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FRONT OFFICE FOOD AND PRODUCT SAFETY

Literature overview on possible adverse effects of black cohosh in cancer patients.

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Literatuuroverzicht van mogelijke schadelijke effecten van zilverkaars bij kankerpatiënten.

Samenvatting.

Extracten met zilverkaars worden oraal gebruikt als kruidengeneesmiddel of kruidensupplement bij menopauzale klachten zoals opvliegers en zweten.
Bureau Risicobeoordeling en Onderzoek (BuRO) heeft geadviseerd om op het etiket van kruidenpreparaten met zilverkaars te waarschuwen dat deze niet gebruikt mogen worden door patiënten die behandeld zijn of behandeld worden voor borstkanker of andere hormoongevoelige tumoren (BuRO, 2020). Omdat hier vragen over gesteld zijn aan BuRO, heeft BuRO het Front Office Voedsel- en Productveiligheid verzocht een literatuuroverzicht op te stellen over de relatie tussen de inname van zilverkaars en mogelijke nadelige effecten in (ex)kankerpatiënten.

In 2013 heeft het RIVM een risicobeoordeling voor zilverkaars uitgevoerd (RIVM, 2013). Deze risicobeoordeling was grotendeels gebaseerd op een beoordelingsrapport van de European Medicines Agency (EMA, 2010). Hierin is geconcludeerd dat een muizenstudie erop wijst dat zilverkaars de kans op uitzaaiingen bij (hormoongerelateerde) kanker vergroot en er niet voldoende onderzoek rond de mogelijke carcinogeniteit van zilverkaars is gedaan om het risico op tumor-promotie uit te sluiten (RIVM, 2013). De EMA heeft zilverkaars opnieuw beoordeeld in 2017 (EMA, 2018a) en geconcludeerd dat patiënten die behandeld zijn of worden voor borstkanker of andere hormoongevoelige tumoren geadviseerd worden om zilverkaarspreparaten niet te gebruiken zonder medisch advies.
Deze herevaluatie van EMA is als startpunt genomen voor dit literatuuroverzicht, en in Embase, Pubmed en Scopus is gezocht naar recentere literatuur (2016-2021) over mogelijke nadelige effecten van het gebruik van zilverkaars bij (ex)kankerpatiënten. Hierbij is ook gezocht naar recente genotoxiciteits- en carcinogeniteitsstudies met zilverkaars.

Op basis hiervan zijn de volgende bevindingen gedaan:

- De EMA heeft in 2017 geconcludeerd dat de veiligheid van het gebruik van zilverkaars door patiënten met borstkanker niet bewezen is, omdat de

beschikbare klinische studies te weinig deelnemers hadden en te kort duurden. Een studie in genetisch gemodificeerde muizen liet een verhoogde incidentie van uitzaaiingen in de longen zien in tumorpositieve dieren behandeld met zilverkaars. Er werd geen effect gezien op de ontwikkeling van de primaire borsttumoren. EMA concludeerde daarom dat kruidengeneesmiddelen met zilverkaars niet zonder medisch advies gebruikt mogen worden door patiënten die behandeld zijn of worden voor borstkanker of andere hormoongevoelige tumoren

- Het literatuuronderzoek naar recentere gegevens levert een klinische studie op in borstkankerpatiënten die 12 weken een zilverkaarspreparaat gebruikten. In deze studie werden geen significante verschillen gezien tussen de controlegroep en de behandelde groep, behalve een hogere incidentie van baarmoederhalscystes in de behandelde groep. De oorzaak en relevantie hiervan zijn onduidelijk.
- EMA gaf in 2018 aan dat adequate genotoxiciteits- en carcinogeniteitsstudies ontbraken. Het huidige literatuuronderzoek leverde enkele studies op. Zilverkaarsextracten gaven negatieve resultaten in bacteriële genmutatietesten, met uitzondering van één test die een twijfelachtig resultaat gaf. Zilverkaarsextracten gaven positieve resultaten in *in vitro* en *in vivo* micronucleustesten. De auteurs concludeerden dat zilverkaars mogelijk een aneugeen werkingsmechanisme heeft. Dit wil zeggen dat zilverkaars het chromosomenaantal kan wijzigen.
- Actein en 26-deoxyactein, belangrijke bestanddelen van zilverkaars, lieten een anti-tumor effect zien in *in vitro* en *in vivo* testen. In deze testen zijn pure bestanddelen getest. De relevantie voor zilverkaarspreparaten is onduidelijk.
- Een carcinogeniteitsstudie in vrouwelijke ratten en muizen wordt momenteel uitgevoerd en beoordeeld door het National Toxicology Program of the United States (NTP). De resultaten hiervan zijn nog niet beschikbaar.

Subject

In July 2020, BuRO published an advice on the health risk of food supplements containing black cohosh (BuRO, 2020). BuRO recommended that manufacturers and distributors of preparations containing black cohosh should warn patients who have been treated or who are undergoing treatment for breast cancer or other hormone-depending tumours not to use black cohosh preparations. BuRO received some questions on this recommendation.

Question

Provide a literature overview on the relation between the intake of black cohosh and possible adverse effects in (ex)cancer patients.

