REPORT

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Request from the Norwegian Food Safety Authority

Risk assessment of holy basil (*Ocimum tenuiflorum* L. and *Ocimum sanctum* L.) and some of the component substances used in herbal teas and food supplements

NIPH
Norwegian Institute of Public Health

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Table of content

Key messages

The Norwegian Food Safety Authority requested the Norwegian Institute of Public Health to assess which amounts of the green parts of the plant holy basil (*Ocimum tenuiflorum* L./*O. sanctum* L.) that could be consumed without risk to human health. This plant is used in teas and food supplements sold on the Norwegian market. Information about potential harmful effects of intake of this plant from two extensive literature searches was used in this risk assessment. Safety from both intake of the plant material as such, as well as some individual substances from this plant, was evaluated.

The content of plant material of holy basil, assumed to be dried, in teas sold on the Norwegian market was stated as 0.6-2.0 g per tea bag. It was assumed that all of the active substances in the plant were extracted by and ends up in the hot water, as a worst-case scenario due to lack of more specific data. The recommended daily doses of food supplements (dried plant material) varied from 60 to 360 mg in one product and from 800 to1800 mg in another product.

Reproductive toxicity, i.e. reduced fertility, appeared to be the most critical adverse effect from intake of holy basil. Intake of one cup of tea or the two food supplements with holy basil plant material may be a risk for embryo implantation loss in pregnant women and for impaired reproduction in males, based on data from animal experiments.

A general safe level for all types of preparations of these basil plants seems to be below 1 mg/kg body weight per day.

Based on the individual substances methyleugenol, estragole and eucalyptol in holy basil, there may be a risk for adverse health effects at intake of one or more cups of tea per day or from one or both food supplements. Based on eugenol, an intake of three cups of tea per day with the highest level of holy basil reported in tea may be a risk. For β-caryophyllene and ursolic acid, there was not a risk from one, two or three cups of tea per day or from the food supplements.

There are a lot of uncertainties in these evaluations, such as regarding whether the few available examples of content of holy basil in teas and food supplements are representative of such products sold in Norway. A question is also how representative the numbers used in the calculations of exposure are for intake of these products in Norway, since composition and levels of the individual substances vary depending on i.a. plant variety, location, methods of cultivation and preparation of the final product. It is also uncertain how relevant the data on adverse effects observed in experimental animals with various extracts of plant material are for risk assessments of other preparations of the plant, such as the dried plant material in teas and food supplements.

In conclusion, based on all the available information, precaution is warranted to limit the intake of teas or food supplements with holy basil for pregnant women, and for both men and women wanting to become parents. Since there were no studies on children specifically, the intake of these products is also better avoided during lactation or by children in general.

Summary

The Norwegian Food Safety Authority (NFSA) requested on April 3, 2020, the Norwegian Institute of Public Health (NIPH) to assess which amounts of the green parts of the plant holy basil (*Ocimum tenuiflorum* L.) that could be consumed without risk to human health. The background for this request was that NFSA needed a risk assessment that could be used both for evaluation of specific products such as teas and food supplements containing this plant material sold on the Norwegian market and as a basis for a decision on general actions regarding products containing this plant.

In 2020, NIPH performed an extensive literature search which covered the period after previous risk assessments by the Technical University of Denmark (DTU) and the Swedish Food Agency, i.e. from January 1, 2018 to April/May 2020. In total, 2786 publications were found in this search. After screening, 70 publications were included. An updated search was performed June 30 - July 3, 2023, to cover new literature since the last search, and 45 publications were included. Additional relevant publications were also included from the reference lists of the publications from both searches or from earlier risk assessments. In total, information from 242 references were used in this risk assessment. In addition to publications on the plant material as such, publications on some individual substances from this plant were assessed. Since the potential reproductive toxicity effects for humans based on observations in several animal experiments were the main concern regarding the safety of this plant, also older reproductive toxicity studies were included for completion of the evidence. Thus, the literature included in this risk assessment was quite extensive, although not strictly systematically collected. Positive health effects of the basil plants or the individual substances were outside the scope of this assessment.

The genus *Ocimum* from the *Lamiaceae* family (in Norwegian 'leppeblomstfamilien') contains annual or perennial aromatic herbs that are native to the tropical and subtropical regions of Asia, Africa and Central South America, and is composed of >65 species. The plant *O. tenuiflorum* L., holy basil, has many different names, both in English and Latin, and it is not entirely clear from the literature which plant names to include in this risk assessment. *O. tenuiflorum* L. is the correct and preferred name, however, many publications still use the name *O. sanctum* L. Data from other basil species were also included when considered relevant. The content of individual chemical substances in the essential oil from holy basil and other basil plants can vary depending on many factors; the species, cultivar, origin, age, part of the plant, environmental conditions such as harvesting season, agronomic techniques, spectral light composition used during cultivation, type of fertilizers, use of elicitors and other conditions in root cultures, and extraction methods and other processing of the final plant product. Also several chemotypes, haplotypes or morphotypes of these plants are described. High heavy metal content in the plants taken up from the earth may in some cases be an additional risk factor in herbal plants and their preparations. It is recommended that the manufacturers of herbal teas and dietary supplements should monitor the chemical (heavy metals, radionuclides and pesticides) and microbial contaminants potentially present in the plant material, ensuring their presence below maximum regulatory limits.

According to information from NFSA, the content of plant material of holy basil, assumed to be dried, in products on the Norwegian market may be 0.6-2.0 g per tea bag. Based on this information and using a default body weight of 60 kg, one cup per day of holy basil tea made with one tea bag will give an exposure of maximum 10-33 mg/kg bw per day. This is based on the assumption that all of the active substances are extracted by and ends up in the hot water, as a worst-case scenario due to lack of more specific data. The estimated intake will be 20-66 and 30-99 mg/kg bw per for 2 and 3 cups of this tea per day, respectively. According to information from NFSA, the recommended daily doses of food supplements (dried plant material) varied from 60-360 mg in one product and from 800-1800 mg in another product. Based on a default body weight of 60 kg, these food supplements would give an estimated exposure of 1-6 mg/kg bw or 13.3-30 mg/kg bw per day of the dried plant material. The content of the individual active substances

methyleugenol, estragole, eugenol, eucalyptol, β-caryophyllene and ursolic acid in basil plants was also calculated from the intake of these teas and food supplements.

Based on the available data, the hazards associated with intake of the plant material as such and the individual substances were evaluated mostly based on data from experimental animals. Methyleugenol and estragole were considered genotoxic and carcinogenic, whereas the plant material as such and the other four substances were not. Based on the available data, reproductive toxicity appeared to be the most critical adverse effects from intake of holy basil. The observed effects were impaired spermatogenesis in males, disturbed estrous cycle and loss of embryo implantation in uterus in females, as well as changes in weight and structure of reproductive organs, changes in hormone levels, reduced sexual mating behaviour and reduced fertility of both males and females. However, no teratogenic effects in the offspring were reported after exposure to basil plants. Two publications on reproductive toxicity in experimental animals after exposure to aqueous extracts of holy basil, considered most relevant for the herbal teas and the food supplements, were used in the risk characterization. One study reported that no embryo implantation sites were detected in the uterus of female rats exposed to 100 mg/kg bw on gestational day (GD) 1-4 (in 3 of 5 rats) or exposed to 200 mg/kg bw on GD 1-7 (in 2 of 5 rats). Another study reported significant decreased sperm counts and motility of spermatozoa, and significant increased mortality of spermatozoa, after 10-50 days of treatment, and decreased weight of reproductive organs after 20-50 days of treatment, in male mice given 250 mg/kg bw of the aqueous extract.

Assuming that the plant material as such is not genotoxic and employing the usual uncertainty factor of 100, a health-based guidance value (HBGV) was estimated for embryo implantation loss as 100 mg/kg bw divided by 100 is 1 mg/kg bw per day. For adverse effects on male reproduction, 250 mg/kg bw divided by 100 is 2.5 mg/kg bw. Thus, even intake of one cup of tea with holy basil may be a risk for embryo implantation loss in pregnant women and for impaired reproduction in men.

Alternatively, the margin of exposure (MOE) was estimated from the daily dose giving an adverse effect in experimental animals divided by the human exposure. MOE values are usually considered safe in risk assessments of non-genotoxic substances assumed to have a threshold of effect if they are above 100. If estimating MOE from the lowest observed adverse effect level (LOAEL) divided by the human exposure, 100 mg/kg bw divided by 10-33 mg/kg bw (for one cup of tea), will give a MOE of only 3-10. Since LOAEL was used instead of the no observed adverse effects level (NOAEL), the MOE values could be 1000 instead of 100, or even 3000, if including an additional factor of 3 since the exposure in the animal experiment was not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 10-33 mg/kg bw, MOE was 8-25, thus, also too low. Estimations based on more than one cup of tea per day, would give even lower MOE values.

Using the same scenarios for the two food supplements, intake of these may be a risk for pregnant women and for impaired reproduction in males. Alternatively, if estimating MOE from the LOAEL divided by the human exposure, 100 mg/kg bw divided by 1-30 mg/kg bw of food supplements, gave a MOE of only 3.3-100. Since LOAEL was used instead of NOAEL, the MOE values could be 1000 instead of 100, or even 3000, for including an additional factor of 3 since the exposure in the animal experiment was not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 1-30 mg/kg bw, MOE was 8-250, thus, also too low.

If taking a more general view independent of type of preparation of the basil plants, based on the reproductive toxicity studies available using various preparations of *O. tenuiflorum* L./*O. sanctum* L. performed in three species and in both genders of experimental animals, it seems reasonable to conclude that they may have adverse effects on reproduction in both males and females in doses in the dose range of 100-4000 mg/kg bw per day when administered during gestation or for 14-

90 days to non-pregnant individuals. This statement is valid in spite of many of the studies being old and with weaknesses. Using an uncertainty factor of 100 to account for a possible higher susceptibility in humans than in animals, this indicates that a general safe level for all types of preparations of these basil plants may be below 1 mg/kg bw per day.

Regarding the risk from intake of the individual substances, for methyleugenol and estragole, there may be a risk at intake of one or more cups of tea per day or from both the food supplements. For eugenol, when using the highest level of holy basil reported in tea and an intake of three cups of tea per day, there may be a risk, but not for intake of one or two cups of tea per day or for intake of the food supplements. For eucalyptol, intake of one, two or three cups of tea per day and one of the food supplements, for both product types with the highest level of holy basil, may be a risk. For β-caryophyllene and ursolic acid, there was not a risk from one, two or three cups of tea per day or from the food supplements.

The estimations done above are imprecise and there are uncertainties related to whether the few examples of content of holy basil in teas and food supplements on the Norwegian market as given by NFSA are representative of the exposure to such products in Norway. There is also uncertainty regarding how representative the numbers used in the calculations of exposure are for the products generally sold in Norway, since there are various information on composition and levels of the individual substances given in various publications, being dependent on variety, location, methods for preparations etc. It is also uncertain how relevant the data on adverse effects observed in experimental animals with various extracts of plant material are for risk assessments of other preparations of the plant, such as the dried plant material in teas and food supplements.

In conclusion, based on the available information, reproductive toxicity appears to be the most critical adverse effect from intake of holy basil (*O. tenuiflorum* L./*O. sanctum* L.). Considering all the uncertainties and taking all the available publications on reproductive toxicity into account, precaution is warranted regarding intake of teas or food supplements with holy basil for pregnant women, and for both men and women wanting to become parents. Since there are no studies on children specifically, the intake of these products is also better avoided during lactation or by children in general.

Higher quality studies performed by independent contract laboratories after OECD guidelines with clearly defined and characterized plant material are needed in order to establish with more certainty the lowest dose able to cause adverse effects, and thus, a safe intake level of holy basil (*O. tenuiflorum* L./*O. sanctum* L.).

Hovedbudskap (norsk)

Mattilsynet bad Folkehelseinstituttet om a vurdere hvilke mengder av de grønne delene av planten hellig basilikum (*Ocimum tenuiflorum* L./*O. sanctum* L.) som kunne inntas uten at det utgjorde en helserisiko. Denne planten er brukt i teer og kosttilskudd solgt i Norge. Informasjon om potensielle skadelige helseeffekter ved inntak av planten fra to omfattende litteratursøk ble brukt i denne risikovurderingen. Helsemessig trygt inntak av plantematerialet som sådan og noen enkelt-stoffer i planten ble vurdert.

Innholdet av plantematerialet fra hellig basilikum, antatt å være tørket, i teer solgt i Norge ble oppgitt å være 0,6-2,0 g per te-pose. Det ble antatt at alle aktive stoffer i planten blir trukket ut og ender opp i det varme vannet, som et verst tenkelig tilfelle, på grunn av mangel på mer spesifikke opplysninger. De anbefalte daglige dosene av kosttilskudd (tørket plantemateriale) varierte fra 60 til 360 mg i et produkt og fra 800 til 1800 mg i et annet produkt.

Skadelige effekter på reproduksjonen, dvs. redusert fruktbarhet, syntes å være den mest kritiske skadelige helseeffekten fra inntak av hellig basilikum. Inntak av en kopp te eller de to kosttilskuddene med hellig basilikum plantemateriale kan utgjøre en risiko for at fostre ikke fester seg i livmoren hos gravide kvinner og for dårligere reproduksjon hos menn, basert på data fra dyreforsøk.

Et generelt trygt nivå for alle typer preparater av hellig basilikum ser ut til å kunne være under 1 mg/kg kroppsvekt per dag.

Basert på de individuelle stoffene metyleugenol, estragol and eukalyptol i hellig basilikum kan det være en risiko for skadelige helseeffekter ved inntak av en eller flere kopper te per dag eller fra det ene eller begge kosttilskuddene. Basert på eugenol vil et inntak av tre kopper te per dag med det høyeste innholdet av hellig basilikum være en risiko. For β-caryofyllen og ursolsyre var det ingen risiko ved inntak av en, to eller tre kopper te per dag eller fra kosttilskuddene.

Det er en stor del usikkerhet i disse vurderingene, blant annet om de få tilgjengelige eksemplene på innhold av hellig basilikum i teer og kosttilskudd er representative for slike produkter solgt i Norge. Et spørsmål er også hvor representative tallene brukt i beregningene av eksponeringen er for inntak av disse produktene i Norge, siden sammensetningen og mengdene av enkelt-stoffene varierer avhengig av blant annet plantevarianten, hvor den vokser, metodene for dyrkning av planten og prepareringen av det endelige produktet. Det er også usikkert hvor relevant dataene om skadelige effekter observert i forsøksdyr med forskjellige ekstrakter av plantematerialet er for risikovurderingen av andre typer preparater av planten, slik som det tørkede plantematerialet i teer og kosttilskudd.

Til konklusjon, basert på all tilgjengelig informasjon er det berettiget å begrense inntaket av teer eller kosttilskudd med hellig basilikum for gravide kvinner, og for både menn og kvinner som ønsker å bli foreldre. Siden det ikke var noen studier tilgjengelig spesifikt om barn, er det best å unngå inntak av disse produktene under amming og for barn generelt.

Sammendrag (norsk)

Mattilsynet bad Folkehelseinstituttet 3. april 2020 om kunnskapsstøtte til å vurdere hvilke mengder av de grønne delene av planten hellig basilikum (*Ocimum tenuiflorum* L.) som kan inntas uten helserisiko. Bakgrunnen for dette oppdraget var at Mattilsynet trengte en risikovurdering som kunne bli brukt både i vurdering av konkrete saker som Mattilsynet jobbet med, slik som teer og kosttilskudd som inneholder slikt plantemateriale i salg på det norske markedet, og som et grunnlag for å vurdere generelle tiltak når det gjelder produkter som inneholder denne planten.

I 2020 gjorde Folkehelseinstituttet et omfattende litteratursøk som dekket perioden etter tidligere risikovurderinger fra Danmarks tekniske universitet (DTU) and Livmedelsverket i Sverige, dvs. fra 1. januar 2018 til april/mai 2020. Totalt 2786 publikasjoner ble funnet i søket. Etter gjennomgang av søkeresultatet ble 70 publikasjoner inkludert. Et oppdatert søk ble utført 30. juni - 3. juli 2023 for å dekke ny litteratur siden det forrige søket, og 45 publikasjoner ble inkludert. I tillegg ble andre relevante publikasjoner inkludert fra referanselistene i publikasjonene fra begge søkene eller fra tidligere risikovurderinger. Totalt ble informasjon fra 242 referanser brukt i denne risikovurderingen. I tillegg til publikasjoner om plantematerialet som sådan ble også publikasjoner om noen enkelt-stoffer fra denne planten vurdert. Siden potensielle skadelige effekter på reproduksjon i mennesker basert på observasjoner i flere dyrestudier var hovedbekymringen når det gjaldt helsefare fra denne planten ble også eldre publikasjoner på reproduksjonstoksisitet inkludert for en mer fullstendig oversikt. Litteraturen inkludert i denne risikovurderingen var dermed omfattende, om enn ikke strengt systematisk samlet. Positive helseeffekter av basilikum-plantene eller enkelt-stoffer var ikke inkludert i denne vurderingen.

Slekten *Ocimum* fra familien *Lamiaceae* (på norsk 'leppeblomstfamilien') omfatter ett-årige eller fler-årige aromatiske urter som hører hjemme i tropiske og subtropiske regioner av Asia, Afrika og sentrale Sør-Amerika, og består av >65 arter. Planten *O. tenuiflorum* L., hellig basilikum, har mange ulike navn både på engelsk og latin, og det er ikke helt entydig fra litteraturen hvilke plantenavn som bør inkluderes i denne risikovurderingen. *O. tenuiflorum* L. er det korrekte og foretrukne navnet, men mange publikasjoner bruker fremdeles navnet *O. sanctum* L. Data fra andre basilikum-arter ble også inkludert når de ble vurdert som relevante. Innholdet av de enkelte kjemiske stoffene i den essensielle oljen i hellig basilikum og andre basilikumplanter kan variere avhengig av mange faktorer; art, kultivar, opprinnelsessted, alder, del av planten, miljøforhold slik som innhøstingssesong, jordbruksteknikker, sammensetningen av spektralt lys brukt under dyrking, type gjødsel, bruk av elisitorer og andre forhold for rot-kulturer, samt ekstraksjonsmetoder og annen prosessering av det ferdige planteproduktet. Også flere kjemotyper, haplotyper eller morfotyper av disse plantene er beskrevet. Høyt tungmetall-innhold tatt opp fra jorden kan i noen tilfeller også være en risikofaktor i urteplanter og preparater av disse. Det anbefales at produsentene av urteteer og kosttilskudd bør overvåke kjemiske (tungmetaller, radionuklider og plantevernmidler) og mikrobielle kontaminanter som kan være til stede i plantematerialet, og sikre at disse er under maksimumsgrensene i lovverket.

Ifølge informasjonen fra Mattilsynet er innholdet i materialet fra hellig basilikum-planten, som er antatt å være tørket, i te-produktene på det norske markedet 0,6-2,0 g per tepose. Basert på denne informasjonen og ved bruk av en standard kroppsvekt på 60 kg vil en kopp per dag av te laget av én tepose med hellig basilikum gi en eksponering på maksimum 10-33 mg/kg kroppsvekt (kv) per dag. Dette er basert på antagelsen om at alle de aktive stoffene er ekstrahert med og ender opp i det varme vannet, som et verst tenkelig scenario på grunn av mangel på mer spesifikke data. Det beregnede inntaket vil bli 20-66 og 30-99 mg/kg kv for henholdsvis inntak av 2 og 3 kopper av slik te per dag. Ifølge informasjonen fra Mattilsynet, varierer de anbefalte daglige dosene av kosttilskudd med tørket materiale fra planten hellig basilikum fra 60 til 360 mg i et produkt og fra 800 til 1800 mg i et annet produkt. Basert på en standard kroppsvekt på 60 kg vil disse

kosttilskuddene gi en estimert eksponering på henholdsvis 1-6 mg/kg kv eller 13.3-30 mg/kg kv per dag av det tørkede plantematerialet. Innholdet av de individuelle aktive stoffene metyleugenol, estragol, eugenol, eukalyptol, β-caryofyllen and ursolsyre i basilikumplanter ble også beregnet fra inntaket av disse teene og kosttilskuddene.

