embryotoxic. In both rabbits and mice a significant increase in stillbirths was observed after intramuscular administration and intraperitoneal injection, respectively. In addition, in mice also a significant increase in foetal resorptions was observed (Yu et al., 1993).

Based on this study, RIVM (2001) previously established a NOAEL of 0.04 mg/kg bw in rabbits, and a NOAEL of 0.08 mg/kg bw in mice (see section 5.3.5). As mentioned, these studies were conducted using intramuscular or intraperitoneal administration. The internal concentration peak of huperzine A could be higher after injection compared to oral exposure, possibly resulting in effects at lower doses. Moreover, the study description was limited and does not comply with current OECD standards. Also, in the rabbit study, a significant effect was observed after administration of a low dose (0.08 mg/kg bw) huperzine A but not at a higher dose (0.2 mg/kg bw). It was assumed that this was a result of the larger group size used at the lower dose (n=6) compared to the higher dose (n=3). The results of this study are considered less reliable. Therefore, the developmental toxicity were not suitable to use as a reference value.
Nevertheless, the NOAELs found in these developmental toxicity studies are in the same order of magnitude as the LOAEL found in a acute oral toxicity study performed by Schmidt and van der Staay (1998), most likely because the oral bioavailability of huperzine A is high.

In the latter study, the LOAEL for acute physiological and behavioural symptoms of cholinergic overstimulation was 0.3 mg/kg bw, the lowest dose tested. The adverse effects were scored by qualitative observations directly after dosing and every 15 minutes until 3 hours after dosing. This is a disadvantage compared to quantitative measurements, as the results are less objective. Another disadvantage of this study is that a racemic mixture of huperzine A (ratio unknown) was used instead of only the natural and potent (-)-eutomer. An in vitro study showed that a racemic mixture was three times less potent than natural huperzine A (Ferreira et al., 2016). As a result, the study would most likely underestimate the effects of natural huperzine A. In a previous risk assessment, RIVM (2001) established an 'estimated NOAEL' of 0.1 mg/kg bw, using a factor three for LOAEL to NOAEL extrapolation.

Another acute oral toxicity study was identified, which studied the inhibition of AChE in rats after huperzine exposure (Cheng & Tang, 1998, see section 5.3.6). The data of this study were used for BMD modelling. An overview of the studies characteristics can be found in Table 8. The lowest BMDL, for 20% AChE inhibition, was 0.6 mg/kg bw (590 µg/kg bw). The goal of the study was to compare the effect of three different AChE inhibitors, namely huperzine A, E2020 and tacrine. For all three inhibitors, the AChE activity was measured 30 minutes after dosing. The choice of timing of the measurements was not substantiated in the study.

The question is if 30 minutes is the optimal window to measure huperzine A induced AChE inhibition. The toxicokinetic parameters, including time to maximal effects, of huperzine A are well studied, but it is difficult to compare different studies as this depends on many factors, including species and strain of animal, dose, route of exposure and measured endpoints. One study is available in literature (Yue et al., 2007; see section 5.1.1), which studied toxicokinetic parameters, including the T_{max} in Sprague-Dawley rats, the same strain of rats as studied in Cheng & Tang (1998). After intragastric administration of 0.5 mg/kg bw huperzine A the T_{max} in plasma and cerebrospinal fluid was reached after 51 and 102 minutes, respectively. The toxicity study of Schmidt & van der Staay (1998) also suggest that 30 minutes might be early for the maximal effect. In the study of Schmidt & van der Staay (1998) the maximal effect after a dose of 0.3 mg/kg bw via oral gavage was observed around one hour post dosing. It has to be noted that in this study a different strain of rats was studied and different endpoints were measured compared to the study of Cheng & Tang (1998).

Table 8 Summary overview of two critical studies, Schmidt & van der Staay (1998) and Cheng & Tang (1998).