Main findings of the literature overview

The European Medicines Agency (EMA) concluded in 2018 that the safety of black cohosh in breast cancer patients is not proven, as the available clinical studies in those patients only covered a short duration and included small numbers of patients.

One study in transgenic mice showed an increased incidence of lung metastasis in tumour-positive animals treated with black cohosh, whereas no effect on primary mammary tumour development was observed.

EMA concluded that possible adverse effects on hormone sensitive tissue cannot be excluded and that patients who have been treated or who are undergoing treatment of breast cancer or other hormone dependent cancer should not use black cohosh preparations without medical supervision.

The current literature search identified one recent clinical study in breast cancer patients, receiving a commercial medicine containing *C. racemosa* extract, again with a short duration (12 weeks). This study revealed no significant differences between the group treated with a black cohosh extract and the control group except for a statistically significant higher incidence of cervical cysts in the treated group. The cause and relevance of this finding is unclear.

EMA also concluded in 2018 that adequate tests on carcinogenicity and genotoxicity have not been performed. The current literature search identified some recent studies on these aspects. Black cohosh extracts gave negative results in several bacterial reverse mutation tests, except for one assay with equivocal results. Black cohosh extracts gave positive results in *in vitro* and *in vivo* micronucleus tests. It was concluded by the authors of these studies that black cohosh extracts might have an aneugenic mode of action (i.e. may change the number of chromosomes).

Actein and 26-deoxyactein – which are (major) constituents of black cohosh extracts – showed anti-tumour activities in *in vitro* and *in vivo* tests. Since purified compounds were tested, the relevance of this finding for black cohosh preparations is unclear.

A two-year carcinogenicity study in female rats and mice is performed by the National Toxicology Program of the United States (NTP) and the data are currently being reviewed and not yet available for this evaluation.

Introduction

Black cohosh (*Cimicifuga racemosa* or *Actaea racemosa)* is a perennial plant of the Ranunculaceae (buttercup family). It is commonly used in food supplements or herbal medicinal products for relief of menopausal symptoms, e.g. hot flushes and excessive sweating. Besides homeopathic medicines, no (herbal) medicinal products with black cohosh are registered in the Netherlands (MEB, 2021).

In 2013, RIVM performed a risk assessment on black cohosh (RIVM, 2013) which was largely based on an assessment report on black cohosh from the European Medicines Agency (EMA, 2010). Three points of concern for the use of food supplements with black cohosh were mentioned in the RIVM risk assessment report:

1. There are indications that black cohosh can cause liver damage in exceptional cases. However, based on the limited information available it cannot be concluded with certainty that black cohosh can cause liver toxicity;

2. The results of a study in mice indicate that black cohosh could increase the risk on metastases in case of (hormone-related) cancer. Insufficient studies are available to exclude the risk on tumour promotion;
3. Black cohosh probably has an oestrogenic effect, but also these data are inconclusive.

EMA re-evaluated black cohosh in 2017 (EMA, 2018a). For herbal medicinal products with black cohosh, the following special warning and precaution for use is described by the European Medicines Agency (EMA) in their herbal monograph (EMA, 2018b): *"Patients who have been treated or who are undergoing treatment for breast cancer or other hormone-dependent tumours should not use Cimicifuga rhizoma* [roots of black cohosh] *preparations without medical advice."*

In 2020, BuRO published an advice on the health risks of food supplements containing black cohosh. BuRO recommended, amongst others, that manufacturers and distributors of preparations containing black cohosh should warn patients who have been treated or who are undergoing treatment for breast cancer or other hormone-depending tumours not to use black cohosh preparations (BuRO, 2020).

The EMA re-evaluation (EMA, 2018a) is used as starting point for the current literature overview. Subsequently a literature search was performed to identify recent literature (2016-2021) on the possible adverse effects of the use of black cohosh preparations by (ex)cancer patients. Also, a search for recent genotoxicity and carcinogenicity studies with black cohosh preparations was performed. EMA considered all literature up to January 2017, and the 2016-2021 period was chosen for the literature search to complement the EMA literature search with a small overlap. The EMA conclusions on these aspects (EMA, 2018a, b) are described below, followed by the search strategy and the summary of the newly available literature data.

Summary conclusions EMA assessment (2018a)
EMA prepared in 2017 an assessment report on black cohosh (*Cimicifuga racemosa* (L.) Nutt., rhizome; EMA, 2018a) to establish a European Union herbal monograph (EMA, 2018b). The assessment report amended the first EMA assessment report on black cohosh (2010) and included a literature search up to January 2017.

The conclusions from the EMA monograph and EMA assessment report (EMA, 2018a; 2018b) on carcinogenic or tumour promoting effects from non-clinical studies and from studies in (ex)cancer patients are copied below.