Helsefaren forbundet med inntak av dette plantematerialet som sådan og de individuelle stoffene ble hovedsaklig vurdert basert på data fra eksperimenter med forsøksdyr. Metyleugenol og estragol ble betraktet som gentoksiske og kreftfremkallende stoffer, mens plantematerialet som sådan og de fire andre enkelt-stoffene ikke ble betraktet som gentoksiske. Basert på de tilgjengelige dataene var reproduksjonstoksisitet den mest kritiske skadelige effekten fra inntak av hellig basilikum. Dette ble observert som svekket spermieproduksjon i hanner, forstyrret brunstsyklus og tap av implanterte fostre i livmor hos hunner, samt forandringer i vekt og struktur av reproduksjonsorganer, forandringer i hormonnivåer, redusert parringsadferd og redusert fruktbarhet både i hanner og hunner. Ingen misdannelser, teratogene effekter, ble rapportert på avkommet etter inntak av plantematerialet. To publikasjoner på reproduksjonstoksisitet i forsøksdyr etter eksponering for vandige ekstrakter av hellig basilikum, som ble vurdert som det mest relevante ekstraktet for urteteer og kosttilskudd, ble brukt i risikokarakteriseringen. En studie rapporterte tap av implanterte embryoer i livmoren hos hunn-rotter eksponert for 100 mg/kg kv på dag 1-4 i svangerskapet (i 3 av 5 rotter) eller eksponering for 200 mg/kg kv på dag 1-7 i svangerskapet (i 2 av 5 rotter). En annen studie rapporterte signifikant nedgang i antall og bevegelse av spermier, og signifikant økt dødelighet av spermier, etter 10-50 dagers eksponering, og lavere vekt av reproduksjonsorganer etter 20-50 dagers eksponering, i hann-mus gitt 250 mg/kg kv per dag av et vandig ekstrakt av hellig basilikum.

Hvis man antar at plantematerialet som sådan ikke er gentoksisk og bruker en standard usikkerhetsfaktor på 100, kan man beregne en helsebasert veiledende verdi (HBGV) for tap av implantering av embryoer i livmoren på 100 mg/kg kv per dag dividert med 100, som blir 1 mg/kg kv per dag. For skadelige effekter på reproduksjonen hos hanner, 250 mg/kg kv per dag dividert på 100, blir 2.5 mg/kg kv per dag. Ut fra dette vil selv inntak av én kopp te som inneholder hellig basilikum være en risiko for implanteringstap av embryoer i livmoren hos gravide kvinner og for svekket reproduksjon hos menn.

Alternativt kan det beregnes en eksponeringsmargin (MOE) ut fra en daglig dose som har gitt skadelige effekter i forsøksdyr dividert med eksponeringen hos mennesker. MOE-verdiene regnes vanligvis som trygge i en risikovurdering av ikke-gentoksiske stoffer som antas å ha en terskel for effekt hvis de er over 100. Når man estimerer MOE fra den laveste dosen hvor det er observert en skadelig effekt (LOAEL) dividert med den humane eksponeringen, 100 mg/kg kv per dag dividert med 10-33 mg/kg kv per dag (for én kopp te), vil dette gi MOE-verdier på bare 3-10. Siden LOAEL ble brukt i stedet for den høyeste dosen hvor det ikke ble observert en skadelig effekt (NOAEL), bør MOE-verdiene være 1000 i stedet for 100, eller til og med 3000, hvis man inkluderer en tilleggsfaktor på 3 siden eksponeringen i dyreforsøket ikke var kronisk, dvs. livslang. Tilsvarende, for 250 mg/kg kv per dag dividert med 10-33 mg/kg kv per dag, gir MOE-verdier på 8-25, som også er for lave. Beregninger basert på mer enn én kopp te per dag vil gi enda lavere MOE-verdier.

Hvis man bruker de samme scenarioene for de to kosttilskuddene, vil inntaket av disse kunne utgjøre en risiko for tap av implantering av embryoer i livmoren og for svekket reproduksjon i hanner. Alternativt, hvis man beregner MOE fra LOAEL dividert med human eksponering, 100 mg/kg bw per dag dividert med 1-30 mg/kg bw per dag av kosttilskuddene, vil dette gi MOE-verdier på bare 3.3-100. Siden LOAEL ble brukt i stedet for NOAEL, bør MOE-verdiene være 1000 i stedet for 100, eller til og med 3000, hvis man inkluderer en tilleggsfaktor på 3 siden eksponeringen i dyreforsøket ikke var kronisk. Tilsvarende, for 250 mg/kg kv per dag dividert med 1-30 mg/kg bw per dag gir MOE-verdier på 8-250, som også er for lave.

Hvis man betrakter dette mer generelt uavhengig av type preparat av hellig basilikum basert på de reproduksjonstoksiske studiene tilgjengelig som har brukt ulike preparater av *O. tenuiflorum* L./*O. sanctum* L. i tre ulike arter og begge kjønn av forsøksdyr, så synes det å være fornuftig å konkludere at de kan ha skadelige effekter på reproduksjon i begge kjønn i dose-området 100-4000 mg/kg kv per dag ved inntak under svangerskapet eller ved inntak i 14-90 dager for ikke gravide. Dette gjelder på tross av at flere av studiene er gamle og har svakheter. Ved å bruke en usikkerhetsfaktor på 100 for å ta hensyn til en mulig større følsomhet i mennesker enn i dyr, kan man anta at et generelt trygt nivå for alle typer preparater av disse basilikum-plantene kan ligge på under 1 mg/kg kv per dag.

Vedrørende risiko fra inntak av de individuelle stoffene, for metyleugenol og estragol kan det være en risiko ved inntak av en eller flere kopper te per dag eller fra begge kosttilskuddene. For eugenol, hvis man bruker det høyeste nivået av hellig basilikum rapportert i te og et inntak av tre kopper te per dag, vil det kunne være en risiko, men ikke for inntak av en eller to kopper te per dag eller for inntak av kosttilskuddene. For eukalyptol, kan inntak av en, to eller tre kopper te per dag og inntak av ett av kosttilskuddene, for begge produkttyper med det høyeste innholdet av hellig basilikum, være en risiko. For β-caryofyllen og ursolsyre var det ikke en risiko ved inntak av en, to eller tre kopper te eller fra kosttilskuddene.

Beregningene som er gjort ovenfor er upresise og det er usikkerhet knyttet til om de få eksemplene på innholdet av hellig basilikum i teer og kosttilskudd på det norske markedet som ble oppgitt av Mattilsynet er representative for eksponeringen for slike produkter i Norge. Det er også usikkert hvor representative tallene brukt i beregningene for eksponering er for slike produkter generelt solgt i Norge siden det er ulik informasjon om sammensetning og nivåer av de individuelle stoffene oppgitt i ulike publikasjoner. Dette avhenger av varietet, voksested, prepareringsmetoder etc. Det er også usikkert hvor relevante dataene for skadelige effekter observert i forsøksdyr med ulike ekstrakter av plantematerialet er for risikovurderinger av andre preparater av planten, slik som det tørkede plantematerialet i teer og kosttilskudd.

Til konklusjon, basert på den tilgjengelige informasjonen ser det ut til at reproduksjonstoksisitet utgjør den mest kritiske effekten ved inntak av hellig basilikum (*O. tenuiflorum* L./*O. sanctum* L.). Ut fra en betraktning av alle usikkerhetsmomentene og alle de publiserte artiklene om reproduksjonstoksisitet virker det fornuftig å være forsiktig med inntak av teer eller kosttilskudd med hellig basilikum for gravide kvinner, og for både menn og kvinner som ønsker å bli foreldre. Siden det ikke er noen studier gjort spesifikt på barn er det også best å unngå inntak av disse produktene under amming og for barn generelt.

Studier med bedre kvalitet utført av uavhengige kontrakt-laboratorier etter OECDs retningslinjer for dyreforsøk med klart definert og karakterisert plantemateriale trengs for å etablere med større sikkerhet den laveste dosen som kan gi helseskadelige effekter, og dermed et trygt inntak av hellig basilikum (*O. tenuiflorum* L./*O. sanctum* L.).

Preface

The Norwegian Food Safety Authority asked the Norwegian Institute of Public Health to assess which amounts of the green parts of the plant holy basil that could be consumed without risk to human health, also based on some of the individual harmful substances in the plant, as well as in relation to potential vulnerable groups. The scientific assessment was to be used for evaluation of specific products as well as a basis for general management of foods containing this plant.

We hope that this report may be useful in their work regarding the use of this plant in foods in Norway. This report will also be of interest for risk managers in other countries.

Oslo, November 2023

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Introduction

Background for the request from the Norwegian Food Safety Authority (NFSA)

NFSA requested on April 3, 2020, the Norwegian Institute of Public Health (NIPH) to assess which amounts of the green parts of the plant holy basil (*Ocimum tenuiflorum* L.) that could be consumed without risk to human health. The reason for this request was that NFSA needed a risk assessment that could be used both for evaluation of specific products such as teas and food supplements containing this plant material and as a basis for a decision on general actions regarding products containing this plant. NFSA requested that new literature published after previous evaluations was taken into consideration.

Holy basil is used, based on NFSA's knowledge, in food supplements (dried leaves/extracts) and herbal teas (dried leaves). It is also used as a culinary herb, flavour or spice in Thai food or other Asian dishes (fresh plant).

Terms of references from NFSA

1. To assess what is the limit for a safe daily or weekly intake of the green parts of holy basil (*Ocimum tenuiflorum* L.) (except the seeds), also including the substances methyleugenol and estragole.
2. To establish a safe exposure level for the green parts of holy basil from food supplements, herbal teas and used as spice, if possible.
3. To evaluate if there are any vulnerable groups.

According to NFSA, various products are in sale in Norway, both as teas and food supplements, containing holy basil in varying amounts. Examples of doses in such products are:

- Content in tea: 2 g holy basil per tea bag or 0.6 g holy basil per tea bag (assumed by NIPH to be dried plant material)
- Recommended daily doses in a food supplement (dried plant) varying from 800 mg to 1800 mg in one product, or from 60 mg to 360 mg in another product (in the labelling on this last product it was stated that this can be used in addition to other products containing holy basil). It was also mentioned in general, that extract(s) of holy basil plant material may be used in food supplements.

No further information was given for these commercial products on which part(s) of the plants were used, where the plants were grown, which extract(s) and their amounts etc. that would affect the content of active and potentially harmful substances in the plant material.

Use of herbal medicines

In many developing countries, populations rely on traditional medicine for primary health care, which is now increasingly being sold also in commercial markets globally as natural remedies, which usually are regarded as safe by the general population. Traditional and natural remedies are adapted and expanded in commercial products to provide alternatives for various conditions and diseases. These products resemble very little the traditional use (Prinsloo et al., 2018). Several cases of adverse effects of such preparations have been reported. Still, in most publications on these compounds, the focus is on demonstrating beneficial effects, and adverse effects are often not examined and reported. Some of these plants are known to contain chemical components that have been identified as genotoxic carcinogens, such as methyleugenol, estragole and safrole. It is still uncertainty regarding the adverse effects to human health from consumption of relatively low levels of such substances from herbs and spices in foods, particularly because of uncertainties in their exposure. However, based on the toxicity data, it is clear that high intake levels, for instances from plant food supplements, can be adverse (Eisenreich et al., 2021). Herbs and botanical formulations, therefore, may not be as safe as they are usually considered because they are

'natural'. The concentrations of the individual compounds in the preparations and their mixture effects, causing synergism or antagonism, metabolism of these compounds, as well as matrix effects, may affect their toxicity. Thus, further toxicity studies performed according to Organization for Economic Cooperation and Development (OECD) guidelines are needed with proper focus on the adverse effects, with sufficient description and characterization of the plant material or extracts, and their content of active substances.

Classical herbal formulations used in traditional medicine are those which are prepared according to the formula given in the traditional books of alternative systems of medicines used for centuries in countries such as India and China. The manufacturers of the classical herbal formulations follow the same formula to prepare the formulations and standardize them as given in the guideline of official monographs. Non-classical herbal formulations have gained momentum in the recent past. These represent the modification of the indigenous classical herbal preparations by changing administration form or route, method of preparation or indications for use. Hence, these modified preparations may get impaired due to incompatibility, instability and impurity leading to serious adverse events. As opposed to classical herbal formulations, the non-classical or proprietary formulations have not been well integrated with modern medicine in most countries and are prepared as per manufacturer's own formula often with ingredients and additives that are not found in the traditional literature. Most of the proprietary herbal preparations consist of a complex heterogeneous mixture. Though some of the materials are included in different official monographs, their chemical markers and chromatographic specifications are not well documented. Thus, there is a lack of scientific evidence pertaining to their long-term safety and efficacy. Besides, the paucity of authentic monographs on the impurity profiling, standardization protocols and lack of guidelines on the fixed-dose combinations are among the major caveats. However, WHO have made guidelines on safety monitoring of herbal medicines in pharmacovigilance systems (WHO, 2004). Impurities in herbal preparations may be deliberately added synthetic drugs, or heavy metals, radionuclides, mycotoxins, pesticides and solvent residues from the plant extraction and processing. According to Silpavathi et al. (2019), more than 16000 suspected case reports of adverse effects of herbal medicinal preparations had so far been reported in the WHO global database. The most frequently reported adverse effects were face edema, hepatitis, hypertension, angioedema, convulsions, dermatitis and death. Adverse reactions associated with medicinal products can be found in WHO global database VigiBase (WHO, 2023).

Substantial efforts have been made by Indian scientists to identify pregnancy interceptive plant preparations from those reported in ancient Indian literature and also from plants not referred to in these old texts (Kamboj, 1988), explaining the high number of publications in that area. However, the lack of botanical identification of plant samples and the activity studies being limited to less than sufficient number of animals have resulted in inconsistent results. For instance, the activity of *Ocimum sanctum* leaves on preventing implantation of the embryo in the uterus was reported as 0-80% in various publications. In spite of this limitation, a few leads of efficient plants were evident, which, however, were not followed up. A more systematic approach as advocated by the WHO and Indian Council of Medical Research (ICMR) Task Forces on Plants for Fertility Regulation to generate reproducible results was suggested.

Previous risk assessments

Holy basil was previously assessed in a European Food Safety Authority (EFSA) Scientific Cooperation (ESCO) report in 2009 and relevant information from this report is included in the text below. EFSA (2014a) excluded *Ocimum tenuiflorum* L. (leaves) from the Qualified Presumption of Safety (QPS) approach for its safety assessment. They concluded that the chemical analytical data of the holy basil plant did not provide adequate compositional information. The chemical(s) of concern for the reproductive effects had not been pinpointed. The information on composition, toxicity and use was insufficient. Moreover, *Ocimum tenuiflorum*'s leaves were reported to contain genotoxic and carcinogenic substances.

Risk assessment of holy basil (*Ocimum tenuiflorum* L./*Ocimum sanctum* L.)

New literature searches

In collaboration with the library at NIPH, an extensive literature search was performed, which covered the period after previous searches done by DTU in Denmark and the Swedish Food Agency, i.e. from 01.01.18 to April/May 2020. The search strings were set up by an information specialist at the NIPH library, and are shown in Appendix 1. Search number 1 on content and concentrations of substances in the plant was performed on April 24, 2020, and search number 2 on toxicity and adverse health effects of the plant or individual substances in the plant was performed on May 4, 2020 (see Appendix 1 for the search dates for the individual databases). Both searches were done in in Medline, Embase, Web of Science, Scopus, Toxline via Pubmed and Crop Protection Compendium. After removal of duplicates, in total 2786 publications were found for both searches in total. The titles and abstracts were screened and divided into two categories; excluded (2581 publications) or retained for further scrutiny in full text (205), of which 70 publications were finally included. In addition to the publications included from the literature searches, other relevant publications were included in this assessment from the reference lists of the publications from the search (96) or from risk assessments and regulations (30), in order to have a more complete database for this assessment covering as many toxicological endpoints as possible. Among these publications, some were used in the actual risk assessment, others contained useful background information.

An updated literature search was performed by the information specialist at NIPH library June 30- July 3, 2023, using the same two search strategies in Medline, Embase, Web of Science and Crop Protection Compendium (see Appendix 2). At this time, Toxnet no longer existed and the references there are included in Medline, and unfortunately, the NIPH Library no longer had access to Scopus. In this updated search, after removal of duplicates between the databases and between searches 1 and 2, 5144 publications were found in total for both searches (see above for the difference between search number 1 and 2). In addition, 7 additional duplicates were detected when going through the abstracts, thus, the final number of hits in this search was 5137. The titles and abstracts were screened and divided into two categories; excluded (4965 publications) or retained for further scrutiny in full text (172), of which 45 publications were included. In addition, one previous risk assessment was included. As before, among these new publications, some were used in the actual risk assessment, others contained useful background information.

Because reproductive toxicity appeared to be the most critical effect, also older publications were included on this endpoint. In addition to the two individual substances mentioned in the terms of reference (methyleugenol and estragole), four other substances (eugenol, eucalyptol, β-caryophyllene and ursolic acid present in holy basil were included because there was quite a lot of data on their toxicity available. Some information was also included on safrole. Thus, the literature finally included in this risk assessment report (309 references) was quite extensive, although not strictly systematically collected. Positive health effects of the basil plants were outside the scope of this assessment and publications on such effects were not included, except for the few that had recorded and reported adverse effects in humans.

Exposure data, regulations and previous risk assessments

Exposure from therapeutic and medicinal use was given in EFSA (2014a), as fresh leaves: 2 g/kg bw per day for 30 days, dried leaves: 300-2000 mg per day as a single dose or 600-2000 mg per day in multiple doses, infusions: 2 g dried leaves per cup of water or 2.5 g dried leaves per day, fresh leaf juice: 10-20 ml. No information on recommended dose levels of other plant parts than leaves was available.

In EU, in addition to the general food law (EU, 2002a), food supplements are regulated by the food supplements directive (EU, 2002b). In Norway, the corresponding regulations are the Norwegian food law (2004) and the Norwegian regulation on food supplements (2004). Because they exert their toxicity upon bioactivation to proximate carcinogens, the European Commission (EC) prohibited the use of the alkenylbenzenes methyleugenol, estragole and safrole as pure compound in foods, including food supplements (EU, 2008, Annex III, Part A). See for instance Martins et al. (2018) for molecular structures of the alkenylbenzenes.

EFSA has published a compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements (EFSA, 2012a). The purpose of the compendium was to assist risk assessors responsible for the evaluation of specific ingredients in food supplements to more easily identify the compound(s) of concern on which to focus the assessment. However, this document lists in alphabetical order botanicals without any judgment on whether they are suitable or not suitable for food applications in Europe and it has no legal or regulatory force pertaining to the legal classification of products or substances.

The margin of exposure (MOE) approach is recommended by several expert groups, i.a. European Food Safety Authority (EFSA), for risk assessments of compounds that are genotoxic and carcinogenic (EFSA, 2005). MOE is the ratio between a reference point usually obtained from epidemiological or experimental animal data on tumour incidence, (i.e. the $BMDL_{10}$, the lower confidence bound of the benchmark dose in mg/kg bw per day that gives 10% extra cancer incidence), or the T25 (the chronic dose in mg/kg bw per day that cause tumours in a given tissue in 25% of the animals during their life-time, after correction for spontaneous tumours in the negative control group) and the estimated daily intake (EDI) in humans. A MOE value lower than 10000 based on $BMDL_{10}$ or lower than 25000 based on T25 is considered to indicate a priority for risk management (EFSA, 2005). When using the recommended daily intake indicated on the labels of the supplements, van den Berg et al. (2011) calculated that the genotoxic and carcinogenic alkenylbenzenes such as estragole and methyleugenol from various plant food supplements generally had a MOE lower than 10000 and often below 100, in some cased even below 10, i.e. in the range of the doses of pure substances causing tumours in experimental animals. However, the situation may be different when using the botanicals in the form of multicomponent extracts, because of the matrix effect (see the Chapter *Hazards from individual substances in basil plants*).

A risk matrix was used to identify chemical hazards that have the highest human health risk from spices and herbs, including basil (van Asselt et al., 2018). Based on both the probability of occurrence evaluated from monitoring data and the European Rapid Alert System for Food and Feed (RASFF) notifications and the severity of the hazard based on toxicological reference values and classification of carcinogenicity, methyleugenol was classified as having medium risk, and estragole as having high risk.