Schmidt & van der Staay, 1998	**Cheng & Tang, 1998**
• Male Wistar rats	• Male Sprague-Dawley rats
• 6 animals per group	• 4 – 12 animals per group
• 0, 0.3, 1, 3 mg/kg bw of racemic mixture of huperzine A	• 0, 0.242, 0.484, 0.969 mg/kg bw of huperzine A
• Administered by oral gavage	• Administered orally
• Qualitative observations of physiological and behavioural symptoms of cholinergic overstimulation	• Quantitative measurements of acetylcholinesterase activity in the brain (cortex, hippocampus, hypothalamus and striatum)
• Directly and every 15 minutes until 3 hours post-dosing	• 30 minutes post-dosing
• LOAEL: 0.3 mg/kg bw	• BMDL: 0.6 mg/kg bw
	• BMR: 20% AChE inhibition

In general, using a BMDL as a reference value has the preference above using a NOAEL or LOAEL, as the uncertainty is large in NOAELs and LOAELs. Furthermore, quantitative measurements of AChE activity have a preference above qualitative observations of cholinergic overstimulation. However, in this case an adverse effect has been observed below the BMDL, as the LOAEL is below the BMDL. Therefore, the studies were compared in more detail to established the most appropriate reference value. A disadvantage of the study of Cheng & Tang (1998), which the BMDL is derived from, is the time of measurements. In the study the AChE activity was only measured at one timepoint, 30 minutes after dosing. There is considerable evidence

that 30 minutes is too soon to reach the peak tissue concentration of huperzine A and peak effect after oral administration. With the available information it is not possible to accurately estimate what level of uncertainty factors would be necessary to take the difference between the measured inhibition and the peak inhibition into account. Furthermore, to take into account the cholinergic adverse effect observed at lower doses, another safety factor would be necessary. A disadvantage of the study of Schmidt & Staay (1998), which the LOAEL was derived from, is the use of qualitative observations, which made it not possible to use the data for BMD modeling. The LOAEL available is more uncertain than a BMDL would have been. Furthermore, in this study a racemic mixture of huperzine A was administered to the animals, which is mostly likely less potent than natural huperzine A. Safety factors would be necessary to take this difference into account.

Taken all the above mentioned arguments into consideration, it was decided to use the LOAEL of 0.3 mg/kg bw established in the study of Schmidt & Staay (1998) as a reference value in the risk assessment, as the AChE activity in the study of Cheng & Tang (1998) was only measured 30 minutes post-dosing and as this is the lowest oral dose in the toxicological data showing an adverse effect.

6 Risk assessment

6.1 Risk assessment

As a first step in the risk assessment, it was investigated whether the presumption of safety can be applied to *Huperzia serrata*. Botanical preparations for which an adequate body of knowledge exists, can benefit from a presumption of safety without any need for further testing (EFSA, 2009; EFSA, 2014). This generally means that when there is a history of safe use and the intended use of the botanical preparation in food supplements does not exceed the historical levels of intake, the intended use in food supplements is assumed to be safe. *H. serrata* has a history of use as traditional Chinese medicine, however, safety is not adequately documented and the level of exposure to *H. serrata* in traditional Chinese medicine is unknown. The presumption of safety could therefore not be applied to *H. serrata* and more information was needed to assess its safety.

It was not possible to establish a HBGV for *Huperzia serrata* or huperzine A, and hence no safe use level could be determined.

For the risk assessment, the Margin of Exposure (MOE) approach was applied using the LOAEL of 0.3 mg huperzine A per kg bw for acute cholinergic effects. A minimal MOE of 1500 was considered necessary to assume no acute adverse health effects would occur. This minimal MOE was based on assessment factors for the intra- and interspecies variation, the quality of the data and the use of a LOAEL instead of a NOAEL as a reference value. An overall assessment factor of 100 is default for intra- and interspecies variation (EFSA, 2012b). EFSA recommends establishing a factor for quality of data on a case to case basis (EFSA, 2012b). WHO recommends a factor between two and ten (WHO, 2009). Since a racemic mixture of huperzine A was administered to the animals and the dataset was incomplete, it was decided to include an extra factor of 5. This is the same factor as used in the risk assessment of 2001, which was partly based on the same study (RIVM, 2001). Furthermore, an additional assessment factor was applied for LOAEL to NOAEL extrapolation. According to EFSA (2012b), this factor should be determined on a case to case basis. In this assessment a factor 3 was applied, the same factor as in the risk assessment of 2001 (RIVM, 2001).