Non-clinical studies:
In the herbal monograph on black cohosh (EMA, 2018b), the preclinical data are summarized as follows: *"Evidence from in-vitro and in-vivo pharmacological studies suggests that Cimicifugae rhizoma* [roots of black cohosh] *extracts do not influence the latency or development of breast cancer. However, contradictory results have been obtained in other* [non-pharmacological] *in-vitro experiments.*
In Cimicifugae rhizoma-treated (isopropanolic black cohosh extract equivalent to 40 mg of root and rhizome), tumour-bearing, female transgenic mice, the percentage of mice with detectable metastatic lung tumours at necropsy was increased compared to those on the control diet. However, in the same experimental model, no increase in primary breast tumour was seen. Influence on breast cancer or other hormone-depending tumours cannot be excluded.
Adequate tests on genotoxicity, carcinogenicity and reproductive toxicity have not been performed."

EMA's conclusion on the use in breast cancer patients:
In the EMA assessment report (2018a) the following conclusion is derived for use in breast cancer patients:

"Especially women undergoing breast cancer therapy are looking for alternatives to HT [hormone treatment]*, which is contraindicated in these patients. Furthermore the antihormonal therapy of breast cancer patients with the anti-oestrogen tamoxifen often induces or aggravates menopausal complaints.*

In some early pharmacological experiments CR [Cimicifuga rhizoma; roots of black cohosh] *extracts exhibited organ specific effects, which resembled effects caused by oestrogen. This is not supported by current evidence.*

Neither the mode of action nor the constituents relevant for the improvement of menopausal complaints are known yet. Since the mechanism of action is not clearly identified, possible effects on the hormone sensitive tissue cannot be excluded.

Based on the preliminary observational data, CR does not appear to adversely impact the risk of breast cancer recurrence or incidence in women with or without a history of breast cancer. However, in contrast to these findings, one in-vitro study using transgenic mice expressing c-erbB2 (MMTV-neu mouse model) showed that CR significantly increased the incidence of lung metastases in tumour-positive animals when compared to those on the control diet (Davis et al. 2008). In the same experimental model, no effect on primary mammary tumour development was observed. Clinical evidence supporting the potential to promote progression of metastatic disease is not available from studies in patients with breast cancer, but influence on breast cancer cannot be completely excluded.

If examined, CR does not influence circulating levels of oestradiol, FSH or LH or appear to exert oestrogenic effects on breast, endometrial or vaginal tissues.

Clinical studies indicate that climacteric symptoms improve under treatment with medicinal products containing CR. Only limited data regarding the efficacy of CR in women with breast cancer are available which do not allow a final assessment. The small number of patients and the short term duration of the few studies are not sufficient to prove safety of CR preparations in patients with breast cancer.

On the basis of available data, the use of CR preparations or combined therapy with tamoxifen for patients with a history of or treated breast cancer or hormone dependent tumours is not recommended and should be avoided."

EMA's overall conclusion (benefit-risk assessment)
The following overall conclusion was derived in relation to (ex)cancer patients by EMA (2018a):
"Possible effects on the hormone sensitive tissue cannot be excluded. Patients who have been treated or who are undergoing treatment of breast cancer or other hormone dependent tumours should not use CR preparations without medical advice."

Literature Search
A literature search[1] was conducted in Embase, Pubmed and Scopus to identify recent literature on the possible adverse effects of the use of black cohosh preparations by (ex)cancer patients, that was not yet available for the EMA evaluation.

In Scopus, "cimicifuga racemosa" was used as search term. In Pubmed, the Medical Subject Headings (MeSH) term 'Cimicifuga'[2] was used. In Embase, the EmTree term 'Actaea racemosa'[3] was used. The search was restricted to human data and to the period 2016-2021[4]. This search resulted in 45 hits in Pubmed, 74 hits in Scopus and 142 hits in Embase. All references found were exported to EndNote and all double references were deleted. This resulted in 200 unique references.

From these 200 references, all conference abstracts (32), review articles (57) and articles in languages other than Dutch, German and English (1) were identified based on title and abstract and excluded. Further, ninety additional references were excluded based on title and abstract because these were either not about black cohosh (5), did not include human data (35), were not about cancer or were not in (former) cancer patients (49). Also one study in male patients with prostate cancer was excluded. The 20 remaining references were selected for full text appraisal.

Based on the full text appraisal, only two references provided information relevant to the research question and were included in the assessment.

As EMA (2018a) indicated that adequate tests on genotoxicity and carcinogenicity have not been performed, an additional search[5] was conducted in Embase, Pubmed and Scopus to check for recent (2016-2021) animal carcinogenicity and genotoxicity data and *in vitro* genotoxicity data. In Pubmed, the MeSH terms 'Cimicifuga' and 'Neoplasms' were combined, and the restriction 'animal data' was applied to search for animal carcinogenicity data. This resulted in 3 hits. To search for genotoxicity data, the MeSH terms 'Cimicifuga' and 'Mutagenicity tests' were used. This resulted in 2 hits. In Embase, the Emtree term 'Actaea racemosa' was combined with the Emtree term 'Neoplasm', where after the restriction to animal data was applied. This resulted in 5 references. To search for genotoxicity data, the Emtree term 'Actaea racemosa' was combined with the Emtree term 'Mutagen testing' or with the Emtree term 'genotoxicity'. This resulted in 5 unique references. In Scopus, the search term 'Cimicifuga racemosa' was used to search in article title, abstract and keywords for references about animal carcinogenicity and genotoxicity. Restriction to animal data and publication years resulted in 50 results. As these were not too many references to go through by hand, it was decided not to add further search terms to limit the search. To identify possible relevant references regarding *in vitro* genotoxicity in Scopus, the search terms 'Cimicifuga racemosa' and 'mutagen*' were used. This resulted in 2 references.
All references found were exported to EndNote and all double references were deleted. This resulted in 60 unique references.
Based on title/abstract screening of these 60 references, 5 conference abstracts, 12 review articles and 39 other references were excluded because they were not relevant for the current question. Four relevant articles were selected for full text appraisal. Based on the full text of one of these *in vitro* studies, one previously excluded *in vivo* study in mice