Risk assessments of 10 plant food supplements (PFS), 23 traditional Chinese medicines (TCM) and 38 herbal teas on the Chinese market containing alkenylbenzenes were performed using the margin of exposure (MOE) approach by Ning et al. (2018). PFS and TCM were in the form of capsules, tablets or powder. The teas were provided as tea bags, powder, whole fruit or leaves. The levels of alkenylbenzenes in botanical preparations were quantified with ultra-performance liquid chromatography (UPLC) analysis after methanol extraction, and hot water extraction for the teas, and the combined estimated daily intake (EDI) was determined using dose additivity, assuming similar mode of actions. It was assumed that 2 g of dry tea plant material was used to prepare a cup of tea of 200 ml. The combined EDI values obtained assuming equal potency of all alkenylbenzenes detected in the PFS, TCM and herbal teas were 0.3 to 14.3, 0.05 to 539.4 and 0.04 to 42.5 μg/kg bw per day, respectively. Calculating combined EDI values taking into account the toxic equivalency (TEQ) approach, using estragole as the reference compound, the values for PFS,

TCM and herbal teas were 0.3 to 7.7, 0.05 to 278.0 and 0.02 to 16.5 µg estragole equivalents/kg bw per day, respectively. The MOE values resulting from consumption of these PFS, TCM and one cup of herbal tea per day during life-time were generally lower than 10000, suggesting a potential risk, and thus, a priority for risk management. For short-term exposure such as two weeks consumption, applying Haber's rule, only one TCM product had a MOE value below 10000. Haber's rule is k = C X T, where C is the concentration or dose of the toxic chemical, T is the period of exposure and k is the toxic outcome, which is considered to be constant (Felter et al., 2011). It was concluded that selected consumption of Chinese botanical preparations raised a concern because of exposure to alkenylbenzenes, especially when exposure was for longer periods of time. The mixture of compounds in these products may result in synergistic or antagonistic effects, for example causing modification of bioactivation and/or detoxification pathways of the genotoxic carcinogens. In addition, such products may also contain many compounds of other chemical groups, having different mode of action than the alkenylbenzenes.

Mahony et al. (2020) evaluated the use of the threshold of toxicological concern (TTC) approach to assess safety of botanical preparations that may contain potentially genotoxic constituents, based on estimation of the fraction that may be genotoxic. A database of 107 chemical constituents of botanicals was compiled and their potential for genotoxicity was evaluated from published data. Forty-three constituents met the criteria for potential genotoxicity. Concentration data on their occurrence in plants provided 2878 data points; the majority of these were in the low ppm (mg/kg) level (range 0.00001–139965 ppm, by dry weight). Wet weight concentrations were converted to dry weight concentrations using a standard factor of 5, assuming that plants contain 80% water. Weibull models of the quantitative distribution data were used to calculate 95th percentile values for chemical concentrations, analysing the dataset according to their presence in botanicals (i) as a single chemical, (ii) as two or more chemicals from the same chemical group, or (iii) as two or more chemicals from different chemical groups. The highest 95th percentile concentration value from these analyses was 1.8%. Using the TTC value of 0.15 µg/person per day for potentially genotoxic substances derived by Kroes et al. (2004), this value of 1.8% was used to derive an adjusted oral TTC value of 10 µg of plant material on a dry weight basis/person per day (0.17 µg/kg bw per day for a person of 60 kg bw) for assessment of potentially genotoxic substances in botanicals. This new TTC value can in principle be used on any botanical preparations or simple extracts, but it is not appropriate to use the TTC value for any extract that is selectively concentrated to give more of a single chemical or chemical class, or when the entire extract is concentrated, which may change the natural ratios of plant chemical constituents. Neither should it be used for any oils or essential oils. It is not applicable to ingested single genotoxic substances in the diet, for which the existing TTC values of 0.15 µg/person per day for DNA-reactive mutagens (potentially genotoxic substances) should be used. This value of 0.15 µg/person per day is considered to be 10-fold more conservative than the MOE. It would not be appropriate to use the TTC approach for risk assessment of a botanical that has well-documented toxic effects, in such cases the assessment should be based on the existing toxicity data. Matrix effects were not taken into consideration here (see the Chapter *Hazards from individual substances in basil plants*). Estragole and methyleugenol were listed in this publication as substances that met the acceptance criteria for plant origin and potential genotoxicity, whereas eugenol did not. In the absence of dermal absorption data, a worst-case assumption can be made assuming 100% absorption of a dermally applied product, which can be compared with the proposed oral TTC value.

The genus *Ocimum* from the *Lamiaceae* family and its variety of basil plants

The genus *Ocimum* from the *Lamiaceae* family (in Norwegian 'leppeblomstfamilien') contains annual or perennial aromatic herbs that are native to the tropical and subtropical regions of Asia, Africa and Central South America, and is composed of >65 species (Saaban et al., 2019).

The plant *Ocimum tenuiflorum* L. has many different names, both in English and Latin, and it is not entirely clear from the literature which plant names to include in this risk assessment. Common names in English on the plant *Ocimum tenuiflorum* L. are i.a. holy basil, sacred basil, monk's basil and tulsi, and it is referred to as the 'Queen of herbs'. According to WHO (2002), the plant *Ocimum tenuiflorum* L. has several synonyms: *Moschosma tenuiflorum* (L.) Heynhold, *O. album* Blanco, *O. anisodorum* Muell., *O. brachiatum* Hasskarl, *O. flexuosum* Blanco, *O. frutescens* Burm., *O. gratissimum* Lour., *O. inodorum* Burm., *O. monachorum* L., *O. nelsonii* Zipp ex Span. and *O. virgatum* Blanco. However, according to EFSA ESCO Report (2009), also *O. sanctum L.* and *O. gratissimum* sensu Lour (non L.) are the same plant as *O. tenuiflorum* L. In this risk assessment, also information on other basil plants is included when considered relevant for basil plants in general, such as *O. basilicum* L., called sweet basil or Thai basil. According to Saaban et al. (2019), the calculated genetic distance between *O. sanctum* L. and *O. basilicum* L. showed that they are closely related species and share the same traits. Although *O. tenuiflorum* L. is the correct and preferred name, many publications still use the synonym *O. sanctum* (EFSA ESCO Report, 2009).

The genus basil, *Ocimum* L., has a large variation in chromosome number and size, believed to be a consequence of centuries of cultivation and selection for desirable traits. Meiosis was examined in five *Ocimum* species from Thailand, from five populations of each species, by Lekhapan et al. (2019). The results revealed that three of these species were cytogenetically related, forming a polyploid series with a base chromosome number of x=13: *O. americanum* L. (2n=2x=26, a new number for Thai plants), *O. basilicum* L. (2n=4x=52) and *O. africanum* Lour. (2n=6x=78, a new number for the genus). Two species with small chromosomes, *O. tenuiflorum* L. (2n=36) and *O. gratissimum* L. (2n=40), seemed probably tetraploids with base numbers x=9 and 10, respectively. Meiotic irregularities in *O. basilicum* and *O. africanum* suggested that the species may be newly formed polyploids undergoing diploidization. Unlike recent polyploids, the meiotic chromosomes of *O. tenuiflorum* and *O. gratissimum* were entirely stable. Statistically significant intra-specific variation in chiasma frequencies was found in *O. basilicum* and *O. africanum* and the variation appeared to be geographically associated.

In addition to various basil species, there are also varieties within the same species, for instance with green or purple leaves. In addition, many other factors determine the content of individual chemical compounds in the essential oil of the basil plants, as described below.

Content of active substances in basil plants

Essential oils (EO) are complex secondary metabolites, rich in volatiles, and the essential oil constituents comprise a diverse family of low molecular weight organic compounds with a wide range of biological activities, which could potentially also be harmful (Bhavya et al., 2018; Burcul et al., 2020). Essential oils are in general approximately 75-100 times more concentrated than dried herbs (Vani et al., 2009). EO may be considered as multicomponent mixtures with up to several hundred individual chemical compounds, which may have additive, synergistic or antagonistic interactions among them (Bunse et al., 2022). In addition, the individual constituents of EO may interact with the rest of the plant matrix, food components or other pharmaceuticals, making predictions of human safety challenging.

Folium Ocimi Sancti consists of the fresh or dried leaves of *Ocimum sanctum* L. (a synonym of *O. tenuiflorum* L.) and contains not less than 0.5% essential oil (EO) (WHO, 2002). The main components are tannins (4.6%) and essential oil (up to 2%). The amounts of the primary constituents of the essential oil vary with eugenol (up to 62%), methyleugenol (up to 86%), and α- and β-caryophyllene (up to 42%). Also present are estragole, linalool and eucalyptol.

According to EFSA (2014a), the leaves of *O. tenuiflorum* L. contain 2% essential oil and 2.02% ursolic acid. The essential oil contains up to 62% of eugenol and up to 86% of methyleugenol, 7-25% estragole and 7-23% eucalyptol. In addition, the leaves contain alkaloids and saponins.

Phytochemical screening of leaves of *Ocimum tenuiflorum* L. extracted with ethanol, methanol, acetone or water revealed the presence of saponins, alkaloids, terpenoids, flavonoids, steroids, anthroquinones, phenols, tannins and glycosides (Saravanakumar et al., 2018).

In two varieties of *O. basilicum* and one variety of *O. sanctum* grown in four locations in southeastern USA, the average content of essential oils was approximately 0.36% and 0.18% in the two plant species, respectively (Zheljazkov et al., 2008). The major constituents in average wt/wt % in essential oil of *O. basilicum* were (-)-linalool (47.3) and eucalyptol (8.0), and some samples also contained eugenol (4.7), but no samples contained estragole. The major constituents in essential oil of *O. sanctum* were eucalyptol (12.8), eugenol (10.4) and estragole (15.3, range 7.02-25.1). Both species also contained β-caryophyllene, 0.4 wt/wt % in *O. basilicum* and 1.4 wt/wt % in *O. sanctum*, and none contained methyleugenol.

Devendran and Balasubramanian (2011) identified 10 compounds in a hydroalcoholic extract (70% v/v) of *O. sanctum* L. leaves in India with gas chromatography-mass spectrometry (GC-MS) analysis. The major components were eugenol (43.8%) and caryophyllene (26.5%). In addition, alkaloids, tannins, saponins, steroids, terpenoids, flavonoids and cardiac glyceride were identified in this extract.

Essential oils are complex low molecular weight secondary metabolites in plants, rich in volatile compounds that have a wide range of biological activities. According to Burcul et al. (2020), based on their chemical structure these active compounds can be divided into four major groups: terpenes, terpenoids, phenylpropenes and "others". They may contain diverse functional groups that may be used for classification as hydrocarbons (monoterpenes, sesquiterpenes and aliphatic hydrocarbons), oxygenated compounds (monoterpene and sesquiterpene alcohols, aldehydes, ketones, esters and other oxygenated compounds) and sulfur and/or nitrogen sulfur-containing compounds (thioesters, sulfides, isothiocyanates, nitriles and 'others').

The plant *Ocimum tenuiflorum* L. has been used in traditional medicine, such as Ayurveda in India, and as fragrance. The characteristic aroma of this and related plants is attributed to its specific combination of volatile phytochemicals mainly belonging to terpenoid and/or phenylpropanoid classes in its essential oils (EO) (Maurya et al., 2019). The essential oil constituents are synthesized and sequestered in specialized epidermal secretory structures called glandular trichomes. There are three types of trichomes; peltate, capitate and hairy trichomes (Maurya et al., 2019). The basil plants show variations in the yield and phytochemical composition of their essential oils. Microscopic analysis of trichomes of *O. basilicum*, *O. gratissimum*, *O. kilimandscharicum* and *O. tenuiflorum* (green and purple cultivars) revealed substantial variations in density, size and relative proportions of peltate and capitate trichomes among them. The essential oil yield is controlled by the population, dominance and size of peltate and capitate glandular trichomes. The essential oil sequestration in the leaves is controlled by the dominance of peltate glandular trichome size over its number and is also affected by the capitate glandular trichome size/number with variations in leaf area albeit at lower proportions. Comparison of results of gas chromatography-mass spectrometry (GC-MS) analysis of essential oils showed that most of the *Ocimum* (*O. basilicum*, *O. tenuiflorum* and *O. gratissimum*) species produce phenylpropanoids (eugenol, estragole) as major volatiles, except *O. kilimandscharicum*, which is a monoterpenoid-rich species. Among the phenylpropanoid-enriched *Ocimum* (*O. basilicum*, *O. gratissimum*, *O. tenuiflorum* purple, *O. tenuiflorum* green), also terpenoids were important constituents in the aroma.

Essential oils were hydrodistilled from fresh leaves of two Holy basil (*Ocimum sanctum*) varieties, white and red, and Tree basil (*O. gratissimum*), Thai basil (*O. basilicum* var. thyrsiflorum) and Lemon basil (*O. citriodorum*) grown in Thailand (Tangpao et al., 2018). Oil physiochemical

characteristics and volatile chromatograms from GC-MS were used to describe the chemical compositions qualitatively and quantitatively. Estragole, eugenol and methyleugenol were among the major volatiles found in the essential oils of these *Ocimum* species. Principal component analysis (PCA) showed that they were grouped based on distinctive anise, citrus aroma (estragole, geranial and neral) or spice-like aroma (methyleugenol, β-caryophyllene and α-cubebene).

A large number of publications report content of various essential oils in basil plants in varying detail. *O. basilicum* and *O. sanctum* leaves contain most chemical constituents followed by inflorescence (in Norwegian: 'blomsterstand') and flowers (Saaban et al., 2019). The main constituents of *O. sanctum* consists mainly of eugenol methyl ether (>34.34%), (E)-caryophyllene (>7.91%), germacrene D (>5.58%), β-elemene (>4.22%) and copaene (>1.49%), whereas *O basilicum* consists of estragole (>35.71%), (E)-β-ocimene (>1.47%), trans-α-bergamotene (>0.83%), τ-cadinol (>0.41%), eucalyptol (>0.25%) and α-caryophyllene (>0.07%) (Saaban et al., 2019). Less than 1% (w/w) of essential oils was extracted with steam distillation from leaves of *O. tenuiflorum* L. cultivated in the Philippines and the most abundant compound identified with gas chromatography/mass spectrometry (GC/MS) was *m*-eugenol (69.1%) (Bugayong et al., 2019). Essential oils from leaves and twigs of *O. tenuiflorum* L. and *O. basilicum* L. cultivated in Italy were isolated with hydrodistillation and characterized with gas chromatography (GC) and GC/MS) (Piras et al., 2018). The major components were methyleugenol (84.7%) and β-caryophyllene (7.4%) in *O. tenuiflorum* L., and linalool (35.1%), eugenol (20.7%) and eucalyptol (9.9%) in *O. basilicum* L. The GC-MS results of leaf-based essential oil from *O. tenuiflorum* bought in Bengaluru, India, showed that the major components present were eugenol and caryophyllene, constituting about 70.5% of the total essential oil composition (Bhavya et al., 2018).

In three *O. basilicum* L. cultivars from Serbia, the EO content in the dry herbs isolated with hydrodistillation and analysed by gas chromatography-flame ionization detector (GC-FID) and GC-MS was 0.65%, 0.41% and 0.62% (Ilić et al., 2019). The main classes of compounds of two of the plants were sesquiterpene hydrocarbons (38.39% and 37.95%), oxygenated monoterpenes (25.44% and 28.04%) and phenylpropanoids (17.43% and 15.71%). The main constituents of both EOs were monoterpene alcohol linalool (13.68% and 15.38%), phenoyl derivate eugenol (10.83% and 8.97%) and sesquiterpene hydrocarbon α-bergamotene (8.12% and 9.25%). In both EOs, epi-bicyclosesquiphellandrene was detected in considerable amount (7.03% and 8.07%). The most abundant compound classes in the third plant were oxygenated monoterpenes (52.07%), sesquiterpene hydrocarbons (24.27%) and phenylpropanoids (10.95%). Linalool was the dominant compound (40.97%), followed by epi-bicyclosesquiphellandrene (8.70%) and estragole (7.92%).

The composition of hydrodistilled essential oils of the fresh aerial parts at the blooming stage of *O. basilicum* L. (four chemovariants), *O. tenuiflorum* L., *O. gratissimum* L. and *O. kilimandscharicum* Guerke in India was analyzed and compared by using capillary gas chromatography (GC/FID) and GC/MS (Padalia et al., 2014). Phenyl propanoids (up to 87.0%) and monoterpenoids (up to 83.3%) were prevalent constituents distributed in the studied *Ocimum* taxa. The major constituents of the four distinct chemovariants of *O. basilicum* were estragole (86.3%), estragole (61.5%)/linalool (28.6%), citral (65.9%) and linalool (36.1%)/citral (28.8%). Eugenol (66.5% and 78.0%) was the major constituent of *O. tenuiflorum* and O*. gratissimum*. Eugenol (34.0%), β-bisabolene (15.4%), (E)-α-bisabolene (10.9%), estragole (10.2%) and eucalyptol (8.2%) were the major constituents of *O. kilimandscharicum*.

The amount of various alkenylbenzenes, such as estragole, methyleugenol, safrole and analogues, was shown to vary greatly among traditional Chinese medicines samples (capsules, tablets or powders), for instance with about five times higher levels of some compounds in one product than in others (Ning et al., 2018).

No publications were found that had examined the levels of the various individual active substances in the plants in tea as drunk, i.e. extracted in the water, or that had examined the levels in food supplements.

Factors affecting the composition of essential oils in basil plants

The content of individual chemical substances in the essential oil in holy basil and other basil plants can vary depending on many factors; the species, cultivar, origin, age, height and part of the plant, environmental conditions such as harvesting season, agronomic techniques, spectral light composition used, type of fertilizers, use of elicitors and other conditions in root cultures and extraction methods and other processing of the final plant product (Sestili et al., 2018). Also several chemotypes, haplotypes or morphotypes of these plants are described. Examples of these factors are shown in the publications that follows.

Leaves and flowers from fourteen varieties of *Ocimum tenuiflorum* L. grown and harvested in Georgia, USA, during the 2015 and 2016 seasons, were compared (Fuller et al., 2018). The main active compound in the holy basil essential oil fraction, eugenol, was quantitated and compared for each variety. Overall, there were significant differences in harvestable weights and essential oil yields among the varieties, and a significant effect of growing season. The eugenol content was highly variable among the varieties examined, with higher eugenol contents in 2016 than in 2015. Both estragole and methyleugenol were found to be in high, but varying, levels throughout the year in essential oils from *O. basilicum* and *O. sanctum* grown in Malaysia, respectively (Vani et al., 2009). Eugenol was found in much lower levels in both plants and only in one of five harvest months, i.e. in November for *O. basilicum* and in June for *O. sanctum*, however, the two plants were analyzed in different years. When *Ocimum sanctum* L. (purple variety) was collected throughout the year in India, it was found that the level of methyleugenol was high in November (76.78 ± 0.54%) versus in April (55.48 ± 0.36%) (Joshi and Sharma, 2021). In April, the accumulation of sesquiterpenes increased and phenylpropanoids decreased.

While the effect of diurnal variability on sampling on essential oil ratios did not appear in five different basil genotypes (species not stated), it caused very significant differences on essential oil components (Aygun et al., 2022). In some genotypes, the content of the essential oil varied more among the plant parts than between the time of harvest.

Türkmen et al. (2021) examined the variation in essential oil content and components in five different genotypes of *Ocimum basilicum* L. harvested in different phenological periods (pre-flowering, full-flowering and post-flowering periods). When the basil was harvested in the pre-flowering period, it could be harvested three times. In plants left to full-flowering, basil genotypes reached full-flowering twice in one vegetation period. The plants left to the post-flowering period were harvested once. The content of essential oil in the plants with multiple harvests was higher in the second and third harvests compared to the first harvest. The essential oil ratios obtained from the leaves were higher than from the flowers. Rana et al. (2021) reported that the content and levels of compounds in essential oil of aerial parts of *Ocimum sanctum* L. varied between the vegetative and the full blooming stage, but not in a consistent way, and were also affected by altitude, temperature and soil physiochemical properties of the collection sites. Kholiya et al. (2022) found that both growth stage (phenological stage), i.e. higher in the full flowering stage than in the seed formation stage, and plant density affected the essential oil yield from *Ocimum basilicum* L.

Various parts, i.e. the stems and leaves of the *Ocimum basilicum* plant, demonstrated differential phytochemistry that could be correlated with bioactivity variations, more notably in anti-oxidant and anti-inflammatory properties (Bensaid et al., 2022). In ethanol extracts, the total phenolic content was significantly higher in leaf extract than in stem extract. In aqueous extracts, the

absolute value of the total phenolic content was higher in leaves than in stems, but the difference was not significant.

Pinto et al. (2019) found that the cropping season affected the concentration and chemical composition of the essential oil (EO) when comparing the leaves of 24 genotypes, consisting of 20 cultivars and 4 hybrids of *Ocimum basilicum* L., cultivated in different cropping seasons, dry season and rainy season, in Brazil. The essential oil content ranged from 0.66% to 3.21% in the dry season and from 0.80% to 4.20% in the rainy season. The major compounds found among the genotypes were linalool, methyl chavicol, neral, geranial, eugenol and methyl (E)-cinnamate, defining the formation of five groups in each season, classified in the following chemotypes (i.e. distinct groups of chemical composition of EO): methyl chavicol (Group 1), citral (neral + geranial) (Group 2), methyl cinnamate (Group 3), linalool (Group 4), and intermediate linalool (Group 5).