For herbal preparations containing *H. serrata* extract, the estimated exposure to huperzine A ranged from 0.7 to 11.4 µg/kg bw for an individual weighing 70 kg.

MOEs were calculated using the LOAEL and the estimated exposure and ranged from 26 to 429 (Table 9). The calculated MOEs for acute exposure to huperzine A via herbal preparations containing *H. serrata* extract were insufficient (well below 1500). Therefore, it can be concluded that the current use of herbal preparations containing *H. serrata* and huperzine A may lead to acute cholinergic adverse effects.

Table 9 The margin of exposure calculated by dividing the Lowest Observed Advere Effect Level (LOAEL) for cholinergic overstimulation by the estimated exposure to huperzine A.

	Estimated exposure (0.7-11.4 µg/kg bw)
LOAEL (300 µg/kg bw)	26 - 429

It is important to consider that this risk assessment is solely based on toxicological data for huperzine A, one of the constituents of *H. serrata*. However, many more of its constituents, including eight other alkaloids with AChE inhibitory activity are present in *H. serrata*. Therefore, cholinergic effects of *H. serrata* extract might be stronger than calculated in this risk assessment. To be able to include the other constituents of *H. serrata* in the risk assessment, concentration and toxicological data are needed of all individual constituents, which are currently not available.

In addition, it has to be noted that in this risk assessment only the acute effects of huperzine A were assessed. None of the repeated dose toxicity studies identified in the literature search were considered suitable to use as a reference value, including the studies indicating that huperzine A is embryotoxic. In addition, no reproductive toxicity studies and no oral toxicity studies with a durations longer than 30 days were identified. Furthermore, the genotoxicity of huperzine A could not be adequately evaluated. Owing to omissions in the toxicological profile, no firm conclusions can be drawn on these aspects and the safety of herbal preparations containing *H. serrata* and huperzine A in pregnant women or after repeated exposure.

6.2 Interactions

Natural Medicines[26] reported two possible interactions of huperzine A, with scopolamine, an anticholinergic drug, and cholinergic drugs. Also interactions with dietary components or medication could occur as a result of huperzine's metabolism or effect on CYP enzymes. Regarding the metabolism of huperzine A, studies in rats indicate the involvement of two CYP enzymes, CYP1A2 and CYP3A1/2 (orthologue of human CYP3A4). When the activity of these CYP enzymes is altered, the toxicokinetics of huperzine A can change. For example, caffeine and omeprazole can alter CYP1A2 activity and might potentially alter the toxicokinetics of huperzine A. In addition, a study suggests that huperzine A may induce CYP3A4, which can potentially alter the kinetics of other substances that are metabolised by this enzyme.

6.3 Sensitive/vulnerable groups

The studies in pregnant rabbits and mice indicate that huperzine A is embryotoxic, thus unborn children might be more vulnerable to the effects of huperzine A (Yu et al., 1993).

Moreover, another study found that older people had a lower clearance of huperzine A than younger people resulting in a higher AUC (Sheng et

[26]https://naturalmedicines.therapeuticresearch.com/databases/food,-herbs-supplements/professional.aspx?productid=764. Accessed in oktober 2021.

al., 2016). As a result of the higher exposure, older people might be more sensitive to effects of huperzine A. Moreover, it should be taken into account that other factors could also differ between the older people and younger people. For example, Alzheimer's disease has a large influence on the cholinergic system (Ferreira-Vieira et al., 2016), thus the effect of huperzine A could be different in someone with Alzheimer's disease compared to a healthy individual. Therefore, younger people might respond differently to huperzine A and could potentially be more or less vulnerable.

The warning phrases on herbal preparations (section 3.6) and the drug interactions (section 6.2) also indicate that certain subpopulations may be more sensitive for the effects of huperzine A.

Not enough information was identified to conduct a risk assessment for specific groups.