[1] Search was conducted 2 February 2021
[2] In Pubmed, black cohosh is indexed under the MeSH term 'Cimicifuga'
[3] In Embase, black cohosh is indexed under the Emtree term 'Actaea racemosa'
[4] EMA considered all literature up to January 2017. The period was chosen to complement the EMA literature search with a small overlap.
[5] Search was conducted 17 February 2021

was selected for full text appraisal as well since it contained relevant information on *in vivo* genotoxicity.

Lastly, a search was conducted in Google Scholar to focus on grey literature, using "zilverkaars' or "cimicifuga racemosa" in combination with "kanker" or "cancer" as search terms. No additional relevant literature was identified.

Results literature search

Human studies with (ex-)cancer patients

Castelo-Branco et al. (2020) published a meta-analysis on the use of isopropanolic *Cimicifuga racemos*a (iCR) extract. The authors searched for clinical studies with iCR extract (irrespective of design) and meta-analyses thereof, published from 1997 to January 2020. They identified 35 clinical studies and one meta-analysis. The clinical studies identified were all published in the period 1987-2015, except for a study by Wang et al. (2019) and a conference abstract by Hofstatter et al. (2018). The identified meta-analysis was published in 2011 (Naser et al., 2011). The authors included all placebo-controlled randomized clinical trials (RCTs) examining efficacy for neurovegetative and psychological climacteric symptoms in their meta-analysis (irrespective of publication date). In total, six RCT's published in the period 1987-2015 were included in their meta-analysis; only one of these RCT's included (ex)cancer patients (Jacobsen et al., 2001). Since the review by Castelo-Branco et al. contains no new studies from the period 2016-2021 – except the study by Wang et al. (2019) that is summarized below – this review is not further considered for the current literature overview.

Wang et al. (2019) investigated the effect of Remifemin® (commercial medicine containing *C. racemosa* extract) in preventing menopausal syndrome induced by luteinizing-hormone releasing hormone analogue (LHRH-a) treatment in breast cancer patients. Pre- and peri-menopausal patients diagnosed with early breast cancer and who underwent treatment with LHRH-a were randomly assigned to receive Remifemin (n=42) or no intervention (control group; n=43). The intervention consisted of 20 mg Remifemin twice a day for 12 weeks. At the start, there were no significant differences of tumour stage, lymph node stage, oestrogen receptor, progesterone receptor and human epidermal growth factor receptor-2 between both groups. The evaluation of effects on preventing menopausal syndrome was done using the Kupperman menopause index (KMI). The KMI scores at week 4, 8 and 12 were higher than at baseline level in both groups, but the increase in the treated group was limited and the KMI scores were statistically significantly lower than in the control group at all time points ($p<0.01$). Complications including endometrial thickening, ovarian cysts, uterine fibroid and cervical cysts were also evaluated, by ultrasound examination. This revealed no significant differences between the two groups besides a higher incidence of cervical cysts in the Remifemin group (9/42) than in the control group (2/43) ($p=0.02$). The authors were not able to give an explanation for this observation. Overall, Wang et al. concluded that the short-term use of *C. racemosa* extract was safe in breast cancer patients, whereas further research is needed to confirm whether long-term use of black cohosh is safe. However, also due to the limited group size and limited number of outcome parameters investigated in this study, RIVM and WFSR consider that no firm conclusions on the safety can be drawn.

Studies of genotoxicity in vitro

An overview table with the *in vitro* genotoxicity assays is provided in Annex 1.

The National Toxicology Program of the United States (NTP) performed four bacterial reverse mutation (Ames) assays with black cohosh (dried) extract (BCE; this lot is referred to as 'NTP-BCE' hereafter) (NTP, 2021). The NTP-BCE was dissolved in dimethyl sulphoxide (DMSO) or suspended in 0.5 % methylcellulose in the different experiments. Three of them were negative and one of them gave equivocal results. The latter results were published by Smith-Roe et al. (2018). One of the studies giving negative results was performed using *Salmonella typhimurium* strains TA100, TA1535, TA1537, TA97, TA98, TA102 and TA104 and evaluated concentrations of NTP-BCE up to 10,000 µg/plate, whereas the other three studies were performed using *S. typhimurium* strains TA100, TA98 and *Escherichia coli* WP2 uvrA pKM101 and NTP-BCE at concentrations up to 6,000 µg/plate. Data (summary tables) from all four assays are available on the NTP website. Besides NTP-BCE, Smith-Roe et al. (2018) tested five other black cohosh extracts in an Ames test using *Salmonella typhimurium* strains TA100 and TA98 and *Escherichia coli* strain WP2 *uvrA* pKM101. All extracts tested negative.