Biosynthesis of essential oil compounds was induced by abiotic stresses in *Ocimum tenuiflorum* L.f. plants by Nguyen et al. (2022). Although this basil grows well in tropical, subtropical and warm temperate regions, cold stress at 15°C and drought stress for five days did not affect normal plant growth. The stress treatments stimulated an increase in major essential oil components, eugenol, methyleugenol and β-caryophyllene, at both the juvenile and pre-flowering stages. The essential oil compound accumulation was related to an increase in eugenol *O*-methyltransferase (OtEOMT) transcript levels.

Many biotechnological methodologies are now used for genetic improvement of production of secondary metabolites in *Ocimum sanctu*m L., such as micropropagation, direct or indirect *in vitr*o regeneration, secondary metabolite enhancement by different types of elicitors, somatic embryogenesis and genetic transformation by various gene transfer approaches like *Agrobacterium tumefaciens*, particle bombardment etc. (Kumari et al., 2022). Genetic engineering for improvement of production of secondary metabolite and its biosynthesis pathways by overexpressing vital genes involved in the metabolite biosynthesis is also used.

Cultural practices influence the formation of essential oils in basil plants. Thokchom et al. (2020) assessed the effect of the arbuscular mycorrhizal fungus (*Rhizophagus intraradices*) inoculation on the production of essential oil in two genotypes of *Ocimum tenuiflorum* L. An arbuscular mycorrhiza is a type of mycorrhiza in which the symbiont fungus penetrates the cortical cells of the roots of a vascular plant forming arbuscules (tree-like structure). Colonization by this fungus resulted in significant increase in the leaf biomass, and up to 59.01% and 86.95% increase in essential oil concentration in the two genotypes of plants, respectively, in comparison with control. Concentration of eugenol, β-caryophyllene and other terpenoids increased in the essential oils of mycorrhizal plants.

Close correlations were found between some essential oil compounds in plants grown under different light spectra. The effects of light spectra on bioactive compounds depended on variety and type of compound. Therefore, through exposure to specific light spectrum it is possible to alter bioactive compound composition in basil plants. For instance, GC-MS analysis showed that essential oil compounds in both green and purple varieties of *O. basilicum* were influenced by spectral light composition (Hosseini et al., 2018). In green basil, chemical composition of essential oil was improved by growing them for 30 days under 70:30 % red:blue light, while in the purple variety red light induced production of limonene, α-pinene and β-myrcene.

Light modification by colour shade net manipulation affects the synthesis of bioactive compounds and also affect quantity and quality of essential oils in basil plants. Using Sweet basil (*O. basilicum* L. cv. 'Genovese') grown in the soil under net-house cover by pearl and red nets (50% shade index) or in unshaded condition (open field-control), it was found that the lowest accumulation of essential oils was observed in the second harvest from unshaded control plants (1.02 mL/100 g), while the highest oil accumulation was achieved in first harvest from red nets (3.23 mL/100 g)

(Milenković et al., 2019). The main constituents found in the oil were linalool (46.7–53.9%), eugenol (9.7–20.9%), 1,8-cineole (8.7–15.3%), epi-α-cadinol (3.3–4.5%) and α-trans-bergamotene (2.2–3.4%). Plants grown under blue shade nets from the second harvest were characterized by the highest eugenol content (20.9%).

High biomass yields of medicinal and aromatic crops are obtained by conventional chemical fertilizers, whereas the production of EOs is more influenced by elicitors that act on secondary metabolic pathways, thus, affecting the content of bioactive substances, as was shown in field trials with *O. basilicum* L. (Burducea et al., 2018). Several types of fertilizers; biosolids from municipal wastewater treatment plants, organic fertilizer based on granulated poultry manure, fertilizer based on microorganisms and a chemical fertilizer were shown to affect the composition of various EOs differently.

Due to low quantities of ursolic acid and eugenol, the major secondary metabolites of *O. tenuiflorum* L., attempts were made to apply biotechnological approaches to increase the content of these compounds (Sharan et al., 2019). Effects of three elicitors, namely yeast extract, methyl jasmonate and salicylic acid, were studied on the hairy root cultures induced by infecting leaves of *O. tenuiflorum* with *Agrobacterium rhizogenes*. The yield of ursolic acid and eugenol varied with the age of the culture, the concentration of the elicitor and the incubation time. The 17-day-old hairy root cultures when treated with yeast extract (50 mg/L) produced the highest amount of ursolic acid of 1.56 mg/g dry weight (5.6-fold higher than control culture) and eugenol content of 0.41 mg/g dry weight (~6.0-fold higher than the control) after 8 days of exposure. Methyl jasmonate at 60 mg/L concentration also enhanced the accumulation of ursolic acid up to 1.43 mg/g dry weight (5.0-fold higher than the control) and eugenol up to 0.1 mg/g dry weight (1.55-fold higher than the control) after 8 days of elicitor treatment. Increased elicitor concentration and extended exposure time as well as age of the culture resulted in reduced accumulation of ursolic acid and eugenol in hairy root cultures.

Different extraction techniques may give different extraction yields of bioactive compounds from basil plants, depending on processing conditions, including solvent polarity, concentration of solvent, temperature and solvent to sample ratio applied. For instance, Soxhlet extraction required 2 hours, sample to solvent ratio of 1:10 and temperature at 60°C to obtain the highest yield (1.7%) of *O. gratissimum* extract, while ultrasonic-assisted extraction (UAE) showed highest yield of *O. gratissimum* extract at a sample to solvent ratio at 1:10 with yield 20.6% using 60% methanol as the best solvent for extraction (Rasit et al., 2019).

Chemical compounds in herbal preparations such as spices may be affected by the processing. They may be degraded by drying, irradiation and microwave treatment, and roasting, frying and stewing may affect the toxicity or effects of traditional herbal medicines (Ning et al., 2018). The large variation found in hot water extraction efficiency of alkenylbenzenes in herbal teas seemed to be related to the form of the teas, as teas made of whole fruits resulted in lower extraction efficiency than grinded leaves or fine cut material (Ning et al., 2018).

Shalaby et al. (2020) compared the quality of *Ocimum basilicum* L. leaves volatile oil dried by indirect mode forced convection solar dryer (IMFCSD) integrated with thermal storage material with the traditional air drying method. The usage of the indirect solar dryer resulted in higher abundances of 31 volatile organic compounds. There was no significant difference in the abundance of methyleugenol, however, there were significant differences in the abundance of most minor components in the favour of IMFCSD. The findings indicated that the IMFCSD is better than natural air drying because of the controlled and stable drying.

The boiling time may affect the bioactivity of herbs such as *Ocimum sanctum* L. in herbal teas. Salamatullah et al. (2021) showed that that *Ocimum sanctum* L. displayed the highest total

polyphenol content, total flavonoid content and antioxidant activity when it was boiled for 5 min, and the lowest total polyphenol content when it was boiled for 15 min.

Jürges et al. (2018) examined whether the chemical diversity underlying the medicinal use of *O. tenuiflorum* had a genetic component, and whether this was detectable using genetic barcoding markers. Based on four plastidic markers, several haplotypes within *Ocimum* could be detected. Haplotype II was congruent with *O. tenuiflorum*, while haplotype I extended over several members of the genus and could not be resolved into genetically separate subclades. The vernacular subdivision of *O. tenuiflorum* into three types (Rama, Krishna and Vana) could only be partially linked with genetic differences; Rama and Krishna were assigned to *O. tenuiflorum*, while Vana belonged to haplotype I. This genetic difference was mirrored by differences in the profiles of secondary compounds.

Differences in the essential oil content, chromosome count and morphology led to consider *O. gratissimum* as a polymorphic complex species often subcategorized into subspecies, varieties and forms. Characterization of O*. gratissimum* complex was carried out by Kumar et al. (2019) using morphological, molecular markers combined with chemical analysis of essential oils in an attempt to resolve the complexity. The molecular marker inter-simple sequence repeat (ISSR) generated a total of 341 loci, of which 290 loci were polymorphic, depicting 84.28% polymorphism. The efficiency of ISSR's markers was revealed through principal component analysis plot that grouped all the accessions according to the morphotypes irrespective of their geographical locations. Chemo profiling of essential oils revealed eugenol (38.6-79.2%) and thymol (47.6-50.7%) as the major essential oil constituents in *O. gratissimum*. Pearson correlation coefficient (r = 0.482) suggested a moderate correlation between the genetic markers and essential oil content, thus depicting that genetic markers may be linked to the essential oil constituents.

The aroma, flavour and pharmaceutical effects of *O. sanctum* L. are caused by its essential oils, which contains most of the monoterpenes and sesquiterpenes. Kumar et al. (2018) showed that *O. sanctum* L. has 81 putative terpene synthase genes (*Osa*TPS), of which 47 were putatively functional genes, 19 partial *Osa*TPS genes and 15 probably pseudogenes. Phylogenetic analysis revealed that these genes could be classified in sub-family clusters TPS-a, -b, -c, -e, -f and –g. The *Osa*TPS gene family is one of the largest gene family of specialized secondary metabolites in the *Ocimum* species.

Essential oils obtained from plants such as oregano, cinnamon and clove with antimicrobial activity are demonstrated to increase the shelf life of various foods such as bread, meat or fish when incorporated into a food packaging material. However, Becerril et al. (2019) showed that microorganisms such as *Aspergillus flavus* and *Escherichia coli* present in food were able to metabolize some of the main compounds released from the essential oils, such as linalool, eugenol and cinnamaldehyde, thus reducing their concentrations and producing new substances such as benzene propanol, cinnamyl alcohol, methyleugenol and styrene. As these substances could be harmful for consumers, more studies are needed in order to guarantee the safety of active materials containing essential oils.

Comment:
These examples of various factors that affect the composition of EO in various basil plants demonstrate the need to control the plant material going into production of teas and food supplements in terms of the content and concentration of active substances, some of which may be harmful to human health above certain levels. It points to the responsibility of manufacturers of such products to analyse, confirm and describe the content of their products in order to secure their safety.

Contamination of the plant material with chemical contaminants, radionuclides or microorganisms

Dinu et al. (2020) studied effects of **heavy metals** on plant growth and metal accumulation in a laboratory experiment where *O. basilicum* L. was cultivated on unpolluted soil and on soil polluted with Cd, Co, Cr, Cu, Ni, Pb and Zn, using inductively coupled plasma optical emission spectrometry. The plants grown in the polluted soil had a visible increase of biomass and intensified green pigment in the leaves. The metals gathered differently in the plant organs: Cd, Co, Cr and Pb accumulated in the roots, while Cu, Ni and Zn accumulated in the flowers. Cr and Pb exceeded the toxic levels in roots. Also, the heavy metal intake depended on the plant development stages; thus, Cd, Cr and Pb accumulated more in mature plant leaves. The Cd and Pb contents were higher than the World Health Organization (WHO) and European Commission (EC) permissible limits. Translocation from roots to flowers and to leaves was observed for Cu, Ni and Zn, indicating a competition between metals. The calculated bioaccumulation factors were insignificant, but Cd and Pb concentrations exceeded the legal limits in the mature plants, thus, being restricted for human or animal consumption.

Heavy metals may also accumulate in plants from natural sources, such as the soil or contaminated irrigation water. Two varieties of *O. sanctum* from a local nursery in Andhra Pradesh, India, were all shown to have levels of Zn, Cu, Mn, Cr, Fe, Pb and Cd within the permissive levels of WHO (Himakar et al., 2018). In contrast, the amounts of Pb (5.59 mg/kg) and Cd (1.38 mg/kg) were exceeding the highest acceptable limits stated in the Codex Alimentarius of the Slovak republic (Pb 5 mg/kg, Cd 0.5 mg/kg) in samples of *O. basilicum* L. from all of three local markets (Harangozo et al., 2018). The content of Cu and Zn was under the highest acceptable limits, 25 mg/kg and 80 mg/kg, respectively. Thus, high heavy metal content may in some cases be an additional risk factor in herbal plants and their preparations.

Hydroponically grown basil (*Ocimum basilicum*) contained no heavy metals except Cu, present at permissible levels, whereas soil-grown basil had content of Pb and Cr above permissible levels as well as Cd and Cu at or below such levels, respectively (Sathyanarayana et al., 2021). The hydroponically grown basil contained no pathogenic bacteria, yeasts or moulds.

Florea et al. (2021) examined transfer of heavy metals in different tea preparations of *Ocimum basilicum* L. and other plants, i.e. infusion 15 min., decoction 15 min. and cold maceration 12h. Pb, Cd, Cu and Cr exceeded the maximum residue levels in the basil plant. The transfer rate varied between the three aqueous extracts for the various metals and plants. In general, using the classical infusion (adding boiling water over raw plant material and then infuse for 15 minutes) was the most suitable method to prepare teas with low transfer of the studied metals. High transfer rate of metals was obtained in cold maceration preparation, being in some situations close to the initial concentrations in plants. Thus, quality control is necessary, so that, by implementing stricter legislation/monitoring programs at national level, the risk of contamination with toxic metals can be reduced.

Zunaidi et al. (2023) found that Mn accumulated more in the leaves than in the stem and root of *Ocimum tenuiflorum* L. Cd, Fe and Mn translocated from the root to the stem, and Cd, Co, Cu, Fe, Mn and Zn were translocated from the root to the leaves.

Biira et al. (2021) measured levels of the naturally occurring **radionuclides** in leaves of *Ocimum gratissimum* (African basil) and found levels of 10.02, 7.37 and 364.99 Bq/kg of ^{226}Ra, ^{232}Th and ^{40}K, respectively.

No publications were found studying content of **pesticide residues** in herbal preparations, but obviously it could be a potential problem with improper use of pesticides or if **pathogenic bacteria** were present after production under unhygienic conditions.

Comment:
It is recommended that the manufacturers of herbal teas and dietary supplements should monitor the chemical (heavy metals, radionuclides and pesticides) and microbial contaminants potentially present in the plant material, ensuring their presence below maximum regulatory limits.

Exposure characterization

Exposure to the plant material

According to information from NFSA, the content of dried plant material of holy basil in products on the Norwegian market may be 0.6-2.0 g per tea bag. Based on this information and using a default body weight of 60 kg, one cup per day of holy basil tea made with one tea bag will give an exposure of approximately 10-33 mg/kg bw per day. This is based on the assumption that all of the active substances are extracted by and ends up in the hot water, as a worst-case scenario due to lack of more specific data. The estimated intake will be approximately 20-67 and 30-100 mg/kg bw per for 2 and 3 cups of this tea per day, respectively (Table 1).

Further, according to information from NFSA, the recommended daily doses of food supplements (dried plant material) varied from 60-360 mg in one product (Food supplement 1) and from 800-1800 mg in another product (Food supplement 2). Based on a default body weight of 60 kg, these two food supplements would give an estimated exposure of 1-6 mg/kg bw or 13-30 mg/kg bw per day of the dried plant (Table 1).

Table 1. Estimated worst-case exposure to holy basil preparations (dried plant material) in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	**1 cup of tea**	**2 cups of tea**	**3 cups of tea**	**Food supplement 1**	**Food supplement 2**
Tea bag	10-33	20-66	30-99	-	-
Food supplement	-	-	-	1-6	13.3-30

Exposure to individual substances in basil plants

In addition to the estimated exposure, synonyms, CAS no. and sources of the individual constituents in basil plants are given in this chapter.

In the following estimations of exposure to the individual substances in basil plants from teas and food supplements, the available data considered most suitable, although not always entirely comparable, were used.

Methyleugenol

Methyleugenol has synonym 4-allyl-1,2-dimethoxybenzene and the CAS no. is 93-15-2 (PubChem, 2020).

Methyleugenol is a natural constituent of essential oils in more than 450 plant species widely used in foodstuffs and as flavouring agents, including basil, bay laurel leaves, nutmeg, pimento, lemongrass, tarragon, basil, star anise and fennel (SCF, 2001a; Prinsloo et al., 2018). Methyleugenol is present in up to 86% in dry leaf extracts of *O. tenuiflorum* L. (WHO, 2002; EFSA ESCO Report, 2009) and in 15-100 ppm (mg/kg) of the plant and 50 ppm in the leaves of *O.*

tenuiflorum, 13-1400 ppm in the plant *O. basilicum* L. and 9835 ppm in shoots of *O. gratissimum L.* (EMA, 2005a). Methyleugenol was found in the highest level of all 16 substances detected with GC-MS in the essential oil from *O. sanctum* throughout the year in Malaysia, with varying amounts at different months (Vani et al., 2009).

Methyleugenol is regulated for use as a flavouring agent in jellies, baked goods, non-alcoholic beverages, chewing gums, relish and ice cream, and as fragrance in several cosmetic products (Prinsloo et al., 2018).

The content of essential oil in the leaves of basil plants is up to 2% and methyleugenol is present in up to 86% in the essential oil of dry leaf extracts of *O. tenuiflorum* L./*O. sanctum* L. (WHO, 2000; EFSA, 2014a). For instance, one cup of tea will give 10-33 mg/kg bw per day of the dried plant material (Table 1), which contains 2% essential oils, which again contains 86% of methyleugenol, giving 0.17-0.57 mg/kg bw per day of methyleugenol.

Exposure to methyleugenol can then be estimated to be as shown in Table 2.

Table 2. Estimated exposure to methyleugenol in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	**1 cup of tea**	**2 cups of tea**	**3 cups of tea**	**Food supplement 1**	**Food supplement 2**
Tea bag	0.17-0.57	0.34-1.14	0.52-1.70	-	-
Food supplement	-	-	-	0.02-0.10	0.23-0.52

Estragole

Estragole is a phenylpropanoid (Burcul et al., 2020) and has the synonyms methyl chavicol and 1-allyl-4-methoxybenzene, and CAS no. 140-67-0 (PubChem, 2020).

Estragole is found in many plant species and their essential oils, such as tarragon, sweet basil (*Ocimum basilicum*), sweet fennel, anise and star anise, among others (Prinsloo et al., 2018). In the leaves of *O. tenuiflorum* L., it was reported 39950 ppm (mg/kg) of estragole, and in essential oil of *O. basilicum* L. it was reported 5-85% of estragole and in the plant it was reported 238-8780 ppm of estragole (EMA, 2005b). It was reported 0.8% essential oil in *O. basilicum* L. and 20-89% estragole in this essential oil, and approximately 0.4% estragole in the part of *O. basilicum* used (EMA, 2014). Estragole was found in the highest level of all 15 substances detected with GC-MS in the essential oil from *O. basilicum* throughout the year in Malaysia, with varying amounts at different months (Vani et al., 2009).

Estragole is used as flavour and fragrance in many foods and food products, in cosmetics such as perfumes, soaps and detergents, and in pharmaceuticals (Prinsloo et al., 2018).

The content of essential oil in the leaves of basil plants is up to 2% and estragole is present in up to 25% in the essential oil of the whole plant (stems, leaves and flowers) of *O. tenuiflorum* L./*O. sanctum* L. (Zheljazkov et al., 2008; EFSA, 2014a).

Exposure to estragole can then be estimated to be as shown in Table 3.

Table 3. Estimated exposure to estragole in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	1 cup of tea	2 cups of tea	3 cups of tea	Food supplement 1	Food supplement 2
Tea bag	0.05-0.17	0.10-0.33	0.15-0.50	-	-
Food supplement	-	-	-	0.005-0.03	0.07-0.15

Eugenol

Eugenol is a phenylpropanoid (Burcul et al., 2020) and has synonyms eugenic acid and 4-allyl-2-methoxyphenol, and CAS no. 97-53-0 (PubChem, 2020).

Eugenol is a component of the defense system of plants and is also a signal molecule between plants and microbes (Prinsloo et al., 2018). In addition to its presence in foods containing basil, such as pesto and other sauces, and in spices (nutmeg and clove), it is also used in fragrances, dentistry (in mouthwash, dental cement, temporary fillings etc.) and in traditional medicine (Martins et al., 2018).

The content of essential oil in the leaves of basil plants is up to 2% and eugenol is present in up to 62% in the essential oil of dry leaf extracts of *O. tenuiflorum* L./*O. sanctum* L. (WHO, 2000; EFSA, 2014a).

Exposure to eugenol can then be estimated to be as shown in Table 4.

Table 4. Estimated exposure to eugenol in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	1 cup of tea	2 cups of tea	3 cups of tea	Food supplement 1	Food supplement 2
Tea bag	0.12-0.41	0.25-0.82	0.37-1.23	-	-
Food supplement	-	-	-	0.01-0.07	0.16-0.37

Eucalyptol

Eucalyptol is a bicylic monoterpenoid with synonym 1,8-cineole (Burcul et al., 2020) and CAS no. 470-82-6) (PubChem, 2020).