6.4 Uncertainties

6.4.1 Exposure

The composition of the plant material used in herbal preparations is not always known and is known to differ between different brands and between batches. This hampers exposure assessment. Furthermore, the estimated exposure in this report is based on normal or recommended use. When users do not adhere to the recommended use, actual exposure to huperzine A may be higher or lower than estimated.. Moreover, no information is available about the frequency of use for the pre-workout supplements. Therefore, the exposure to huperzine A could be over- or underestimated, when pre-workout supplements are not used once a day.

In addition, the ingredient list is not always clear for all herbal preparations, especially for the pre-workout supplements.

6.4.2 Toxicity

This risk assessment focussed only on huperzine A as the active constituent from *H. serrata*. However, currently there are eight other alkaloids isolated from *H. serrata*, which can also inhibit AChE activity. These constituents were not included in this risk assessment since both exposure as well as toxicological data are lacking. Still, it should be noted that the presence of the other alkaloids with AChE activity in herbal preparations containing *H. serrata* extract may add to the toxicity.

Many of the available toxicity studies were published in a patent application or study results were only briefly mentioned without the underlying data. For these studies the available information was too limited to adequately evaluate the toxicity of huperzine A.

In the study of Schmidt & van der Staay (1998), which is used to derive the reference value, a synthetic huperzine A is used. This synthetic version is a mixture of (-) huperzine A and (+) huperzine A, with unknown ratio of enantiomers. Natural huperzine A, for example found in an extract of *H. serrata*, only contains the (-) eutomer. In one study it

was found that natural huperzine A is three times more potent compared to the a racemic huperzine A (Ferreira et al., 2016). In herbal preparations, an extract of *H. serrata* is used, meaning that it only contains (-)huperzine A and this likely to be more potent compared to the compound studied in Schmidt & van der Staay (1998), which would underestimate the toxicity. To take this into account, an additional uncertainty factor was included in the minimal MOE, but it is still a source of over- or underestimation of the toxicity.

The toxicological dataset for huperzine A is incomplete. No reproductive toxicity studies and no oral toxicity studies with a durations longer than 30 days were identified. Also, the genotoxicity of huperzine could not be adequately evaluated. Furthermore, the developmental toxicity studies which indicate that huperzine A is embryotoxic, were not considered suitable to use as a reference value. Owing to omissions in the toxicological profile, no firm conclusions can be drawn on these aspects and the safety of herbal preparations containing *H. serrata* and huperzine A in pregnant women or after repeated exposure.

7 Conclusions and recommendations

Use of herbal preparations containing *H. serrata* extract and huperzine A that are currently available on the Dutch market may lead to acute cholinergic adverse effects, including increased salivation, muscular weakness, cramps, lacrimation, diarrhoea, paralysis and blurred vision. In addition, there are data indicating that huperzine A is embryotoxic.

It is noted that only the acute effects of huperzine A could be evaluated in this risk assessment. Data on repeated exposure were not sufficient to evaluate the risk of prolonged exposure to huperzine A.

Based on the possible acute adverse effects of huperzine A, RIVM advises consumers to not use herbal preparations containing *H. serrata* and huperzine A, especially not during pregnancy.