Smith-Roe et al. (2018) tested 15 different BCEs and powders in an *in vitro* micronucleus assay. These included the NTP-BCE and BCE from commercial Remifemin® tablets (with a similar chromatographic profile to that of NTP-BCE) and root powder from different species of cohoshes (black, Chinese, red, yellow). Human TK6 lymphoblastoid cells were grown in medium containing either 120 (physiologically more relevant to human blood levels) or 3000 nM folic acid. After incubation for 24 hr in the absence of rat liver S9 mix, NTP-BCE induced dose-dependent increases in the frequency of micronucleated cells (%MN), which became statistically significant from 95 µg/mL onwards. This was observed both in medium containing 120 nM and 3000 nM folic acid. However, the absolute increase in %MN was lower in the medium containing 3000 nM compared to the medium containing 120 nM. After incubation for 4 hr in the presence of S9 mix, followed by a 20 hr recovery time, the induction of MN was lower compared to the induction in the absence of S9 mix, for both folic acid concentrations. To investigate whether this attenuation in response in the presence of S9 was due to detoxification of BCE or to a shorter exposure time, cells were exposed to NTP-BCE for 4 hr in absence of S9 mix followed by a 20 hr recovery time. A dose-dependent increase in %MN was again observed in cells grown in medium containing 120 nM folic acid, which was statistically significant from 125 µg/mL onwards. In contrast, only the highest dose of 500 µg/mL showed a statistically significant but small increase in %MN in cells grown in medium containing 3000 nM folic acid. The increases in %MN were higher after 24 hr of incubation than after 4 hr for the same concentrations. Based on their results, Smith-Roe et al. suggested that there may be a detoxification as the induction of MN was attenuated in the presence of S9. Also, BCE may affect the folate metabolism pathway, as excess folic acid appeared to attenuate the BCE-induced MN response.
After incubation of other BCE samples for 24 hr in the absence of S9 mix, all samples induced statistically significant increases in %MN when grown in medium containing 120 nM folic acid. One BCE sample, in addition to the NTP-BCE, was also positive in cells grown in medium containing 3000 nM, and 4 BCE samples had an equivocal result.
A benchmark dose analysis was performed by Smith-Roe et al. on the results of the micronucleus assay to rank the cohosh samples by their potency. The NTP-BCE showed the highest potency, and the root powder suspensions tended to have less potency than the extracts. However, the variation was less than an order of magnitude.

In addition, a multiflow DNA damage assay – which assesses biomarkers of DNA damage, cell divisions and cytotoxicity – was conducted in human TK6 lymphoblastoid cells to investigate the mode-of-action (MoA). Two BCE extracts were tested, the NTP-BCE and a BCE reference material (BC XRM; supplier ChromaDex). Both BCE extracts showed a

pattern of response that was consistent with an aneugenic MoA, including an increase in phospho-histone H3-positive events, translocation of p53 to the nucleus (at 24 hr), and no induction of γH2Ax. Based on all the results, the authors conclude that black cohosh extracts contain an aneugen, a genotoxic compound that can alter the chromosomal number (Smith-Roe et al., 2018).

The results of Smith-Roe et al. were confirmed by Bernacki et al. (2019). NTP-BCE and BC XRM induced micronuclei in TK6 cells. Also the aneugenic mode of action was confirmed using the Multi-Flow DNA Damage Assay (evaluating several biomarkers that are useful for distinguishing between clastogenic and aneugenic mode of action) and the results pointed towards an effect on tubulin binding. Furthermore, a follow-up assay, the novel MultiFlow Aneugen Molecular Mechanism Assay, was used to further investigate the aneugenic action. For this experiment, TK6 cells were exposed to NTP-BCE and BC XRM over a range of concentrations in the presence of fluorescent 488 Taxol. After 4 hr, nuclei from lysed cells were stained with a nucleic acid dye and labeled with fluorescent antibodies against phospho-histone H3 (p-H3) and Ki-67. Whereas BCEs did not affect p-H3:Ki-67 ratios (a signature of aneugenic mitotic kinase inhibitors), 488 Taxol-associated fluorescence (a tubulin binder-sensitive endpoint) was affected. More specifically, 488 Taxol-associated fluorescence was reduced over the same concentration range that was previously observed to induce micronuclei. Bernacki et al. concluded that the results provide direct evidence that BCEs destabilize microtubules *in vitro*, and that this is the molecular mechanism responsible for the aneugenicity findings.

Studies of genotoxicity in vivo

The NTP performed three micronucleus assays in female mice and one in female rats with NTP-BCE and positive results were obtained in all assays (NTP, 2021). Data (summary tables) from these studies are available on the NTP website. The study in rats and one of the studies in mice were already published by Mercado-Feliciano et al. (2012) and are not included in this literature overview because they are not in the selected time period (2016-2021). It is not clear when the other two studies in mice were conducted. Female mice were exposed to 0, 30, 100, 300 or 1000 mg NTP-BCE/kg bw for 90 days or one year. Blood samples were collected 24 hr after the last treatment and the number of micronucleated polychromatic erythrocytes (MN PCE)/1000 PCE and the number of MN normochromatic erythrocyte (MN NCE/1000 NCE) were calculated. A significant increase in both parameters was observed in both experiments (NTP, 2021).