Eucalyptol is used as flavouring and in over-the-counter drugs (SCF, 2002; Bhowal and Gopal, 2015).

The content of essential oil in the leaves of basil plants is up to 2% and eucalyptol is present in up to 25% in the essential oil of the whole plant (stems, leaves and flowers) *O. tenuiflorum* L./*O. sanctum* L. (Zheljazkov et al., 2008; EFSA, 2014a).

Exposure to eucalyptol can then be estimated to be as shown in Table 5.

Table 5. Estimated exposure to eucalyptol in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	1 cup of tea	2 cups of tea	3 cups of tea	Food supplement 1	Food supplement 2
Tea bag	0.05-0.17	0.10-0.33	0.15-0.50	-	-
Food supplement	-	-	-	0.005-0.03	0.07-0.15

β-Caryophyllene

β-Caryophyllene is a bicyclic sesquiterpenoid (C_{15}) in the isoprene biosynthesis pathway in plants (Burcul et al., 2020; Bastaki et al., 2020). It has a synonym (-)-trans-caryophyllene and CAS no. 87-44-5 (PubChem, 2020). β-Caryophyllene oxide (also called β-caryophyllene epoxide (Bastaki et al. (2020)) has CAS no. 1139-30-6, β-caryophyllene epoxide (synonym caryophyllene α-oxide) has CAS no. 13877-94-6 and β-caryophyllene alcohol (BCPA) has CAS no. 472-97-9 (PubChem, 2020).

The content of essential oil in the leaves of basil plants is up to 2% and β-caryophyllene is present in up to 2.4% in the essential oil of the whole plant (stems, leaves and flowers) *O. tenuiflorum* L./*O. sanctum* L. (Zheljazkov et al., 2008).

Exposure to β-caryophyllene can then be estimated to be as shown in Table 6.

Table 6. Estimated exposure to β-caryophyllene in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	1 cup of tea	2 cups of tea	3 cups of tea	Food supplement 1	Food supplement 2
Tea bag	0.005-0.02	0.01-0.03	0.01-0.05	-	-
Food supplement	-	-	-	0.0005-0.003	0.006-0.01

Ursolic acid

Ursolic acid is a pentacyclic triterpene acid of the ursane type, with synonym 3β-3-hydroxy-urs-12-en-28-oic acid and CAS no. 77-52-1 (PubChem, 2020).

Ursolic acid is widely spread in herbs, flowers, fruits and vegetables and can be isolated from various medicinal plants, including plants in the *Lamiaceae* family (López-Hortas et al., 2018). The content of ursolic acid varies between the different parts of plants, and is particularly high in leaves, flowers/inflorescences and in fruit peel versus in the roots, stems and rhizomes, also being dependent on developmental stage, environmental conditions, seasonal variations and pathogen infections. The content of ursolic acid in leaves of *Ocimum sanctum* was found to be 0.4% (w/w) (Chan et al., 2019). Among eight *Ocimum* species grown in the northeastern Brazil, the ursolic acid content in leaves after maceration and ethanol extraction was highest in *O. tenuiflorum* (2.02%), in *O. gratissimum* 1.04%, and in three variants of *O. basilicum* the content ranged from 0.27% to 0.38% (Silva et al., 2008).

The content of ursolic acid was 2% in leaves after maceration and ethanol extraction in *O. tenuiflorum* L./*O. sanctum* L. (Silva et al., 2008; EFSA, 2014a). This value for used to calculate exposure, lacking a value for aqueous extraction.

Exposure to ursolic acid can then be estimated to be as shown in Table 7.

Table 7. Estimated exposure to ursolic acid in tea or food supplements on the Norwegian market (range, in mg/kg bw per day).

Holy basil product	1 cup of tea	2 cups of tea	3 cups of tea	Food supplement 1	Food supplement 2
Tea bag	0.20-0.66	0.40-1.32	0.60-1.98	-	-
Food supplement	-	-	-	0.02-0.12	0.27-0.60

Hazard identification and characterization

Hazards from the plant material and preparations and extracts thereof

Human studies

From previous risk assessments

Two clinical studies were reported in WHO (2002), in which no adverse reactions had been reported. The first study by Agrawal et al. (1996) examined the effects of treatment with holy basil leaves on fasting and postprandial (i.e. after eating a meal) blood glucose and serum cholesterol levels in 40 patients (25 men and 15 women, age 41-65 years (mean 52.5) with non-insulin-dependent diabetes mellitus (NIDDM) in a randomized, placebo-controlled, crossover single blind trial in India. The patients apparently consumed leaves of *O. sanctum* and *O. album*, stated to be two closely related species of holy basil, in sachets of dried leaf powder made from 2.5 g of fresh leaves. The placebo was spinach leaf powder treated the same way. The powder of apparently one sachet was dissolved in 200 ml of water and drunk daily on an empty stomach early in the morning. After a five day run-in period where all subjects consumed the holy basil leaves, twenty patients consumed the holy basil leaves for four weeks and the other twenty patients consumed the spinach leaves, thereafter their exposure was changed to the opposite exposure for the next four weeks. Results indicated a significant decrease in fasting and postprandial blood glucose levels during treatment with holy basil leaves compared to during treatment with placebo leaves. The mean cholesterol levels showed a mild reduction during the basil treatment period. The compliance was excellent and no adverse effects of holy basil or placebo were observed. In the second study by Sharma (1983), which was without controls, oral administration of an aqueous extract of dried Folium Ocimi Sancti (leaves of *O. sanctum* L.) to 20 patients with asthma increased lung vital capacity and relieved laboured breathing, without reporting of adverse effects.

Saxena et al. (2012) tested the efficacy of an extract of the whole plant *Ocimum tenuiflorum* Linn. (OciBest™) in a randomized double-blind, placebo-controlled study of management of general stress. The participants of both sexes (aged 18-65 years) received either capsules of OciBest™ as 1200 mg of actives (term not explained) per day (n = 71) or placebo (microcrystalline cellulose) (n = 79) for six weeks. None of the patients from the groups given OciBest™ or placebo reported any adverse effects.

Comment:
There was no information about how adverse effects were recorded during the study.

In a double-blind randomized controlled trial, Mondal et al. (2011) studied effects on the immune system of healthy men and women (age 18-60 years) of 300 mg of a 70% ethanol extract of *O. sanctum* Linn. leaves or placebo (sucrose) in similar looking capsules for four weeks on an empty stomach, followed by a wash-out period of three weeks before the cross-over to the next

treatment. Twelve persons were given placebo first and basil extract after, ten persons got the treatment in the opposite order. Statistically significant increases in the levels of IFN-γ and IL-4, and percentages of T-helper cells and NK-cells, were observed after exposure to the basil extract vs. the placebo group, showing that the basil extract had an immunomodulatory role. No significant adverse effects of the intervention were noted amongst study individuals during the study period of 11 week except in two subjects. One of them complained of nausea while the other had loose motions, after the first visit to the laboratory. These two subjects could not complete the study and their data were excluded from the analysis. The compliance rate was >95%. To determine any side effects of Tulsi, biochemical parameters were also evaluated at the same time points. It was observed that intervention with basil extract did not cause significant changes in body mass index, blood pressure, fasting blood sugar, liver and renal function tests, however, the data were not shown.

Comments:
There was no information about how adverse effects were recorded in the study. It is not known if these changes in immune parameters are beneficial or could be adverse.

Jamshidi and Cohen (2017) reviewed 26 clinical studies of mostly *O. tenuiflorum* L. and *O. gratissimum* L. in humans (n = 3-200 participants, of which only four studies were in healthy persons). None of the studies described how adverse effects were collected. Seventeen of the 26 studies reported no adverse effects and 8 studies did not describe or refer to any adverse effects. One study reported occasional nausea in 16 obese adults taking 250 mg capsule of leaf extract before meals twice daily.

Comment:
It was noted that M. M. Cohen receives remuneration as a consultant and advisor to Organic India Pty. Ltd., which is a company that manufactures and distributes holy basil products.

From the literature searches
In a publication on a meta-analysis of six randomized clinical trials involving effects of holy basil (plant search terms *Ocimum*, *Ocimum sanctum*, *Ocimum tenuiflorum*, *Ocimum gratissimum*) on fasting glucose and lipid profile in adults with metabolic disease (Jamshidi et al., 2018), it was reported that overall holy basil was well-tolerated in intervention participants with no major adverse events reported. However, half of the included trials did not describe any adverse events, two studies did not find any adverse effects, and one study by Satapathy et al. (2017) where 16 adults (17-30 years, both sexes) received one 250 mg capsule of leaf extract of *Ocimum sanctum* Linn. twice daily for 8 weeks reported presence of occasional nausea (number of patients not specified). No other adverse effects were reported or no effects were found on the levels of the liver enzymes aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT) in this study.

In a publication documenting adverse reactions to herbal medicine on the island of Mauritius in the Indian Ocean, Mahomoodally et al. (2018) listed *Ocimum sanctum* L. with moderate or mild adverse reactions in two cases. After intake of a concoction made from six fresh and crushed leaves of the plant boiled in half a litre of water for 30 minutes taken for Type 2 diabetes as one cup (about 400 ml) once per day, severe dizziness was experienced. When six fresh leaves were boiled in 600 ml of water for 15 minutes to maintain general physical and mental health and drank as above, throat irritation (pharyngitis) was reported. The plant was given an index of severity of adverse reactions (ISAR score) of 0.333, listed with gastrointestinal disorders and nervous system disorders.

Lopresti et al. (2022) performed a randomized, double-blind, placebo-controlled trial investigating the effects of an *Ocimum tenuiflorum* hydroalcoholic extract of leaves (Holixer™) for

eight weeks on stress, mood and sleep in adults of both sexes experiencing stress (n = 100, age 18-65 years). The participants were given two capsules, taken twice daily, with the plant extract (in total 125 mg) or placebo. The tolerability of capsule intake was assessed every two weeks online regarding adverse effects believed to be associated with capsule intake. Participants were also asked to contact researchers if they experienced any adverse effects. Two participants withdrew from the study due to self-reported adverse effects associated with capsule intake; one person in the *Ocimum* group withdrew due to increased agitation, and one in the placebo group withdrew due to ongoing nausea. No serious adverse events were reported by participants and the frequency of adverse effects was similar in both groups (12 on plant extracts (digestive disturbances (5), headache (3), vivid dreams (1), skin rash (1), agitation (1), reduced libido (1)), 10 on placebo (digestive disturbances (5), headache (2), vivid dreams (1), skin rash (1), sleep disturbances (1)). There were no reports of any adverse events in 83% of participants.

Comments:
For a person with 60 kg body weight, the dose used in this study was 2.1 mg/kg bw per day. It is not known how comparable the extraction of active substances, and thus, the biological activity, is with this hydroalcoholic extract compared to an aqueous extract.

No other new human studies were found in the literature search that had a sufficient description of the holy basil preparation used and/or that had reported that they investigated adverse effects of the holy basil.

Mutagenicity and genotoxicity

Several preparations were made from fresh leaves of *O. sanctum* Linn. and tested for mutagenicity in *Bacillus subtilis* H17 (*rec*+) and M45 (*rec*-) strains with both the standard streak method and the cold method by Ungsurungsie et al. (1982). Washed and ground fresh leaves (crude form), mixed with water (10 g spice in 50 ml water) and either boiled for 1 hour and filtered giving the water-heated residue and the water-heated filtrate, the latter either remaining untreated or dried at 85°C before being dissolved as 0.5-1 g in 1 ml water, or either mixed with water and treated as above but macerated for 5 days at room temperature instead of being boiled and the water-macerated filtrate being separated from the water-macerated residue and dried at 45°C instead of 85°C. None of these preparations were mutagenic in either *Bacillus subtilis* H17 (*rec*+) or M45 (*rec*-) strains.

Chandrasekaran et al. (2013) investigated the possible genotoxic potential of the methanolic/aqueous extract of the whole plant *Ocimum sanctum* L. (OciBest™) using the *in vitro* genotoxicity tests bacterial reverse mutation test (Ames II™ test), chromosome aberration test in CHO-K1 cells and micronucleus (MN) test in CHO-K1 cells. The tests were carried out in compliance with the OECD test guidelines nos. 471, 473 and 487, respectively. The results showed that OciBest™ (7.9-2500 µg/ml) did not increase the number of histidine revertant colonies in *Salmonella typhimurium* strains (TA98 and TAMix) with and without exogenous metabolic activation (S9). OciBest™ (10-100 µg/ml) did not induce or increase the occurrence of structural chromosomal aberrations or MN formation, with and without S9, at the tested dose range in both 4-hour and 18-hour exposures. Thus, OciBest™ was not genotoxic in bacterial reverse mutation, chromosomal aberration and MN tests.

Comment:
In this publication, it was stated that the trademark of the product under study was owned by the company in which all the authors worked.

Acute, subacute and subchronic toxicity

Some new animal studies were found in the literature searches which were examining various positive effects of holy basil extracts (from *O. sanctum* L. or *O. tenuiflorum* L.) for 2-6 weeks, but

the authors did not mention if they had looked for adverse effects or not. In addition, since the content of the extracts examined was inadequately described and there were issues with lack of proper control groups, use of other treatments in addition to the plant extracts or insufficient numbers of animals per group, these studies were not useful in this risk assessment and were not included.

The acute, subacute and subchronic toxicity studies of the basil plant material are summarized in Table 8 and described in more detail below the table.

Table 8. Acute, subacute and subchronic toxicity studies with oral exposure to various preparations of O. tenuiflorum L./O. sanctum L.

Study	Species/strain/sex	Effects	Plant material	Dose/day (Control group)	Extract	NOAEL/ Comments
Acute toxicity						
Lagarto et al. (2005)	Adult Wistar rats, males and females (n = 3/sex)	No significant toxic effects were reported	Extract of *O. tenuiflorum* L.	A single dose of 2000 mg/kg bw given by gavage, observed for 14 days	Lyophilized aqueous extract	Performed according to OECD guideline no. 423.
Shetty et al., 2008	Adult Wistar albino rats, males and females (n = 3 or 6/sex?)	The doses up to 4 g/kg bw did not produce any toxicity or mortality. No further information on the results of the higher doses	Leaves of *O. sanctum* Linn. (shade-dried and powdered)	Increasing doses of from 400 mg/kg bw up to 6 g/kg bw of the extracts orally for 14 days	Both aqueous (distilled water) and alcoholic extract (95% ethanol), diluted in the vehicle gum acacia in saline	The acute toxicity study is only explained very briefly and the text in the publication is not clear.
Chanda et al. (2013)	Adult Charles Foster (CF) rats, females (n = 5)	No effects on body weight or the limited serum biochemical parameters measured with any extracts, treated rats were inactive and drowsy for 30 min., but recovered after 2 h (not stated with which extract(s))	Three different leaf extracts of *O. sanctum* Linn.	Single oral dose of 2 g/kg, observed for 7 days, controls were given distilled water or the respective vehicles	Aqueous, hydro-alcoholic (50% v/v ethanol), alcoholic (95% v/v ethanol), the last two extracts given in carboxymethyl cellulose (CMC)	Claimed to be performed according to OECD guideline no. 423, but there are deviations from this guideline, i.e. only observed for 7 days instead of 14 days.

Chandrasekaran et al. (2013)	Adult Wistar albino rats, females (n = 5)	Normal body weight development, no treatment-related toxic effects	Whole plant *O. sanctum* L. (OciBest™)	Single gavage of 5 g/kg, observed for 14 days, controls were not mentioned	Extract made with methanol and water	Performed according to OECD guideline no. 420.
Gautam and Goel (2014)	Adult Swiss albino mice, males and females (n = 6/sex)	No deaths, adverse symptoms or effects on the central nervous system or the autonomous nervous system observed with any dose	Leaf extract of *O. sanctum* L.	Single oral administration of 200, 600 and 2000 mg/kg bw suspended in 0.5% CMC in distilled water (vehicle), observed for 14 days, controls were given 0.5% CMC in distilled water	50% ethanolic extract	Performed according to OECD guideline no. 423.
Widjaya et al. (2021)	Adult male Wistar rats (n = ?)	No toxic effects compared with controls were reported	Extract of *O. sanctum*	1500, 3000 and 5000 mg (per kg bw?). Administration way and duration of exposure and observation not stated	96% ethanol extract from macerated dried *O. Sanctum* mixed with 2.0% solution of CMC	
Jayapal et al. (2022)	Adult Wistar albino rats, females (n = 3)	No effects with 300 mg/kg bw. With 2000 mg/kg bw, total white blood cell count and basophil count were	Essential oil of *O. sanctum* L.	A single dose of 300 and 2000 mg/kg bw given by gavage,	Hydro-distilled extract	Performed according to OECD guideline no. 423. LD50 was >2000 mg/kg bw

		higher, and blood creatinine, blood direct bilirubin and serum SGPT were lower, than in controls. Two rats showed transient hind-limb paralysis. One rat had focal peripheral inflammation and hemorrhage in the liver. Another rat had focal hemorrhage and mild steatosis in the liver, a few congested glomerulus and sparse interstitial lymphatic infiltration in the kidneys and mild interstitial inflammation in the myocardium. No rats died		observed for 14 days		
Subacute toxicity						

Chanda et al. (2013)	Adult Charles Foster (CF) rats, females (n = 5)	Significantly increased body weight and serum triglycerides with 300 mg/kg bw at day 28 with aqueous extract, and significantly decreased SGPT with 50 and 300 mg/kg bw at day 28 with the alcoholic extract	Three different leaf extracts of *O. sanctum* Linn.	Oral doses of 5, 50 or 300 mg/kg, given for 28 days, controls were given distilled water or the respective vehicles	Aqueous, hydro-alcoholic (50% v/v ethanol), alcoholic (95% v/v ethanol), the last two extracts given in CMC	The study was claimed to be performed according to OECD guideline no. 407, but there are deviations from this guideline, i.e. only five females were used per group, instead of five rats of each sex per group. NOAEL was indicated at 50 mg/kg bw and 5 mg/kg bw per day for the aqueous and alcoholic extracts, respectively.
Gautam and Goel (2014)	Adult Charles-Foster albino rats, males and females (n = 3/sex)	No mortality or changes in behaviour, body weight, relative organ weights, food and water consumption, hematological and biochemical profiles, no abnormalities in gross examinations or histopathology	Leaf extract of *O. sanctum* L.	200, 400 and 800 mg/kg bw suspended in 0.5% CMC in distilled water (vehicle), 10 ml/kg bw, given daily by gavage for 28 days, controls were given 0.5% CMC in distilled water	50% ethanolic extract	Performed according to OECD guideline no. 407. NOAEL was considered 800 mg/kg bw by the authors. The study was claimed to be performed according to OECD guideline no. 407, but n = 3 of each sex instead of 5, the data were given for both sexes together instead of separately, not all the recommended organs were collected, including accessory sex organs, and there were not macroscopic and histopathological examinations of all the

						organs mentioned in the guideline.
Raina et al. (2015)	Adult Wistar rats, males and females (n = 5/sex)	No changes in body weight, food and water consumption, motor activity, sensory reactivity or foot splay measurements. No significant changes in hematological, pathological and biochemical parameters, or histopathology of tissues (liver, kidney, spleen, heart and testis/ovary) in either sex, with a few exceptions not considered treatment-related, no clinically significant changes at macroscopic and microscopic levels.	Whole plant extract of *O. sanctum* L. (probably OciBest™) suspended in de-mineralized water	250, 500 and 1000 mg/kg bw per day by gavage for 28 days, and a recovery groups given 1000 mg/kg bw per day or distilled water as control, however, the length of the recovery period was not stated, controls were given vehicle distilled water (n = 5)	Methanolic/ aqueous extract	The study was claimed to be performed according to OECD guideline no. 407, but the data were not given with sufficient decimals after comma, making group comparisons impossible, several data were given as 0.0 ± 0.0 for organ weights, thyroid weight data was lacking and this guideline has not sufficient sensitivity to identify substances with weak (anti)androgenic or (anti)oestrogenic modes of action. NOAEL was considered 1000 mg/kg bw by the authors. Three of the authors of this publication work in the company owning the trademark OciBest™, although they declared no conflict of interest in this publication.
Widjaya et al. (2021)	Adult male Wistar rats (n = ?)	No toxic effects compared with	Extract of *O. sanctum*	100, 300 and 500 mg (per kg	96% ethanol extract from	

		controls were reported		bw?). Administration way and duration of exposure and observation not stated	macerated dried *O. Sanctum* mixed with 2.0% solution of CMC	
Subchronic toxicity						
Lagarto et al. (2005)	Adult Wistar rats, males and females (n = 9-10/sex)	No significant toxic effects attributable to the test substance, with absence of mortality and normal weight increase, food consumption, hematology, biochemistry, relative organ weights and histopathology of organs and tissues. A few significant differences vs. controls were observed; decreased % of neutrophiles and increased % of lymphocytes with 500 and 1000 mg/kg only in males, increased urea with 1000 mg/kg and increased glucose with 500 and 1000 mg/kg only in females	Extract of *O. tenuiflorum* L.	250, 500 and 1000 mg/kg bw per day, given for 90 days	Lyophilized aqueous extract, oral exposure	Performed according to OECD guideline no. 408. The authors considered 250 mg/kg bw per day as the NOAEL. The study was written in Spanish with only the abstract in English, thus, all details were not evaluated in this publication.