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Appendix A

Search strategy

Database	Keywords
PubMed	"Huperzine A; "Selagine"; "Huperzia serrata"; "Lycopodium serratum"; "Urostachys serratus"; "Toothed clubmoss"; "Chinese clubmoss"; "Qian Ceng Ta"; toxic*; intoxic*; toxin*; poison*; genotox*; neurotox*; hepatotox*; cytotox*; immunotox*; mutagen*; carcinogen*; phototox*; embryotox*; risk*; safe*; photocytotox*; acute
Scopus	Huperzine-A; Selagine; Huperzia-serrata; Lycopodium-serratum; Urostachys-serratus; Toothed-clubmoss; Chinese-clubmoss; Qian-Ceng-Ta; *toxic*; *toxin*; poison*; mutagen*; carcinogen*; risk*; safe*; acute
Embase	'physical disease'/exp/mj/dm_co,dm_si; 'mental disease'/exp/mj/dm_co,dm_si; toxic*:ti; intoxic*:ti; toxin*:ti; poison*:ti; genotox*:ti; neurotox*:ti; hepatotox*:ti; cytotox*:ti; immunotox*:ti; mutagen*:ti; carcinogen*:ti; phototox*:ti; embryotox*:ti; risk*:ti; safe*:ti; photocytotox*:ti; 'risk'/exp; 'toxicokinetics'/exp/mj; 'pharmacokinetics'/exp/mj; 'metabolism'/exp/mj; 'toxic substance'/exp; 'toxicity and intoxication'/exp; 'exposure'/exp; 'huperzine a'/exp/dd_to; 'huperzine a'/exp/dd_ae; 'huperzine a':ti; 'selagine':ti; 'huperzia serrata':ti; 'lycopodium serratum':ti; 'urostachys serratus':ti OR 'toothed clubmoss':ti; 'chinese clubmoss':ti; 'qian ceng ta':ti; huperzine a'/exp/mj; 'huperzia serrata'/exp/mj
Toxcenter	Huperzine-A; Selagine; Huperzia-serrata; Lycopodium-serratum; Urostachys-serratus; Toothed-clubmoss; Chinese-clubmoss; Qian-Ceng-Ta)/TI; ?toxic?; ?toxin?; poison?; mutagen?; carcinogen?; risk?; safe?; acute; person#; human?; volunteer#; man; men; woman; women; boy#; girl#; child?; infant#; worker#; employee#; case; cases; rat; rats; mouse; mice; dog#; hamster#; pig#; monkey#; rabbit#; mammal#

Appendix B

Overview of the toxicokinetic parameters

Table 10 Toxicokinetic paramers of huperzine A after a single dose of 13.9 GBq via different routes of administration in rats (Wang et al., 1988)

Route of administration	C_{max} (dpm/ml)	T_{max} (min)	AUC (10^{-7}* (dpm*min)/ml)	$t_{1/2}$ (h)	bioavailability (%)
Intravenous	-	-	2.6±0.9	2.5±1.6	-
Intragastric	98569±12153	21±12	1.8±0.8	3.4±3.4	96.9

maximum concentration C_{max}; time to reach the maximum concentration T_{max}; area under the curve AUC; plasma half-life $t_{1/2}$

Table 11 Toxicokinetic parameters of huperzine A after a single dose via different routes of administration in blood plasma of dogs and rats

Animal	Route of administration	Dose (mg/kg)	C_{max} (ng/ml)	T_{max} (min)	AUC_{0-t} (ng*h/ml)	$t_{1/2}$ (h)	Bioavailability (%)	Reference
Dog	Intravenous	0.01	5.6±1.6	5±0.0	16.0±5.2	5.0±0.3	-	1
Dog	Oral	0.1 (mg)	2.6±0.6	75±30	12.9±3.2	5.7±2.3	94.4	1
Dog	Oral	0.5 (mg)	9.8±1.0	180	-	5.9±1.3	-	2
Rat	Intravenous	0.167	132.3±31.3	2±0	112.8±78.2	1.8±0.8	-	3
Rat	Intravenous	0.5	285.6±105	2±1.2	219.7±40.0	2.0±0.8	-	3
Rat	Intragastric	0.5	71.6±24.7	51±22.8	217.3±82.9	2.5±0.8	-	3
Rat	Intranasal	0.167	60.0±19.3	16±7.8	101.9±29.2	1.4±0.6	-	3
Rat	Intranasal	0.5	104.1±34.3	23±8.4	200.6±33.6	1.8±0.2	-	3
Rat	Intravenous	0.5	257±57	3±3	18233±18233 (µg*min/L)[27]	1.0±0.2	-	4
Rat	Intranasal	0.5	190±37	41±7	21592±5034 (µg*min/L)[36]	0.8±0.2	-	4

(maximum concentration C_{max}; time to reach the maximum concentration T_{max}; area under the curve AUC; plasma half-life $t_{1/2}$; 1. Chu et al., 2006; 2. Ye et al., 2008; 3. Yue et al., 2007; 4. Wang & Chen, 2009)

[27] Values in AUC_{inf}, equal to 1295520 (i.n.) and 1093980 (i.v.) ng*h/ml

Table 12 Toxicokinetic parameters of huperzine A after multiple dose levels via different routes of administration in blood plasma dogs