Cora et al. (2017) performed a study to investigate a functional cobalamin or folate deficiency as possible explanation for the hematological changes, pointing at megaloblastic anemia, observed in the NTP-BCE subchronic mouse toxicity study (Mercado-Feliciano et al., 2012). To this aim, B6C3F1/N female mice (32/group) were exposed by gavage to vehicle (0.5% methylcellulose; control group) or 1000 mg/kg NTP-BCE for 92 days. No micronucleus assay was performed as part of this study, but blood smear evaluation revealed increased Howell–Jolly bodies (a marker of chromosomal damage), which supported the increase in micronucleated erythrocytes observed by Mercado-Feliciano et al. (2012). The mean number in treated rats was more than two-fold higher than in control rats. Bone marrow smears were not evaluated in this study, but the authors referred to preliminary NTP data (report in preparation, not published yet). These indicate dysplastic changes in the metarubricytes, including low incidences of multilobulated nuclei, micronuclei, and nuclear to cytoplasmic asynchrony of the erythroid series. Also macrocytosis (enlargement of red blood cells) and decreased red blood cell count was observed (Cora et al., 2017).

Studies on carcinogenicity and anti-tumour activities

A two-year gavage study of the National Toxicology Program of the United States (NTP) in rats and mice is completed and the data are currently being reviewed (NTP, 2021). Reference to this two-year study is made in the recent papers on genotoxicity (Bernacki et al., 2019; Cora et al., 2017; Smith-Roe et al., 2018) but no information with regards to the study results has been provided.

Two recent animal studies were identified (Wu et al., 2016 and Yue et al., 2016) that studied the (anti)carcinogenic effects of the compounds actein and 26-deoxyactein. Actein is a major constituent of black cohosh extracts.

Wu et al. (2016) studied the *in vitro* and *in vivo* anti-tumour activities of the tetracyclic triterpenoids compounds actein and 26-deoxyactein isolated from rhizome of *Cimicifuga foetida* L. *In vitro*, a modified MTT method was used to assay the cytotoxicity of actein and 26-deoxyactein in 12 human tumour cell lines. Actein and 26-deoxyactein inhibited the proliferation of the 12 human cancer cell lines tested, with 50% inhibitory concentrations (IC_{50}) between 12.29 and 88.39 µg/mL. Flow cytometry (FCM) was used to assay the cell cycle distribution of the HL-60 tumour cells, which was the most susceptible cell line with the lowest IC_{50}. This showed that human leukaemia HL-60 cells were arrested at G1 phase after treatment with either actein or 26-deoxyactein (6.25–25 µg/mL) for 48 h.
In vivo, mouse sarcoma S180 and human lung cancer A549 cells were implanted subcutaneously in, respectively, ICR-mice and nude mice to establish implanted tumour models. Actein or 26-deoxyactein was administered at doses of 3, 9 and 27 mg/kg bw in the mouse sarcoma S180 model (10 animals per group) during 10 days. In the human lung cancer A549 model the animals were administered actein or 26-deoxyactein at doses of 10 and 30 mg/kg bw (6 animals per group) for 6 days. The route of administration is not described in the article. The tumour growth was studied.
Both actein and 26-deoxyactein significantly inhibited the growth of the implanted sarcoma S180 in a dose-dependent manner. The growth inhibition rates (based on tumour weight) of the implanted S180 were respectively 40%, 44% and 53% after treatment with actein (3, 9, 27 mg/kg bw). Correspondingly, the inhibition rates for 26-deoxyactein were respectively 41%, 53% and 69%. Actein and 26-deoxyactein also markedly inhibited the A549 tumour growth, with a reduction in tumour volume of 38% and 55% for actein, and 35% and 49% for 26-deoxyactein at, respectively, the 10 and 30 mg/kg bw dose. In addition, immunohistochemistry was used to measure CD31 (endothelial cell specific biomarker)-positive expression in the A549 lung tumour and subsequently to analyze the micro vessel density (MVD). Actein and 26-deoxyactein (10, 30 mg/kg) significantly reduced the CD31-positive expression and MVD in the A549 tumour.
Wu et al. concluded that both actein and 26-deoxyactein have significant anti-tumour activities *in vitro* and *in vivo*, which is associated with cell cycle arrest and angiogenesis inhibition.