		(considered more pharmacological than toxic effects), and increased ALAT only in 500 mg/kg dose only in females				
Chronic toxicity						
Widjaya et al. (2021)	Adult male Wistar rats (n = ?)	No toxic effects compared with controls were reported	Extract of *O. sanctum*	100, 300 and 500 mg (per kg bw?). Administration way and duration of exposure and observation not stated	96% ethanol extract from macerated dried *O. Sanctum* mixed with 2.0% solution of CMC	

Lagarto et al. (2005) studied **acute** and **subchronic (90 days) toxicity** (stated to be according to OECD guidelines nos. 423 and 408, respectively) in adult male and female Wistar rats of a lyophilized aqueous extract of *Ocimum tenuiflorum* L. In the oral **acute toxicity study**, a single dose of 2000 mg/kg bw was given by gavage and the rats (n = 3/sex) were observed for 14 days. For the **subchronic toxicity study**, doses of 250, 500 and 1000 mg/kg bw per day were administered for 90 days (n = 9-10/sex). Toxicity and body weight were evaluated for both assays. In the **subchronic toxicity test**, the food consumption, hematology (hemoglobin, hematocrit, erythrocyte count, total and differential leucocyte count) and clinical biochemistry (glucose, alanine aminotransferase, aspartate aminotransferase, cholesterol, urea nitrogen, total bilirubin and creatinine) was evaluated. Very few significant differences vs. controls were observed; decreased percentage of neutrophiles and increased percentage of lymphocytes were observed with 500 and 1000 mg/kg only in males, increased urea level with 1000 mg/kg and increased glucose level with 500 and 1000 mg/kg were observed only in females, which were considered more pharmacological than toxic effects, and increased alanine aminotransferase (ALAT) only in the 500 mg/kg dose only in females. Gross necropsy and histopathological examinations of organs and tissues (heart, kidney, liver, spleen, brain, lung, stomach, intestine, thymus, adrenals, thyroid/parathyroid, pancreas, salivary glands, cervical ganglion, testicles, seminal vesicles, prostate and ovaries) were carried out and relative weight of each organ was determined (heart, kidney, liver, spleen, brain, lung, thymus, adrenals, prostate, testicles and ovaries). The acute and subchronic toxicity tests showed no significant toxic effects attributable to the test substance, with absence of mortality and normal weight increase, food consumption, hematology, biochemistry, relative organ weights and histopathology of organs and tissues. The authors considered the lowest dose (250 mg/kg bw per day) as the NOAEL in the subchronic study.

Comment:
The study was written in Spanish with only the abstract in English, thus, all details were not evaluated in this publication.

Shetty et al. (2008) performed an **acute toxicity study** of both an aqueous and an alcoholic extract of the leaves of *Ocimum sanctum* Linn., shade-dried, powdered and dissolved in either distilled water or 95% ethanol, giving about 20-25% and 10-15% yield, respectively. The extracts were diluted in the vehicle gum acacia in saline and given to adult Wistar albino rats of both sexes (n = 3 or 6/sex?) via an i.g. tube. The rats were dosed orally with increasing doses of 400 mg/kg bw up to 6 g/kg bw of the extracts for 14 days. The doses up to 4 g/kg bw did not produce any toxicity or mortality. No further information on the results of the higher doses was given.

Chanda et al. (2013) performed oral **acute** and **subacute toxicity studies** of three different leaf extracts of *O. sanctum* Linn. (aqueous, hydroalcoholic (50% v/v ethanol), alcoholic (95% v/v ethanol) given as single oral administrations of 2 g/kg bw to adult female Charles Foster (CF) rats (n = 5) and observed for 7 days. The last two extracts were given in carboxymethyl cellulose (CMC). The controls were given distilled water or the respective vehicles. In the **acute studies**, no significant effects on body weight or the limited serum biochemical parameters measured (creatinine, glutamate oxalacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides) were seen with any extracts. The treated rats were inactive and drowsy for 30 minutes, but recovered after 2 hours (not stated with which extract(s)). Gross pathology showed no observable changes. In the **subacute studies**, the adult female CF rats (n = 5) were treated with 5, 50 or 300 mg/kg bw dose for 28 days with the same extracts as in the acute study. The same parameters as in the acute studies were measured. Gross pathology showed no observable changes. With the aqueous extract, body weight and serum triglycerides were significantly increased with 300 mg/kg bw at day 28, and with the alcoholic extract, SGPT was significantly decreased with 50 and 300 mg/kg bw at day 28 (based on the Result tables),

indicating NOAEL at 50 mg/kg bw and 5 mg/kg bw per day for the aqueous and alcoholic extracts, respectively.

Comments:
The acute study was claimed to be performed according to OECD guideline no. 423, but there are deviations from this guideline, i.e. the rats were only observed for 7 days instead of 14 days. The subacute study was claimed to be performed according to OECD guideline no. 407, but there are deviations from this guideline, i.e.at least 10 animals (five female and five male) should have been used at each dose level.

Chandrasekaran et al. (2013) investigated **acute toxicity** of the methanolic/aqueous extract of the whole plant *Ocimum sanctum* L. (OciBest™) suspended in demineralized water in adult female Wistar albino rats (n = 5) treated orally with a single gavage with 5 g/kg of OciBest™ and observed for signs of toxicity for 14 days. The study was carried out in compliance with the OECD test guideline no. 420. The treatment caused no mortality, abnormal clinical signs or any significant gross pathological changes at necropsy. The body weight gain was regarded as normal. The authors conclude that the results did not show any treatment-related toxic effects in the rats.

Comment:
In the publication, it was stated that the trademark of the product under study was owned by the company in which all the authors work.

Gautam and Goel (2014) studied the **acute** and **subacute toxicity** of orally administered 50% ethanolic leaf extract of *Ocimum sanctum* Linn (OSE). The extract was prepared from 500 g of dried, crushed and powdered leaves in 1 litre of 50% ethanol for 3 days and then filtered, and the whole procedure was repeated twice. The obtained extracts were pooled and dried, and contained about 5% (w/w) of OSE. In the **acute toxicity study**, performed according to OECD guideline no. 423, four groups of adult male and female Swiss albino mice (n = 6/sex) were treated once with doses of 0, 200, 600 and 2000 mg/kg bw OSE suspended in 0.5% CMC in distilled water (vehicle and negative control), and general behaviour, adverse effects and mortality were recorded for up to 14 days. In the **subacute toxicity study**, claimed to be performed according to OECD guideline no. 407, adult male and female inbred Charles-Foster albino rats received OSE (with the same vehicle and negative control as in the acute study) by daily gavage at the doses of 200, 400 and 800 mg/kg bw per day (n = 3/sex) for 28 days, and biochemical, hematological and histopathological changes in tissues (liver, kidney, spleen, heart and testis/ovary) were determined. OSE did not produce any adverse symptoms, deaths or effects in the central nervous system or the autonomous nervous system in the acute toxicity test. Subacute treatment with OSE did not show any change in body weight, relative organ weights (per 100 g bw), food and water consumption, and hematological and biochemical profiles. In addition, no change was observed in macroscopic and microscopic investigations of vital organs (liver, spleen, kidney, heart and testis/ovary) in the rats. The highest dose (800 mg/kg bw per day) was considered the NOAEL in the subacute study by the authors.

Comment:
This subacute study has several weaknesses, i.a. n = 3 of each sex per group, which should have been 5 according to OECD guideline no. 407, the data were reported for both sex together instead of separately, not all the recommended organs were collected, including accessory sex organs, and the macroscopic and histopathological examinations were lacking for some of the organs mentioned in the guideline.

Raina et al. (2015) examined **subacute toxicity** of a methanolic/aqueous preparation of the aerial parts of *O. sanctum* Linn. (assumed by the description to be what is called OciBest™ in other publications by the same authors) in adult male and female Wistar rats according to OECD

guideline no. 407. The rats received *O. sanctum* extract (OSE) by oral gavage at the doses of 250, 500 and 1000 mg/kg bw per day (n = 5/sex) for 28 days. The vehicle used as negative control was distilled water (n = 5). Recovery groups were given 1000 mg/kg bw per day or distilled water as control, however, the length of the recovery period was not stated. The effects of OSE on clinical, hematological, biochemical and histopathological parameters were evaluated. The rats treated with OSE did not show any change in body weight, food and water consumption, motor activity, sensory reactivity or foot splay measurements. There were no significant changes in hematological, pathological and biochemical parameters, or histopathology of tissues (liver, kidney, spleen, heart and testis/ovary) among rats of either sex, with a few exceptions. The dose 1000 mg/kg OSE showed significant increase in hemoglobin (16.5 ± 1.5 vs. 14.1 ± 0.4 g/dl) in males and an increase in mean corpuscular hemoglobin (MCH) (19.8 ± 0.8; 18.7 ± 0.5, in pg) and mean corpuscular hemoglobin concentration (MCHC) (41.8 ± 1.1; 39.3 ± 0.7 in g/dl) in male and female rats, respectively, in comparison to their respective controls (MCH: 17.7 ± 0.3; 17.4 ± 0.3; MCHC: 37.8 ± 0.5; 36.1 ± 0.2). However, these small and transient differences were not significantly different vs. control in the high dose (1000 mg/kg) recovery group, and were not considered biologically significant. In the female rats only, significantly decreased aspartate aminotransferase (AST) and chloride were seen with 500 mg/kg, decreased γ-glutamyltranspeptidase (GGT) level with 500 and 1000 mg/kg and decreased glucose with 250 mg/kg, which were not observed in the high dose recovery group and not considered biological significant. Urine parameters (appearance, blood, nitrate, leucocyte, glucose, ketone, pH, protein and specific gravity) in both male and female rats were comparable to their respective controls. In addition, no clinically significant changes were observed in the vital organs of rats at macroscopic and microscopic levels. Thus, the increase in absolute adrenal weight with high dose and in relative adrenal weight with low dose of OSE in females were considered incidental. Even though a mild consolidation of lungs was observed (at which doses was not stated), the histopathological analysis did not reveal any microscopic changes, and thus, it was considered to be incidental/spontaneous and not attributed to the treatment. The authors stated that the results showed that oral administration of OSE was not toxic to male and female Wistar rats up to the highest dose tested (1000 mg/kg bw per day) for 28 days, thus, this dose was considered the NOAEL.

Comments:
However, this study has several short-comings. The data were not given with sufficient decimals after comma, making it impossible to compare the numbers from the various groups. In addition, there were several data that were given as 0.0 ± 0.0 for organ weights, for instance for the adrenals in Tables 5a and 6b. The data on thyroid weight was lacking, which should have been included for studies of potential endocrine disrupting substances according to OECD guideline no. 407. This test guideline has not sufficient sensitivity to identify substances with weak (anti)androgenic or (anti)oestrogenic modes of action. It was also noted that three of the authors of this publication work in the company owning the trademark OciBest™, although they declared no conflict of interest in this publication.

Widjaya et al. (2021) investigated **acute, subacute and chronic toxicity** of a 96% ethanol extract from macerated dried *Ocimum Sanctum* mixed with 2.0% solution of carboxymethyl cellulose in male Wistar rats. The doses in the acute toxicity study were 1500, 3000 and 5000 mg (per kg bw?), and in the subchronic and chronic studies 100, 300 and 1000 mg. No effects were observed in the acute toxicity study on morbidity or mortality, behavioural changes, other symptoms or blood chemistry markers with any doses compared with controls. Also in the subacute and chronic toxicity studies, no significant effects were observed in any doses versus controls. Histopathological assessment on sections of the liver, kidney, heart, pancreas, lung and lymph in light microscopy did not reveal anatomical abnormalities in any organ sections in any dosage groups compared to the control group.

Comments:
Which parts of the plant used were not stated in this publication. The doses, administration way and duration of exposure, as well as number of rats per group, in the acute, subchronic and chronic studies were not, clearly or at all, reported in this publication.

Jayapal et al. (2022) investigated **acute toxicity** of the essential oil of *Ocimum sanctum* L. at 300 and 2000 mg/kg bw given by oral gavage to female Wistar albino rats according to the OECD Guideline 423 for testing of chemicals (n = 3/group). The 14-day acute oral toxicity test showed that hydrodistilled *Ocimum sanctum* L. essential oil was not toxic at 300 mg/kg bw but has some toxic effects at 2000 mg/kg bw. With this dose, total white blood cell count and basophil count were higher, and blood creatinine, blood direct bilirubin and serum glutamate pyruvate transaminase (SGPT) were lower, than in controls. Two rats showed transient hind-limb paralysis. One rat had focal peripheral inflammation and hemorrhage in the liver or another rat had focal hemorrhage and mild steatosis in the liver. This last rat also showed a few congested glomerulus and sparse interstitial lymphatic infiltration in the kidneys and mild interstitial inflammation in the myocardium. However, no animals died during the 14-day acute oral toxicity test with 2000 mg/kg bw and therefore the LD5 dose of *Ocimum sanctum* L. would be more than 2000 mg/kg bw in female Wister albino rats.

No general toxicity was reported in mice after a 50% ethanol extract of the leaves of *O. sanctum* Linn. was injected once either i.p. (1 g/kg bw) (Dhar et al., 1968) or i.d. (10 g/kg bw) (Mokkhasmit et al., 1971, cited in WHO, 2002).

Carcinogenicity

No long-term carcinogenicity studies were found on the plant *O. tenuiflorum/O. sanctum*. Safety concerns of this plant exists regarding the promutagenic and procarcinogenic alkenylbenzenes such as methyleugenol and estragole contained in the plant. However, in *O. basilicum* L. data indicated that the tumourigenic potential of alkenylbenzenes may be counteracted by other basil plant matrix constituents such as the polyphenol nevadensin, and it was stated that consumption of this and probably other basil plants in food is safe (Sestili et al., 2018). Regarding essential oils containing genotoxic and carcinogenic alkenylbenzenes used in semi-purified food supplements, the situation may be different (see the Chapter *Hazard from individual substances in basil plants*). According to Müller et al. (2021), nevadensin was a selective topoisomerase I poison. In the Comet assay, an increase in DNA strand breaks, a strong arrest in G_2/M cell cycle phase and caspase activation were found with nevadensin treatment of HT29 cells. DNA topoisomerases are essential nuclear enzymes, highly associated with DNA integrity.

Reproductive and developmental toxicity

Reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as adverse effects on development of the offspring. Adverse effects on sexual function and fertility include changes in the structure and function of the male and female reproductive systems and modifications in any other functions that are dependent upon the integrity of the reproductive systems. Adverse effects on development of the offspring means any effects of chemicals which interfere with normal development of the conceptus either before or after birth, which are induced during pregnancy or results from parental exposure. Adverse effects on or via lactation are also included in reproductive toxicity, although classified separately from other reproductive effects (US OSHA, 2020).

Quite a high number of studies of effects on reproduction in experimental animals of various preparations of *O. tenuiflorum* or *O. sanctum* were available since this plant has been studied as a natural contraceptive, especially in India, based on its traditional use as a human abortifacient, as mentioned in many publications (i.a. Kamboj, 1988; EFSA, 2014a). Since the potential reproductive toxicity effects for humans based on observations in several animal experiments

were the main concern regarding the safety of this plant, also older reproductive toxicity studies were included for completion, in addition to the few new studies found in the literature searches.

The reproductive and developmental toxicity studies of the plant material are summarized in Table 9, and described in more detail below the table.

Table 9. Reproductive and developmental toxicity studies with exposure to various preparations of O. tenuiflorum L./O. sanctum L. and related basil plants.

Study	Species/strain/sex	Effects	Plant material	Dose/day* (Control group)	Extract used	Comments
Saha and Kasinathan (1965)	Adult? albino rats (strain?), males (n = ?)	Impaired spermatogenesis, antifertility effects	*O. sanctum* leaves in diet	?	?	Full text publication not available.
Vohora et al. (1969)	Adult albino rats (strain?), females (n = 5)	No implantation sites found in the reported percentage of rats: 1) 60%, 2) 40%, on GD10, not **teratogenic**	*O. sanctum* L. leaves	1) 100 mg/kg bw on GD 1-4, 2) 200 mg/kg bw on GD 1-7, no controls included	Aqueous extract	Implantation loss observed with aqueous extract.
Batta and Santhakumari (1970)	Adult rats (strain?), females (n = 5-7)	Loss of implantation sites reported for benzene extract was 80%, for petroleum ether (both doses) 60%, for ether 20%, for acetone 40% and for ethanol 43%. Fetal loss within normal range, normal sex ratio, no **teratogenic** effects	*O. sanctum* leaves, air-dried under shade and extracted, dried extract reconstituted in various solvents	Petroleum ether (100 and 200 mg/kg bw), benzene (200 mg/kg bw), ether (100 mg/kg bw), acetone (100 mg/kg bw), ethanol (100 mg/kg bw), all exposed on GD1-5 and implantations sites examined on GD10, controls were given vehicles	Petroleum ether, benzene, ether, acetone or ethanol used as extractants	Varying loss of implantation sites with various organic extracts, benzene being the worst.

				without the extracts		
Kasinathan et al. (1972)	Adult albino mice (strain?), males (n = 10 or 15)	Decreased weight of testes, prostate gland and adrenal gland, impaired spermatogenesis, hypertonic environment in seminal vesicles, all males infertile when mated with fertile females, similar results after 30 or 90 days	10% fresh leaves of *O. sanctum* L. in food for 30 or 90 days	465 mg leaves per day, controls (n = 5) given the same diet without *O. sanctum* leaves	No extract used	The results were not tested statistically.
Seth et al. (1981)	Adult rats (strain?), males (n = 5, 7, 10)	Mean testes weight: 150 mg/kg significantly higher and 200 mg/kg significantly lower than controls (and 100 and 150 mg/kg), mean sperm count: all groups significantly lower than controls (all doses statistically similar on average)	Shade-dried leaves of *O. sanctum* orally for 15 days	100, 150 and 200 mg/kg bw, controls (n = 6) were given the same concentrations of propylene glycol, not benzene	Cold benzene, dissolved in propylene glycol	
Khanna et al. (1986)	Adult albino rats (strain?), males and females (n = 6-16)	4000 mg/kg bw: estrous cycle progressively disturbed with time, loss of pregnancies and fertility, with no mating in the 3. month, without **congenital malformations** in pups, 2000 mg/kg bw: similar effects, but to a lesser degree, 2000 and 4000 mg/kg bw: significantly	Shade-dried powdered leaves and soft stem of *O. sanctum* L. in pellets (2.5 g) along with normal diet for three months	200, 2000 and 4000 mg/kg bw, controls (n = 10) were given 0.9% saline	No extract used	Effects seen also when no extract was used. Large difference in effects of 4000 mg/kg bw vs. 2000 or 200 mg/kg bw (dose-response).

		decreased sperm count, percentage of motile sperm and the absolute weight of male reproductive organs, mostly with a dose-response. 200 mg/kg bw: some significant differences from controls (decreased testes weight, slightly reduced sperm count and percentage of motile sperm)				
Kantak and Gogate (1992)	Adult Wistar rats, males (n = 8)	Mean values of sexual behaviour scores (grooming – 1 point (p), pursuit - 2 p, mount – 3 p, intromission – 4 p, ejaculation – 5 p) significantly decreased with 200 and 400 mg/kg bw per day. No structural changes in testis, epididymis and seminal vesicles were seen	Extract of shade-dried leaves of *O. sanctum* L. ground with food, given for 15 days	100, 150, 200 and 400 mg/kg bw per day, controls (n = 8) were given the same diet without *O. sanctum* leaves	Type of extract not stated	Reduced sexual mating behaviour in males.
Reghunandanan et al. (1995)	Adult Wistar rats, males (n = 20)	Sperm count and glutamyl transpeptidase (GTP) activity (index of Sertoli cell function) significantly decreased, lactate dehydrogenase (LDH) (marker of germ cell	Powder of extracted shade-dried leaves of *O. sanctum* L. dissolved in 8 parts of propylene	300 mg/kg bw of the extract, controls (n = 20) were given vehicle, not benzene	Benzene extract	The extract reduced spermatogenesis by impairment of Sertoli cell function without affecting the germ cells.