Route of administration	**Dose**	**Days**	**C_{max} (ng/ml)**	**T_{max} (h)**	**AUC_{0-t} (ng*h/ml)**	**$t_{1/2}$ (h)**	**Reference**
Oral	0.5 (mg)	5	10.1±1.1	3	108.5±8.8	-	1
Intramuscular	10 (mg/kg)	15	0.4±0.1	48±25	92.6±4.5	54.8±5.6	2

(maximum concentration C_{max}; time to reach the maximum concentration T_{max}; area under the curve AUC; plasma half-life $t_{1/2}$; 1. Ye et al., 2008; 2. Wang et al., 2004)

Table 13 Toxicokinetic parameters of huperzine A after a single dose via different routes of administration (intravenous, intragastric, intranasal) in cerebrospinal fluid of rats.

Administration	**Dose (mg/kg)**	**C_{max} (ng/ml)**	**T_{max} (min)**	**AUC_{0-t} (ng*h/ml)**	**$t_{1/2}$ (h)**	**Reference**
Intravenous	0.167	36.9±7.3	4±2	37.9±10.2	1.1±0.3	1
Intravenous	0.5	78.6±9.5	7±4	129.6±8.9	1.7±0.4	1
Intragastric	0.5	21.2±6.4	102±27	67.6±4.1	3.6±0.3	1
Intranasal	0.167	23.9±5.8	38±16	48.4±8.8	2.0±0.7	1
Intranasal	0.5	40.5±11.0	40±16	89.9±12.2	2.0±0.7	1
Intranasal	0.5	43±10	21±8	4300±616 (µg*min/L) [28]	1.0±0.2	2
Intravenous	0.5	68±5	5±4	4119±792 (µg*min/L) [37]	0.8±0.2	2

(maximum concentration C_{max}; time to reach the maximum concentration T_{max}; area under the curve AUC; plasma half-life $t_{1/2}$; 1. Yue et al., 2007; 2. Wang & Chen, 2009)

[28] Values in AUC_{inf}, equal to 258000 (i.n.) and 247140 (i.v.) ng*h/ml

Appendix C

BMD model Cheng & Tang (1998)

Approach
PROAST (version 70.1, released on 19-10-2020), a program developed by RIVM was used for benchmark dose (BMD) modeling. The study contains data on the AChE inhibition by huperzine A for four different brain parts, which were handled as one endpoint and included as covariates. The group size used was four. As the benchmark response (BMR) 20% AChE inhibition was taken. Four different models were used in the analysis; hill, exponential, inverse exponential and lognormal DR. In total, 200 bootstrap runs were conducted. The results showed a benchmark lower and upper confidence limit, the BMDL and BMDU.