Yue et al. (2016) studied the beneficial effects of actein in breast cancer treatment by investigating the effects of actein on angiogenesis *in vitro* and *in vivo* using human microvascular endothelial cells (HMEC-1), a mouse matrigel plug assay[6] and a breast tumour-bearing mouse model.
The results showed that actein significantly inhibited the proliferation, reduced the migration and reduced the motility of the endothelial cells *in vitro*. *In vivo* results showed that oral administration of actein at 10 mg/kg bw for 7 days inhibited blood vessel

[6] The mouse Matrigel plug assay is an *in vivo* technique to detect the new blood vessel formation as reflected by the hemoglobin content of the transplanted gel plugs.

formation in the mouse matrigel plug assay, as measured by a reduced hemoglobin content. In the breast tumour-bearing mouse model, oral administration of actein (10-15 mg/kg bw) for 28 days resulted in decreasing mouse 4T1 breast tumour sizes. The tumour weights of the actein treatment groups were significantly decreased by 21.2% and 36.4% respectively for the 10 and 15 mg/kg dose when compared with the control group. Moreover, reduced angiogenic protein (CD34 and Factor VIII) expression and down-regulated metastasis-related VEGFR1 and CXCR4 gene expression were observed in the breast tumours of the actein treated mice. Furthermore, metastasis to lungs and livers was reduced as demonstrated by a decreased tumour burden of respectively 57.5% and 43.4% in lungs and livers of the 15 mg/kg actein-treated group, when compared with the control group.
It was suggested by Yue et al. that the oral administration of actein in tumour-bearing mice may inhibit the angiogenesis in the tumours, which then may reduce the blood supply for the tumours and suppress the tumour growth.

It is noted that purified compounds were tested, the relevance of this finding for black cohosh preparations is therefore unclear.

Summary of the results
(in bold the sentences which are the main findings of the literature overview)

EMA assessed the use of black cohosh as medicinal product (EMA, 2018a,b) and evaluated the literature data available until January 2017 on – amongst others – adverse effects in (ex)cancer patients. Based on preliminary observational data, black cohosh did not appear to adversely impact the risk of breast cancer recurrence. **EMA concluded in 2018 that the safety of black cohosh in breast cancer patients is not proven, as the available clinical studies in those patients only covered a short duration and included small numbers of patients.**

One study in transgenic mice showed an increased incidence of lung metastases in tumour-positive animals treated with black cohosh, whereas no effect on primary mammary tumour development was observed. Although no clinical evidence was available to support the potential to promote progression of metastatic disease, an influence on cancer (including breast cancer) could not be completely excluded. On the basis of the available data, **EMA concluded that possible effects on the hormone sensitive tissue cannot be excluded and that patients who have been treated or who are undergoing treatment of breast cancer or other hormone dependent cancer should not use black cohosh preparations without medical supervision.**

A literature search was performed over the period 2016 – February 2021. **The current literature search identified one recent clinical study in breast cancer patients, receiving a commercial medicine containing *C. racemosa* extract, again with a short duration (12 weeks). This study revealed no significant differences between the group treated with a black cohosh extract and the control group except for a statistically significant higher incidence of cervical cysts in the treated group. The cause and relevance of this finding is unclear** (Wang et al., 2019). A recent review by Castelo-Branco et al. (2020) confirmed that no new clinical studies are available except this study by Wang et al. (2019).

EMA also concluded in 2018 that adequate tests on carcinogenicity and genotoxicity have not been performed. The current literature search identified some recent studies on these aspects. The additional search was conducted in Embase, Pubmed and Scopus to

check for recent carcinogenicity and genotoxicity data over the period 2016 - February 2021. **Black cohosh extracts gave negative results in several bacterial reverse mutation tests, except for one assay with equivocal results.** NTP has performed four bacterial reverse mutation assays with black cohosh extract (NTP-BCE). Three of them were negative and one study gave equivocal results (NTP, 2021). The latter study was also published by Smith-Roe et al. (2018), who additionally described the negative results of five other black cohosh extracts in bacterial reverse mutation assays.
Black cohosh extracts gave positive results in *in vitro* and *in vivo* micronucleus tests. The NTP performed three *in vivo* micronucleus assays in female mice and one in female rats with NTP-BCE, and an increase in micronucleated cells was obtained in all assays. *In vitro* micronucleus tests with NTP-BCE and several other (black) cohosh products were all positive, in line with the *in vivo* results (Smith-Roe et al., 2018; Bernacki et al., 2019). Based on experiments on the expression of several biomarkers of DNA damage, cell divisions and cytotoxicity, **it was concluded by the authors of these studies that black cohosh extracts might have an aneugenic mode of action (i.e. may change the number of chromosomes)** (Smith-Roe et al., 2018 and Bernacki et al., 2019).

Actein and 26-deoxyactein – which are (major) constituents of black cohosh extracts – showed anti-tumour activities in *in vitro* and *in vivo* tests. This included the inhibition of tumour growth in mouse tumour models implanted with mouse sarcoma S180 or implanted with human lung cancer A549 cells (Wu et al., 2016) and decreased breast tumour sizes in a breast tumour-bearing mouse model (Yue et al., 2016). In the latter study metastasis to lungs and livers was also reduced. The inhibition of tumour growth appears to be associated with cell cycle arrest and angiogenesis inhibition. **Since purified compounds were tested, the relevance of this finding for black cohosh preparations is unclear.**

A two-year carcinogenicity study in female rats and mice is performed by the National Toxicology Program of the United States (NTP) and the data are currently being reviewed and not yet available for this evaluation.