		function) was not significantly affected	glycol, 1.0 part of water and 0.1 parts of ethanol as vehicle, given by i.p. injection, terminated 48 h later			
Reghunandan et al. (1997)	Adult albino rabbits (strain?), males and females (5 treated males and 7 treated females, and 3 of each sex as controls, were used for histology, 2 treated males and 4 treated females were mated)	Histopathological changes in the reproductive organs of males (testis and epididymis) and females (uterus and ovaries), and infertility that was reversed in one month, producing litters of normal size	Freshly plucked tender *O. sanctum* leaves	1 g/kg bw of fresh leaves twice a week for one month, controls were given only the normal diet	The leaves were given in addition to normal diet	
Panda and Kar (1998)	Adult Swiss albino mice, males (n = 10)	No significant effects on body weight, but the relative weights (g/100 g bw) of all the sexual organs were significantly increased	*O. tenuiflorum* L. leaf extract	500 mg/kg bw per day, based on the dry weight, given orally by gastric intubation for 15 days	Aqueous extract of shade-dried leaves (yield 10-12% of air-dried powder), dissolved in distilled water	
Sardessai et al. (1999)	Adult rats (strain?), females (n = 15)	Estrous cycle disrupted after the administration of extract and finally the rats showed only the diestrus phase, Lordosis quotient (LQ, a standard index of	Extract of fresh *O. sanctum* Linn. leaves	Leaf extract 80 mg in 15 ml of 5% glucose per day for two weeks, thereafter only	Type of extract not stated	

		the relative sexual receptivity of a female in the presence of males) decreased in 2. week and remained suppressed further after the leaf extract was replaced by water for two weeks, indicating lasting effect on female mating behaviour		water for 2 weeks, controls (n = 15) were the same rats before given the leaf extract		
Ahmed et al. (2002a)	Adult Wistar albino rats, males (n = 5)	Significantly decreased total sperm count, total number of motile sperm, forward velocity of sperm and fructose levels in cauda epididymal fluid and seminal plasma, and a significant increase in percentage of abnormal sperm. All effects partially recovered after 8 days and fully recovered after 16 days	*O. sanctum* L. leaves shade-dried, powdered, extracted and mixed with propylene glycol (1 ml per day)	250 mg/kg bw extract for 48 days, or with 8 or 16 days recovery after 48 days exposure, controls (n = 5) received only propylene glycol, not benzene	Benzene extract	The gradual recovery after treatment stopped indicated that the extract had reversible anti-fertility effects in males.
Ahmed et al. (2008)	Adult Wistar albino rats, males (n = 10, except for fertility test (n = 5))	Decreased relative weights of testis, epididymis and seminal vesicles, reduced total count, cell diameters and nuclei diameters of germ cells and Leydig cells, and cauda epididymis were affected (principal, clear and basal cells were highly disturbed). Fertility	*O. sanctum* L. leaves shade-dried, powdered, extracted and mixed with propylene glycol (1 ml per day)	250 mg/kg bw extract for 48 days, or with 8 or 16 days recovery after 48 days exposure, controls (n = 10 or 5) received only	Benzene extract	The results indicated reversible anti-spermatogenic and anti-fertility effects in males.

		test showed no implantations in any female rats on GD8. After withdrawal of treatment, partial recovery after one week and complete recovery after two weeks were seen, resulting in normal spermatogenesis and fertility based on number of implantations, number and body weight of pups		propylene glycol, not benzene		
Leigh and Fayemi (2008)	Adult Wistar albino rats, males (n = 6)	Mild to severe congestion and edema with the low dose as well as germinal tissue erosion of the seminiferous tubules with the high dose, significant spermatozoa abnormalities with both doses	Fifty grams of fresh leaves of *O. gratissimum* L. blended in 100 ml distilled water and filtered through filter paper to crude extract	5 x 10^{-4} and 10 x 10^{-4} ml/kg bw of the filtrate given thrice weekly or once daily? (both stated in the publication) for 5 weeks, controls (n = 6) received distilled water	Aqueous extract	The findings indicated anti-fertility properties.
Ahmed et al. (2009a)	Adult Wistar albino rats, males (n = 5)	Cauda epididymis had significant reduction in epithelial height and nuclei diameter of epithelial cells, the nuclei were pycnotic and the height of stereocilia reduced and	*O. sanctum* L. leaves shade-dried, powdered, extracted and mixed with propylene	250 mg/kg bw extract for 48 days, controls (n = 5) received the same volume of polypropylene	Benzene extract	The results showed reduced male fertility, which the author stated could be caused by reduction in androgen status (however, hormones were not

		principal, clear and basal cells were highly disturbed. The lumen was devoid of sperm and filled with lymphocytes and debris of degenerated sperm. Fertility test showed no implantations in any female rats on GD8	glycol (1 ml per day)	glycol, but not benzene		measured) or a direct effect on cauda epididymis and the sperm.
Ahmed et al. (2009b)	Adult Wistar albino rats, males (n = 5)	Decrease in total sperm count, sperm motility, forward velocity and increase in the percentage of abnormal sperms in a dose-dependent manner after all three doses, most of the sperms appeared morphologically abnormal, also dose-dependently	*O. sanctum* L. leaves shade-dried, powdered, extracted and mixed with propylene glycol	150, 200 or 250 mg/kg bw in 1.5, 2.0 and 2.5 ml of polypropylene glycol for 15 days, controls (n = 5) received the same volume of polypropylene glycol, but not benzene	Benzene extract	The authors hypothesized that the adverse effects on the sperm may have resulted from a general disturbance in the proteins and alteration in cauda epididymal milieu.
Sethi et al. (2010)	Adult albino rabbits, males (n = 10)	Significant decreased sperm count, marked increased testosterone level, but decreased follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in serum	Fresh leaves of *O. Sanctum L.* in standard diet for 30 days	2 g/rabbit per day, controls (n = 10) received standard diet	No extract used	The results suggested an effect on reproduction in male rabbits.
Ahmed et al. (2011) (partly the same results	Adult Wistar albino rats, males (n = 5)	Decrease in sperm count, sperm motility, forward velocity and increase in	*O. sanctum* L. leaves shade-dried,	250 mg/kg bw in 1 ml of polypropylene	Benzene extract	The adverse effects on the sperm may have resulted from a general

as presented in Ahmed et al. (2009b))		abnormal sperm, with morphologically changes in plasma membrane and acrosomal membrane of spermatozoa. Fertility test showed no implantations in any female rats on GD8	powdered, extracted and mixed with propylene glycol	glycol for 48 days, controls (n = 5) received the same volume polypropylene glycol, but not benzene		disturbance in the proteins and alteration in cauda epididymal milieu.
Pragya et al. (2012)	Adult Swiss albino mice, males (n = 6)	Significantly decreased sperm counts, motility of spermatozoa and pH in seminal plasma, significantly increased mortality of spermatozoa, for all time periods	Leaf extract of *O. sanctum* L. fed for 10, 20 or 30 days	250 mg/kg bw (0.1 ml extract), controls (n = 6) given distilled water	Aqueous extract	Fertility was not tested.
Mankapure et al. (2013)	Adult Wistar albino rats, males (n = 5)	Duration-dependent decrease in the relative wet weight of testis, caput epididymis and cauda epididymis and deranged histoarchitecture of the testis and epididymis After recovery, 80% of normal organization of seminiferous tubules, spermatids and spermazoa appeared, indicating that spermatogenesis was partially restored	Pellets of leaves and soft stems of *O. sanctum* L., for 24, 48 or 72 days, or treatment for 72 days and recovery up to 120 days	4000 mg/kg bw, controls (n = 5) as pellets	Pellets also contained wheat flour, groundnut oil and honey, which were also given to the controls (n = 5), no extract used	The treatment with pellets of the plant material appeared to affect testicular and epididymal structure and the effects were partially reversible. No statistical testing was performed.
Verma et al. (2016) (apparently partly the same	Adult Swiss albino mice, males (n = 6)	Significantly decreased sperm counts and motility of spermatozoa, and significantly increased	Leaf extract of *O. sanctum* L. fed for 10, 20, 30, 40 or 50	250 mg/kg bw (0.1 ml extract), controls (n = 6)	Aqueous extract	Adverse effects on sperm were observed. Fertility

data as in Pragya et al. (2012))		mortality of spermatozoa, for all time periods. Decreased weight of reproductive organs after 20-50 days treatment	days, and a recovery group (50 days treatment, further 90 days without treatment)	were given 0.1 ml distilled water		was reversed to normal after 90 days recovery.
Srinivasulu and Changamma (2017)	Adult Wistar rats, males (n = 6)	Significantly decreased weight of testes, epididymis, seminal vesicles, significant reductions in sperm count and motility, significantly increased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, and significantly decreased testosterone levels	*O. sanctum* L. leaves dried, crushed, powdered and extracted	500 mg/kg bw per day for 20 days, controls received distilled water for 20 days (n = 6)	Extracted with 95% ethanol for 3 days	Adverse effects on sperm were observed and hormone levels were affected.
Raina et al. (2018)	Adult Wistar rats, males (n = 12), females (n = 24)	1000 mg/kg did not induce any adverse effects on the reproductive performance of male and female rats. No mortality or major signs of clinical toxicity were seen. The data and gross pathological and histopathology observations in parents did not reveal any treatment-related adverse	Extract of the whole plant *O. sanctum* Linn. (OciBest™), solubilized in distilled water at a volume of 10 ml/kg	250, 500 and 1000 mg/kg bw, males treated 12 weeks before and during the mating period (29 days), females treated 2 weeks before mating until the end of	Aqueous/methanolic extract	One-generation reproductive toxicity study performed after OECD guideline no. 415 and GLP. The authors concluded that the NOAEL was 1000 mg/kg bw, both for F0 and F1. Five of the authors of this publication work in the company owning the trademark OciBest™.

		effects in F0. No treatment-related adverse effects were seen in F1 offspring. Histopathology revealed that for 250, 500 and 1000 mg/kg OciBest™, the numbers of pups that showed histopathological changes were 7, 12, and 3, respectively, which were considered incidental (hepatocyte degeneration, necrosis, congestion in the brain and alveoli)		lactation, controls (n = ?) given 10 ml/kg of distilled water		
Poli and Challa (2019)	Adult Wistar albino rats, females (n = 6)	Testosterone levels were significantly decreased and serum progesterone and estradiol levels increased, the tissue somatic index (w/w %, per 100 g bw) was increased significantly in ovary, uterus and vagina	*O. sanctum* Linn. leaf extract	500 mg/kg bw per day for 15 days	Apparently an alcoholic extract (no further information)	The study was not performed according to OECD guidelines for reproductive studies, such as guideline no. 415: One-Generation Reproduction Toxicity Study. The study did not clearly demonstrate the relationship between the hormone profiles and the changes in estrous cycle, and the rats were not mated to test their fertility, thus, it is uncertain whether the observed small changes in estrous cycle may affect the reproduction.

*Administration route was oral (by gavage, as pellets or mixed in the diet), except in Reghunandanan et al. (1995), given i.p., or was not stated, but was likely oral (Vohora et al., 1969; Batta and Santhakumari, 1970).

Saha and Kasinathan (1965) investigated the effects on **fertility and impaired spermatogenesis** of leaves of *Ocimum sanctum* along with normal diet fed to male albino rats (strain not known). The dose per day and how the leaves were prepared is not known, since the full text publication was not available. According to other publications referring to this publication, the results suggested that failure in pregnancies might be due to the impairment of spermatogenesis.

Vohora et al. (1969) tested an aqueous extract of leaves of *Ocimum sanctum* Linn. in female albino rats (strain and administration route not stated, n = 5). Of five rats, three rats (60%) had **no implantation sites** on day 10 of pregnancy after a dose of 100 mg/kg bw on days 1-4 of pregnancy (one rat died during days 1-10 of pregnancy). One rat delivered pups (n = 6). In another set of five rats, two rats (40%) had no implantation sites on day 10 of pregnancy after a dose of 200 mg/kg bw on days 1-7 during pregnancy. Two rats delivered pups (both n = 10). None of the pups had evidence of any **teratogenic effects** up to the age of one month. The authors described the potential use of *O. sanctum* extract as encouraging regarding its abortifacient action.

Batta and Santhakumari (1970) studied **antifertility activity** in adult fertile female rats (strain and administration route not stated). Leaves of *O. sanctum* were air-dried under shade and the plant material was extracted with petroleum ether (doses 100 and 200 mg/kg bw per day, n = 5), benzene (200 mg/kg bw per day, n = 5), ether (100 mg/kg bw per day, n = 5), acetone (100 mg/kg bw per day, n = 5) and ethanol (100 mg/kg bw per day, n = 7). Control vehicles were obtained under similar conditions. Female rats in proestrus phase of the reproductive cycle were placed overnight with proved fertile male rats, and the next morning they were examined for the presence of spermatozoa in the vaginal smears (day 1 of pregnancy). These rats were administered the extracts from day 1-5 of pregnancy by oral administration. On day 10 of pregnancy, laparotomy was performed and the number of implantation sites was recorded. The abdominal cavity was sutured and the rats were allowed to deliver at full term. On the day of delivery, the number of pups and their sex were determined and the pups were examined for macroscopic teratogenic effects. Benzene extract of *O. sanctum* showed 80% antifertility activity (based on the number of implantation sites on day 10 of pregnancy) and petroleum ether extract of *O. sanctum* (both doses) showed 60% antifertility activity, whereas the other extracts showed less than 50% activity (ether 20%, acetone 40% and ethanol 43%). The vehicle did not show any antifertility activity. The fetal loss in dams given any of the extracts without *O. sanctum* leaves was within the normal range for this rat colony. The sex ratio of the pups was not changed with any of the extracts, with more females than males at birth. No macroscopic **teratogenic effects** were observed in any of the pups.

Kasinathan et al. (1972) investigated **reproductive toxicity effects** of 10% fresh leaves of *O. sanctum* L. ground with food (calculated as 465 mg leaves per day) for 30 (n = 10, treated; n = 5, controls) or 90 (n = 15, treated; n = 5, controls) days in adult male albino mice (strain not stated). Presumably, the controls were given the same diet without *O. sanctum* leaves. Testes, prostate gland and adrenal gland decreased in weight in the treated vs. controls. Upon histological examination of the testes, the spermatogonial cells were adhered together in some spaces of the tubular lumina, the spermatid bundles were not properly developed, the sperms were scattered all through the lumen and interstitial cells were sparse and degenerated, suggesting impairment of spermatogenesis in the treated mice. In the seminal vesicles, there was a significant decrease in pH, a reduction of mucoproteins (necessary to form the vaginal plug), increased alkaline phosphatase and acid phosphatase in the treated mice (which normally are low) and increased reducing substances (fructose, ergothioneine and glutathione), creating a hypertonic environment, possibly adversely affecting sperm motility. These changes were not seen in the testes. No changes were seen in sodium, potassium, calcium or citric acid levels. The histological and biochemical changes were the same after 30 and 90 days. The treated mice were mated with fertile females. Although vaginal plugs were noted in some females, no pregnancies occurred.

Comment:
The results were not tested statistically.

Seth et al. (1981) investigated effects of shade-dried leaves of *O. sanctum* extracted with cold benzene for 7 days in doses of 100, 150 and 200 mg/kg bw in propylene glycol given orally for 15 days on **anti-spermatogenic activity** in adult male rats (strain not specified) with proven fertility. The controls were given the same concentrations of propylene glycol. The numbers of rats per group were 6, 7, 5 and 10 for the controls and the three increasing doses, respectively. Using log-transformed data, the mean of the groups differed significantly in testes weight ($P < 0.01$) and sperm count ($P < 0.05$), but there were no significantly differences in weight of epididymis, seminal vesicle, prostate or vas deferens. In the case of testes weight, the mean value of the group with dose 150 mg/kg was significantly higher and that of the group with dose 200 mg/kg was significantly lower than the control group. The group with dose 200 mg/kg showed significant lower mean testes weight compared with the groups with doses 100 and 150 mg/kg. For sperm count, control mean was significantly higher than the mean values in the all the three treated groups ($P < 0.05$), while the three treated groups were not statistically different. The percentages of motile sperm were not evaluated statistically, but were zero in 6/7 (86%), 5/5 (100%) and 6/10 (60%) rats in the treated groups with increasing doses, respectively, whereas it ranged from 50-90% in 6/6 control rats.

Khanna et al. (1986) studied effects of shade-dried powdered leaves and soft stems of *O. sanctum* Linn. in 20, 200 and 400 mg/100 g bw (200, 2000 and 4000 mg/kg bw) of tulsi powder in pellets (2.5 g) along with the normal diet for three months (30 days are also mentioned, but the relevance is not clear) on the **reproductive performance** of adult male and female albino rats (strain not specified, n = 6-16/sex). The controls were apparently given 0.9% saline (n = 6-10/sex) (route not specified). Both males and females with proven fertility were given five chances to mate. Female rats given the 400 mg/100 mg bw for three months initially showed 4-6 normal estrous cycles, but as the treatment continued the pattern of estrous was disturbed and the estrous stage persisted for 5-10 days during which the rats did not mate. This situation became progressively worse, and during the second month of treatment 5/10 rats mated. Three of these rats went to term producing 1, 5 and 8 pups of an average birth weight (5.5 g) **without any congenital malformations**, whereas two rats lost their pregnancies. During the third month of the treatment none of the rats mated. With the 20 mg/100 g bw dose, one rat lost the pregnancy and the rest delivered normally with an average litter size. The weight of ovaries and uterus was not significantly reduced with the 400/100 g bw mg dose (n = 6). In the males, with the 200 and 400 mg/100 g bw doses they mated less frequently than controls (ca. 30-40% successful matings), with the 400 mg/100 g bw dose 61.5% of mated females went to term and resulted in average litter size of 4.7 ± 0.3 and mean birth weight of pups of 5.5 g. The 200 mg/100 g bw dose gave slightly better results than 400 mg for successful full-term pregnancies and litter size, but slightly lower results for percentage of successful matings (the statistical differences between 200 and 400 mg/100 g bw were not stated). The dose of 200 (n = 6) and 400 (n = 16) mg/100 g bw significantly decreased sperm count, percentage of motile sperm and the absolute weight of male reproductive organs (testes, epididymis, seminal vesicle and prostate), mostly with a dose-response. The 20 mg/100 g bw dose also significantly decreased testes weight, and apparently also slightly decreased the sperm count and percentage of motile sperm. No disruption of spermatogenesis was observed histologically in any groups.

Kantak and Gogate (1992) studied effects of short-term (15 days) administration of extract of shade-dried leaves of *Ocimum sanctum* Linn. ground with food (100, 150, 200 and 400 mg/kg bw per day) on **reproductive behaviour** in adult male Wistar rats (n = 8) with proven reproductive activity. The controls were the same rats before they were given the diet with *O. sanctum* L. leaves. Reproductive behaviour towards estrogen-primed ovarectomised females was observed for 10 minutes on alternate days 7-8 times, of which the last four were used for statistical analyses. The

mean values of sexual behaviour score (grooming – 1 point, pursuit - 2 points, mount – 3 points, intromission – 4 points and ejaculation – 5 points) were significantly decreased with the 200 and 400 mg/kg bw per day doses (both $P < 0.01$), but not with 100 and 150 mg/kg bw per day. The latency responses for the various behaviour scores were prolonged, but were not statistically significant. No effects on food or water intake or in motor activity were observed. Structural changes in testis, epididymis and seminal vesicles were not apparent in these rats.

Reghunandanan et al. (1995) studied the effect of a powder made of benzene-extracted shade-dried leaves of *Ocimum sanctum* Linn. dissolved in 8 parts of propylene glycol, 1.0 part of water and 0.1 parts of ethanol as vehicle. The dose 300 mg/kg bw of the extract (n = 20) or the vehicle (n = 20) was given by i.p. injections to adult male Wistar rats. After 48 hours, the rats were sacrificed and testes removed and weighted. The body weight and testicular weight were not affected by the treatment. Testicular sperm count (millions/testis) was significantly decreased ($P < 0.001$), indicating a disturbance in spermatogenesis. Glutamyl transpeptidase (GTP) activity, an index of Sertoli cell function, was significantly reduced ($P < 0.001$). Lactate dehydrogenase (LDH), a marker of germ cell function, was not significantly affected by the treatment. The results indicated that the extract **reduced spermatogenesis** by impairment of Sertoli cell function without affecting the germ cells.