Console output

```
response:  Mean.per.mg.protein
ANALYSIS WITH EXPONENTIAL MODELS

model   converged     npar   loglik   aic
 full model      1       17      54.91          -75.82
 full-v   1       20      55.05          -70.1
 null model      1       2       -74.11         152.22
 null model-a    1       5       34.8    -59.6
 Expon. m3-      1       4       -73.65         155.3
 Expon. m3-a     1       7       52.88          -91.76
 Expon. m3-ab            1       10      54.07          -88.14
 Expon. m5-a     1       8       53.06          -90.12
 Expon. m5-ab            1       11      54.32          -86.64
 ---------------------------------------------------
Best model with covariates is: Expon. m3-a
However Expon. m5-a is a reasonable model as well
-----------------------------------------------------
selected model: Expon. m3-a
  estimate for  var- :  0.01122
  estimate for  a-Cortex :  1.378
  estimate for  a-Hippocampus :  1.725
  estimate for  a-Hypothalamus :  1.779
  estimate for  a-Striatum :  9.16
  estimate for  CED- :  899.3
  estimate for  d- :  1.247
-----------------------------------------------------
 calculating confidence intervals ....

the CED (in orig. units) and the 90 % confidence interval is:
 899.3
 711 - 1120

response:  Mean.per.mg.protein
ANALYSIS WITH HILL MODELS
```

```
model   converged     npar   loglik   aic
 Hill m3-a       1       7       52.88          -91.76
 Hill m3-ab      1       10      54.07          -88.14
 Hill m5-a       1       8       53.06          -90.12
 Hill m5-ab      1       11      54.35          -86.7
 ---------------------------------------------------
 Best model with covariates is: Hill m3-a
 However Hill m5-a is a reasonable model as well
 ---------------------------------------------------
selected model: Hill m3-a
 estimate for  var- :  0.01122
 estimate for  a-Cortex :  1.378
 estimate for  a-Hippocampus :  1.725
 estimate for  a-Hypothalamus :  1.779
 estimate for  a-Striatum :  9.16
 estimate for  CED- :  899.3
 estimate for  d- :  1.249
---------------------------------------------------
 calculating confidence intervals ....

the CED (in orig. units) and the 90 % confidence interval is:
899.3
711 - 1120

response:  Mean.per.mg.protein
ANALYSIS WITH INVERSE EXPONENTIAL MODELS

model   converged     npar   loglik   aic
 Inv.Expon. m3-a      1       7       52.96          -91.92
 Inv.Expon. m3-ab     1       10      54.22          -88.44
 Inv.Expon. m5-a      1       8       53.03          -90.06
 Inv.Expon. m5-ab     1       11      54.37          -86.74
 ---------------------------------------------------
 Best model with covariates is: Inv.Expon. m3-a
 However Inv.Expon. m5-a is a reasonable model as well
 ---------------------------------------------------
selected model: Inv.Expon. m3-a
 estimate for  var- :  0.01119
 estimate for  a-Cortex :  1.377
 estimate for  a-Hippocampus :  1.724
 estimate for  a-Hypothalamus :  1.777
 estimate for  a-Striatum :  9.151
 estimate for  CED- :  897
 estimate for  d- :  0.2192
---------------------------------------------------
 calculating confidence intervals ....

the CED (in orig. units) and the 90 % confidence interval is:
 897
 702 - 1140
response:  Mean.per.mg.protein
ANALYSIS WITH LOGNORMAL DR MODELS
model   converged     npar   loglik   aic
 LN m3-a         1       7       52.93          -91.86
```

```
 LN m3-ab      1       10      54.16          -88.32
 LN m5-a       1       8       53.04          -90.08
 LN m5-ab      1       11      54.39          -86.78
 ---------------------------------------------------
 Best model with covariates is: LN m3-a
 However LN m5-a is a reasonable model as well
  ---------------------------------------------------
selected model: LN m3-a
estimate for  var- :  0.0112
estimate for  a-Cortex :  1.377
estimate for  a-Hippocampus :  1.724
estimate for  a-Hypothalamus :  1.778
estimate for  a-Striatum :  9.155
estimate for  CED- :  897.8
estimate for  d- :  0.4137
---------------------------------------------------
 calculating confidence intervals ....

the CED (in orig. units) and the 90 % confidence interval is:
897.8
706 - 1130

 ----------  CES =  -0.2  --------------------------------
The colors in the plot relate to the following subgroups:
      color             mark       subgroup
1     black   upward triangle       Cortex--
2       red            cross  Hippocampus--
3     green          diamond Hypothalamus--
4 dark blue downward triangle     Striatum—

Calculating confidence intervals by model averaging, this may make
some time ....

 The weights used in model averaging are:

   model weight
1    EXP 0.2419
2   HILL 0.2419
3 INVEXP 0.2620
4   LOGN 0.2543

Start of MA bootstrap runs ...

run 1 – 200

ATTENTION:
       There are NAs in the vector of bootstrap CEDs, this indicates a
problem in interpolation

The model-average BMD confidence interval is:
      subgroup BMDlower.ma BMDupper.ma

1       Cortex        657       1290
2  Hippocampus        616       1200
```