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Annex 1 *In vitro* genotoxicity tests with Black cohosh extracts

Test article	Assay	Species	Time of exposure	Concentrations	Results	Reference
BCE	Bacterial mutagenicity assay (Ames test)	*S. typhimurium* TA100, TA1535, TA1537, TA97, TA98, TA102 and TA104		0 – 10,000 µg/plate (+/- S9)	Negative	NTP website (accessed March 2021). Study number A13815.
BCE	Bacterial mutagenicity assay (Ames test)	*S. typhimurium* TA100 and TA98 *E. coli* WP2 *uvrA* pKM101		0, 187.5, 375, 750, 1500, 3000, 6000 µg/plate (+/- S9)	Negative	NTP website (accessed March 2021). Study number G00058X.
BCE	Bacterial mutagenicity assay (Ames test)	*S. typhimurium* TA100 and TA98 *E. coli* WP2 *uvrA* pKM101		0, 187.5, 375, 750, 1500, 3000, 6000 µg/plate (+/- S9)	Negative	NTP website (accessed March 2021). Study number G00058Y.
NTP-BCE	Bacterial mutagenicity assay (Ames test)	*Salmonella typhimurium* TA100 and TA98 *Escherichia coli* WP2 *uvrA* pKM101		0, 187.5, 375, 750, 1500, 3000 and 6000 µg/plate (+/- S9)	Equivocal[a]	NTP website (accessed March 2021). Study number G00058D; Smith-Roe et al., 2018
Five different BCE extracts.	Bacterial mutagenicity assay (Ames test)	*S. typhimurium* TA100 and TA98 *E. coli* WP2 *uvrA* pKM101		0, 187.5, 375, 750, 1500, 3000 and 6000 µg/plate (+/- S9)	Negative[b]	Smith-Roe et al., 2018
NTP-BCE	Micronucleus assay	Human TK6 lymphoblastoid cells	24 hr 4 hr 4 hr	0, 10, 30, 45, 65, 95, 110, 125 µg/mL (-S9) 0, 30, 62.5, 125, 250, 500, 750, 1000 µg/mL (+S9) 0, 30, 62.5, 125, 250, 500 µg/mL (-S9)	Positive[c] Positive[c] Positive[c]	Smith-Roe et al., 2018

14 different BCE extracts and BC root powders	Micronucleus assay	Human TK6 lymphoblastoid cells	24 hr	0, 10, 25, 50, 75, 100, 125, 175, 250 µg/ml (-S9) 0, 125, 250, 300, 400, 500, 750, 1000 µg/mL (in case of not reaching limit of cytotox) (-S9)	Positive[c]	Smith-Roe et al., 2018
NTP-BCE	Micronucleus assay	Human TK6 lymphoblastoid cells	24 hr	0, 0.98, 1.95, 3.91, 8.81, 15.63, 31.25, 62.5, 125, 250 and 500 µM	Positive[d]	Bernacki et al., 2019
BC XRM	Micronucleus assay	Human TK6 lymphoblastoid cells	24 hr	0, 0.98, 1.95, 3.91, 8.81, 15.63, 31.25, 62.5, 125, 250 and 500 µM	Positive[d]	Bernacki et al., 2019

BC = black cohosh; BCE = black cohosh extract; BC XRM = a BCE reference material; NTP-BCE = BCE lot used in the carcinogenicity study of NTP; S9 = liver 9000 × g supernatant fraction.

[a] Samples were tested in triplicate using the pre-incubation method with and without rat liver S9. An equivocal result was obtained in *S. typhimurium* strain TA98 in the presence of S9 mix.

[b] Six cohosh extracts, including NTP BCE and BCE reference material (BC XRM), were tested in a bacterial mutagenicity assay. Samples were tested in triplicate using the pre-incubation method with and without rat liver S9 homogenate. No cytotoxicity was observed.

[c] Human TK6 lymphoblastoid cells were grown in medium with either 120 or 3000 nM folic acid for 72 hr. Cells were then exposed to NTP BCE for 24 hr without rat liver S9 mix, to NTP BCE with rat liver S9 mix for 4 hr with 20 hr recovery period, to NTP BCE without rat liver S9 mix for 4 hr with 20 hr recovery period, or to other BCE samples for 24 hr without S9 mix. Each BCE sample was tested at 7-8 concentrations. Each concentration was evaluated in triplicate, except the cells incubated for 4 hr with NTP BCE which were evaluated in duplicate. If recommended limit of cytotoxicity (55%±5%) was not reached, the samples were retested with higher concentrations. DMSO was used as vehicle control and mitomycin C as positive control in tests without S9 mix and cyclophosphamide as positive control in tests with S9 mix. 20,000 cells from each sample were analyzed for frequency of micronuclei (%MN). Results were considered positive if both the trend test was significant and at least one dose group showed a statistically significantly increased response compared to the control, or if 2 or more dose groups showed a statistically significantly increased response compared to the control. Results were considered equivocal if only the trend test was significant, or a single dose group showed a statistically significantly increased response compared to control.

[d] Two independent experiments were performed. DMSO was used as vehicle control and MEB (0.25 µM) and MTX (0.01 µM) were used as positive controls. Micronuclei were analyzed using flow cytometric analysis. Data from two experiments were pooled for the analyses.