3	Hypothalamus	590	1230
4	Striatum	595	1290

Fitted models

model	converged	loglik	npar	AIC
full model	1	54.91	17	-75.82
full-v	1	55.05	20	-70.1
null model	1	-74.11	2	152.22
null model-a	1	34.8	5	-59.6
Expon. m3-	1	-73.65	4	155.3
Expon. m3-a	1	52.88	7	-91.76
Expon. m3-ab	1	54.07	10	-88.14
Expon. m5-a	1	53.06	8	-90.12
Expon. m5-ab	1	54.32	11	-86.64
Hill m3-a	1	52.88	7	-91.76
Hill m3-ab	1	54.07	10	-88.14
Hill m5-a	1	53.06	8	-90.12
Hill m5-ab	1	54.35	11	-86.7
Inv.Expon. m3-a	1	52.96	7	-91.92
Inv.Expon. m3-ab	1	54.22	10	-88.44
Inv.Expon. m5-a	1	53.03	8	-90.06
Inv.Expon. m5-ab	1	54.37	11	-86.74
LN m3-a	1	52.93	7	-91.86
LN m3-ab	1	54.16	10	-88.32
LN m5-a	1	53.04	8	-90.08
LN m5-ab	1	54.39	11	-86.78

Model weights

EXP	0.2419
HILL	0.2419
INVEXP	0.262
LOGN	0.2543

Results of individual models

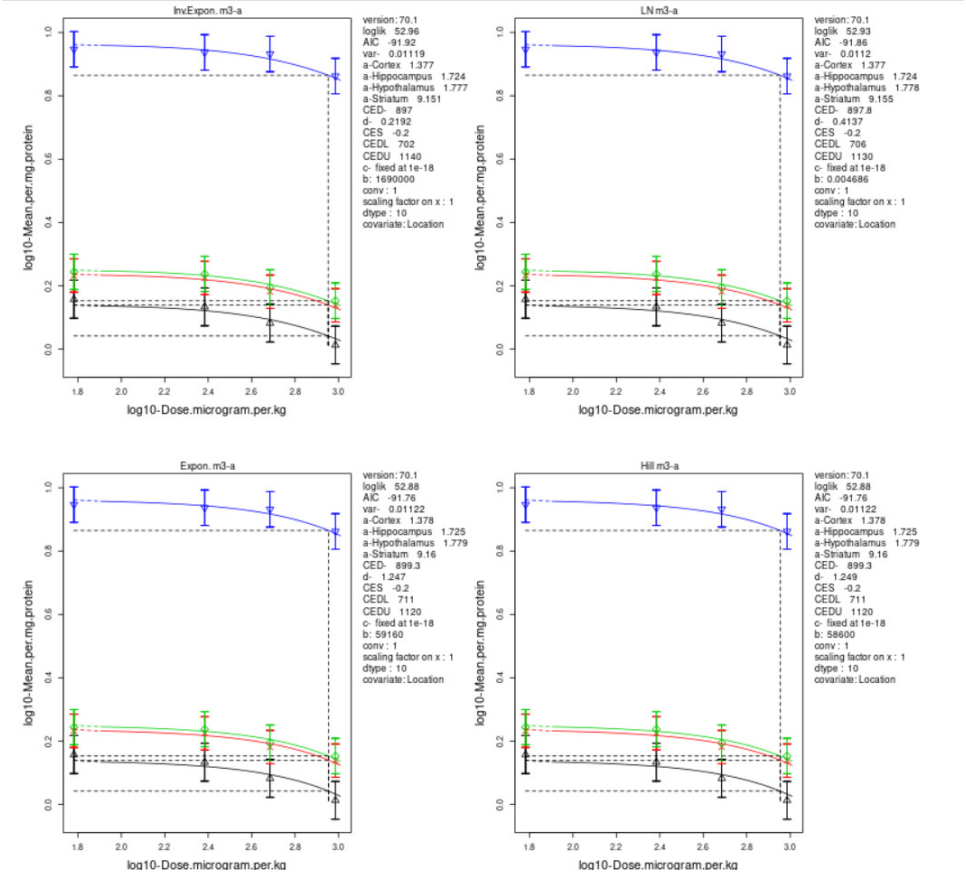

Figure 2 The results of the four different models; inverse exponential (above left), lognormal DR ((above right), hill (below right) and exponential (below left).

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