Reghunandan et al. (1997) studied the effect of fresh leaves of *Ocimum sanctum* on **fertility** in adult (2 year-old) albino rabbits of both sexes with proven fertility. Males (n = 5) and females (n = 7) were given freshly plucked tender leaves of *O. sanctum* orally (1 g/kg bw) twice per week (Mondays and Thursdays) in addition to normal diet in one month, corresponding to 57.1 mg dried leaves/kg bw per day if assuming 20% dry matter from the fresh leaves (DTU, 2012). A dose of 1 g/kg bw fresh leaves daily gave vaginal bleeding in the females, and therefore the administration was changed to twice a week. The control groups consisted of 3 males and 3 females given standard diet. Three rabbits of each sex from the treated and control groups were terminated and ovaries, uterus, testis and epididymis were examined histologically. The treated rabbits showed significant changes in all the studied organs. In the males, degeneration of seminiferous epithelium and Sertoli cells in the testis, reduced Leydig cell count, degenerative changes in the duct of epididymis in the lining of epithelial and sub-epithelial areas, and absence or fewer spermatozoa compared with in the controls, were observed. In the females, the uterus showed folding in the lining of the lumen and the rest of the endometrium was devoid of any glands. All the layers of the uterine wall showed congestion and oedema, and increased vascularity was also seen in the uterine wall, compared with the controls. The ovaries had hardly any primary and secondary follicles, and had only some large Graffian follicles. Haemorrhagic corpus luteum contained large areas with red blood cells. This indicated the occurrence of ovulation, but no implantation of the eggs in the uterus. The remaining 2 males and 4 females were mated immediately after end of the treatment, but no pregnancies occurred. When the mating was repeated one month after the end of the treatment, four litters of normal size were delivered by each rabbit.

Panda and Kar (1998) studied the effects of *Ocimum tenuiflorum* L. leaf extract (0.5 g/kg bw per day) orally by gastric intubation for 15 days in adult male Swiss albino mice (n = 10). The controls were given distilled water (n = 10). The **weight of sexual organs** (testis, seminal vesicle, Cowper's gland, vas deferens and epididymis) was recorded at termination. No significant effects were seen on the body weight, but the relative weight (g/ 100 g bw) of all the sexual organs was increased significantly.

Sardessai et al. (1999) studied the alterations in **reproductive behaviour** in terms of Lordosis quotient (LQ) after oral administration of fresh *Ocimum sanctum* Linn. leaf extract in adult female albino rats (n = 15, strain not stated). LQ is defined as a standard index of the relative sexual receptivity of a female animal in the presence of males, given by the number of times the female

adopts a posture of lordosis (inward curve of the spine) in a specified time period divided by the number of times a male mounts her. The estrous cycle was studied every day by taking vaginal smears. All rats had normal estrous cycle. Every day the females were presented to male rats of proven reproductive activity for 10 minutes, and LQ was measured. After 8 days (control period), these rats were given leaf extract orally (80 mg in 15 ml of 5% glucose per day) for two weeks. Thereafter, the treatment was discontinued and they were given only water for another 2 weeks. During this entire period, their estrous cycle was studied and reproductive behaviour in terms of LQ was measured. The estrous cycle was disrupted after the administration of the extract and finally the rats showed only the diestrus phase. In control rats, LQ was maximum during proestrus phase followed by estrus, metestrus and diestrus phase. After administration of the leaf extract, LQ decreased ($P < 0.05$) in the second week and this decrease continued further even after the leaf extract was replaced by water for two weeks ($P < 0.001$), indicating lasting effect. Thus, in this study the estrous cycle of the rats was disrupted from the second week of leaf extract administration and finally showed a continuous diestrus phase.

Ahmed et al. (2002a) assessed the **anti-fertility effects** of a benzene extract of *Ocimum sanctum* Linn. leaves (shade-dried, powdered, extracted with benzene and mixed with 1 ml propylene glycol per rat, administration route not stated) on sperm parameters and fructose contents in adult male Wistar albino rats. The rats were given 250 mg/kg bw of benzene extract for 48 days (one spermatogenic cycle) and terminated the day after, or were allowed to recover without treatment for 8 or 16 days after the 48 days exposure (n = 5). The controls were given 1 ml propylene glycol for 48 days and autopsied 24 h later (n = 5). Relative to the controls, the rats given the leaf extract had significantly decreased total sperm count, total number of motile sperm and forward velocity of sperm. Also, a significant increase in percentage of abnormal sperm and a significant decrease in fructose levels in cauda epididymal fluid and seminal plasma, providing energy for the spermatozoa, were seen. In the rats allowed to recover for 8 days, all the effects on the parameters affected above were partially reversed, but still significantly different from the controls. After 16 days of recovery, all these parameters were no longer significantly different from those in controls. The gradual recovery after treatment stopped indicated that the extract had reversible anti-fertility effects without apparent toxic side effects. The effects of *O. sanctum* on male and female reproductive systems were also summarized in a review by Ahmed et al. (2002b).

Ahmed et al. (2008) assessed the **reproductive toxicity effects** of a benzene extract of *Ocimum sanctum* Linn. leaves (shade-dried, powdered, extracted with benzene and mixed with 1 ml propylene glycol) on the ultrastructural changes in the epithelial cells of the cauda epididymis, its subsequent recovery in the seminiferous epithelium and fertility of adult male Wistar albino rats. The rats were given 250 mg/kg bw of benzene extract by gavage for 48 days and terminated the day after, or were allowed to recover without treatment for 8 or 16 days after the 48 days exposure (n = 5). The controls were given 1 ml propylene glycol orally per day for 48 days (covering the spermatogenic cycle in rats). The results indicated decreased relative weights (mg/100 g bw) of testis, epididymis and seminal vesicles, whereas other accessory organs (vas deferens, ventral prostate, Cowper's gland, coagulatory gland and ampullary gland) were not affected. Total count, cell diameters and nuclei diameters of germ cells and Leydig cells were reduced. Cauda epididymis exhibited significant reduction in epithelial height and nuclei diameter of epithelial cells. Cells showed vacuolization with signs of degeneration. Ultrastructural studies with electron microscope revealed that, in general, the cauda epididymis was affected and in particular, the principal, clear and basal cells were highly disturbed. Further, there were decreases in the size of lipid droplets, mitochondria, Golgi complex, endoplasmic reticulum and accumulation of lysosomal bodies. Fertility performance tests using female rats of proven fertility with regular estrous cycle and in proestrus or estrus phase showed no implantations in any rats on day 8 of pregnancy after mating with *O. sanctum*-treated males (n = 5). Moreover, after withdrawal of treatment partial recovery after one week and complete recovery after two weeks

were observed, resulting in normal spermatogenesis and fertility, based on number of implantations, number of pups and body weight of pups after one day and one week, suggesting that the effects of the treatment were transient and reversible. Thus, according to the authors the data indicated that the plant had reversible anti-spermatogenic and anti-fertility effects.

Comments:
The absolute weight should have been reported for the reproductive organs. It was stated in the publication that benzene was separated and the obtained extract was allowed to dry and stored in a desiccator at 4°C. The controls did receive only propylene glycol, and did not receive any benzene. Could the observed effects be caused by residual benzene in the extract and not by the plant substances?

Leigh and Fayemi (2008) studied effects of a crude aqueous extract of *Ocimum gratissimum* Linn. on testicular histology and spermiogram in adult male Wistar albino rats (n = 6). Fifty grams of fresh leaves were rinsed, blended thoroughly in 100 ml distilled water and filtered through filter paper. Doses of 5×10^{-4} and 10×10^{-4} ml/kg bw of the filtrate were given orally to two groups of rats thrice weekly or daily? (both are stated in the publication) for 5 weeks. The controls received distilled water (n = 6). Histopathology revealed mild to severe congestion and edema with the low dose as well as germinal tissue erosion of the seminiferous tubules with the high dose. Sperm motility, sperm concentration, % livability (live-dead ratio) and semen volume did not show significant changes with any dose in the treated rats. However, differences in spermatozoa abnormalities between treated (both doses) and control groups were highly significant ($P < 0.01$). The authors concluded that the findings indicated **anti-fertility** properties of *Ocimum gratissimum*.

Ahmed et al. (2009a) assessed the effects on **structural changes in epithelial cells of the cauda epididymis** and **fertility** of a benzene extract of *Ocimum sanctum* Linn. The leaves were shade-dried, powdered, extracted with benzene and mixed with propylene glycol in doses of 250 mg/kg bw in 1 ml of polypropylene glycol, and given by gavage to adult male Wistar albino rats (n = 5) for 48 days and sacrificed 24 hours later. The controls received 1 ml of propylene glycol. Ultrastructural observations in electron microscope showed that cauda epididymis had significant reduction in epithelial height and nuclei diameter of epithelial cells, the nuclei were pycnotic and the height of stereocilia reduced. Principal, clear and basal cells in cauda epididymis were highly disturbed. The lumen was devoid of sperm and filled with lymphocytes and debris of degenerated sperm. In a fertility test, the treated males were mated with female rats of proven fertility (n = 5). The fertility test showed no implantations in any female rats on GD8.

Ahmed et al. (2009b) assessed the dose-dependent effects on the **morphological changes in the cauda epididymal spermatozoa** and **sperm parameters** of a benzene extract of *Ocimum sanctum* Linn. The leaves were shade-dried, powdered, extracted with benzene and mixed with propylene glycol in doses of 150, 200 or 250 mg/kg bw in 1.5, 2.0 and 2.5 ml of polypropylene glycol, respectively, and given by gavage to adult male Wistar albino rats (n = 5) for 15 days and sacrificed 24 hours later. The controls received 'the same volume' (which volume not stated) of propylene glycol. The sperm parametric study showed decrease in total sperm count, sperm motility, forward velocity and increase in the percentage of abnormal sperms in a dose-dependent manner after all three doses vs. controls. Scanning electron microscope observations illustrated the disturbance in plasma membrane as well as acrosomal membrane. Most of the sperms appeared morphologically abnormal in the mid region of the tail with formation of balloon-like cytoplasmic droplet. These effects were also dose-dependent. The authors stated that the effects may have resulted from a general disturbance in the proteins and alteration in cauda epididymal milieu probably due to androgen deficiency consequent upon anti-androgenic property of *Ocimum sanctum* leaves.

Comments:
Androgens were not measured in this study. The controls did receive only propylene glycol, and did not receive any benzene. Could the observed effects be caused by residual benzene in the extract and not the plant substances?

Sethi et al. (2010) studied the effects of fresh leaves of *Ocimum Sanctum Linn.* (OS) on male **reproductive function** (sperm count and reproductive hormones) in albino rabbits (n = 10). The rabbits in the test group received standard chow diet supplementation of 2 g of fresh leaves of OS per rabbit for 30 days, while the control group was maintained on standard chow diet. Sperm count and levels of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured in serum by chemiluminescence. A significant decrease was noted in sperm count in the rabbits given OS (110.2 ± 5.7 million/ml) vs. controls (162.2 ± 7.0), P < 001. Serum testosterone levels were markedly increased (>1500.0 ng/dl) vs. controls (303.6 ± 18.2), while FSH and LH levels were both significantly reduced in OS-treated rabbits, being 0.13 ± 0.03 mIU/ml vs. 0.64 ± 0.05, and undetectable levels vs. 0.53 ± 0.03 mIU/ml), in OS-treated and controls, respectively. The results suggested an effect of OS on male reproduction.

Ahmed et al. (2011) investigated the effect of a benzene extract of *O. sanctum* leaves (shade-dried, powdered, extracted with benzene and mixed with propylene glycol) on the cauda epididymal sperm parameters, morphology and their organelles at the ultrastructural level in adult male albino Wistar rats. The treated group received benzene extract of *O. sanctum* leaves (250 mg/kg bw per day) for 48 days by gavage (n = 10) and the controls received 1 ml propylene glycol orally per day for 48 days (n = 10). Five male rats from each group (with proven fertility) were used for the fertility test. The results showed that the female rats mated with the benzene extract-treated males had no implantation sites on day 8 of pregnancy, whereas after mating with the control males, the number of implantations was 10.20 ± 1.07 on day 8 of pregnancy and number of pups obtained was 9.60 ± 1.08. Twenty-four hours after the last dose, the remaining control (n = 5) and treated (n = 5) rats were sacrificed and the cauda epididymal plasma was used for sperm analysis, scanning electron microscopy (SEM) and transmission electron microscopic (TEM) studies. Sperm analysis of the test group exhibited significant (P ≤ 0.001) decrease in the sperm count (56%), motility (45%), forward speed (49%) and increase in sperm anomalies (544%) when compared to controls. SEM and TEM observations in treated rats indicated morphological changes in plasma membrane and in the acrosomal membrane of spermatozoa, formation of a balloon-like cytoplasmic droplet in the mid-region of abnormal tails and disorganization or degeneration of mitochondria of sperm mitochondrial sheaths. The authors stated that the effects observed may result from a general alteration in the cauda epididymal milieu, probably due to androgen deficiency consequent to the anti-androgenic property of *O. sanctum* leaves.

Comments:
Androgens were not measured in this study. The controls did receive only propylene glycol, and did not receive any benzene. Could the observed effects be caused by residual benzene in the extract and not the plant substances? The results from 250 mg/kg bw per day here are the same as also presented in Ahmed et al. (2009b).

Pragya et al. (2012) studied **antifertility effects** of an aqueous leaf extract of *Ocimum sanctum* Linn. in male Swiss albino mice (n = 6). The aqueous extract of the plant was made by grounding 10 g of fresh leaves with 10 ml distilled water and the homogenized mixture was filtered and centrifuged at 5000 rpm for 10 min. The supernatant was collected and diluted with 30 ml of distilled water to obtain a concentration giving the dose of 250 mg/kg bw per day, which was orally administered as 0.1 ml for 10, 20 or 30 days. The control mice were given 0.1 ml of distilled water, but it was not stated when they were terminated (n = 6). Treatment with aqueous leaf extract of *Ocimum sanctum* L. caused significant decreased sperm counts after 10, 20 and 30 days treatment vs. the controls. Motility of the spermatozoa also declined significantly in the treated

group after 10, 20 and 30 days. The pH in seminal plasma decreased significantly during all time periods, which may affect spermatozoa motility. Mortality of spermatozoa increased significantly in the treated groups of mice during all time periods. Thus, the authors concluded that significantly decreased sperm count, motility and seminal pH, and increased mortality of spermatozoa, altered the seminal quality, which may lead to infertility among the treated mice.

Comments:
No statistical methods were described in this study. The fertility of the treated males was not tested. Importantly, in this publication, the mean ± SEM numbers given for sperm mortality (in %) for controls and mice treated for 10 and 30 days, are exactly the same numbers as given in Verma et al. (2016), but there called sperm abnormality (%). Only for treatment for 20 days, there are different numbers in these two publications. For the other measured parameters (sperm counts and % motility), the tendencies in the data were the same, but not the numbers, between these two publications. Thus, it is not clear if some of the data are from the same or two separate but identical experiments.

Mankapure *et al*. (2013) studied the **reproductive effects** of pellets of leaves and soft stems from *Ocimum sanctum* Linn. (OSE), 400 mg/100 g bw per day (4000 mg/kg bw per day) for 24, 48 or 72 days, or treatment for 72 days and recovery up to 120 days, on the testis and epididymis in male Wistar albino rats (n = 5). The pellets also contained wheat flour, groundnut oil and honey, which were also given to the negative control rats (n = 5). They reported that this high dose of OSE caused duration-dependent decrease in the relative wet weight of testis (mg/100 g bw). The relative wet weight of caput epididymis was slightly reduced after 24, 48 and 72 days versus controls, whereas the relative wet weight of cauda epididymis decreased in 24 and 72 day treatment groups and showed no difference from the control after 48 days. The diameter of seminiferous tubules was also decreased, with corresponding increase in the interstitium, arrested spermatogenesis, and also derangements in the histoarchitecture of the testis and epididymis. Epididymal tubules regressed, and the luminal spermatozoa formed a coagulum. In the recovery group, the body weight was unaffected. The testis and cauda epididymis partly regained normal relative wet weights, and the diameters of testis, caput and cauda epididymidis increased and were partially restored to normal. Histological observations estimated >80% of normal organization of seminiferous tubules, and spermatids and spermazoa appeared in the lumen in the recovery group, indicating that spermatogenesis was partially restored. Thus, this high dose of OSE leaves appeared to affect testicular and epididymal structure reversibly.

Comments:
The absolute weight should have been reported for the reproductive organs. No statistical testing of any of the comparisons was reported.

Verma et al. (2016) examined **anti-fertility effects** of *O. sanctum* Linn. in male Swiss albino mice (n = 6). An aqueous extract of the plant was made by grounding 10 g of fresh leaves with 10 ml distilled water and the homogenized mixture was filtered and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and diluted with 30 ml of distilled water to obtain a concentration giving the dose of 250 mg/kg bw per day, which was orally administered as 0.1 ml for 10, 20, 30, 40 or 50 days. The control mice were given 0.1 ml of distilled water, but it was not stated when they were terminated (n = 6). The treatment showed significantly decreased sperm counts and percentage of sperm motility after 10, 20, 30, 40 and 50 days of treatment vs. the controls. Abnormality of spermatozoa increased significantly in the treated group of mice after 10, 20, 30, 40 and 50 days of treatment. The results also showed that *O. sanctum* treatment caused significantly decreased weight of the reproductive organs; in testis, epididymis, seminal vesicle and vas deferens at 30, 40 and 50 days of treatment, and in ventral prostate at 20, 30, 40 and 50 days of treatment, compared with the controls. A recovery group of mice received 50 days of treatment and was further maintained for 90 days without any treatment to check the

reversibility of the effects. All the mice showed normal fertility rate after the recovery period (no further details were given on this mating). Apparently, the data indicated that all effects were reversible, however, it is not clear if statistical testing was done. Thus, *O. sanctum* adversely affected fertility in mice. The authors concluded that *O. sanctum* could be used as a potent and reversible anti-fertility agent.

Comments:
No statistical methods were described. Importantly, in this publication, the numbers given for sperm abnormality (%) for controls and mice treated for 10 and 30 days, are exactly the same numbers as given in Pragya et al. (2012), but there called mortality (in %). Only for treatment for 20 days, there are different numbers in these two publications. For the other measured parameters (sperm counts and % motility), the tendencies in the data were the same, but not the numbers, between these two publications. Thus, it is not clear if some of the data are from the same or two separate, but identical experiments.

Srinivasulu and Changamma (2017) studied the **reproductive effects** of *Ocimum sanctum* L. (OSE) leaf extract (dried, crushed and powdered leaves extracted with 95% ethanol for 3 days) given as 500 mg/kg bw per day orally for 20 days on spermatogenesis in adult male Wistar rats (n = 6). The controls were given distilled water for 20 days (n = 6). OSE did not affect the body weight. Administration of OSE leaf extract decreased weights (presumably absolute weights) of paired testes, paired epididymis, paired seminal vesicles and prostate gland; the first three significantly at $P < 0.01$. Administration of OSE leaf extract caused reductions in the sperm count (29.6%), motility (20.2%) and viability (11.8%), of which the two first parameters were significant at $P < 0.01$. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were significantly increased (+28.27 (P = ?) and +35.8% ($P < 0.01$), respectively), and testosterone levels significantly ($P < 0.01$) decreased (-38.6%), in the serum of OSE-treated rats compared to controls.

Raina et al. (2018) performed an **one-generation reproductive toxicity** study of aqueous/methanolic extract of *Ocimum sanctum* Linn. (OSE) (OciBest™) on the male and female reproductive performance of adult Wistar rats according to the Organization for Economic Cooperation and Development (OECD) guideline no. 415 and good laboratory practice (GLP). OciBest™ was an extract of the whole plant from Tamil Nadu, India, and was identified and authenticated at the National Institute of Science and Communication and Information Resources, New Delhi. The rats were orally gavaged with OciBest™ solubilized in distilled water at a volume of 10 ml/kg at dose levels of 0, 250, 500 and 1000 mg/kg bw. The control rats were given 10 ml/kg of distilled water. Males (n = 48) were administered OciBest™ for 12 weeks before and during the mating period (29 days), and terminated at the end of the mating period. Nulliparous, non-pregnant females (n = 96) received OciBest™ for 2 weeks before mating until the end of the lactation period (day 21), when they were terminated and subjected to full, detailed gross necropsy. The F1 offspring were evaluated and euthanized at the end of lactation, when they were subjected to external and internal macroscopic examination, and further histopathological examinations of organs with macroscopic findings were done. OciBest™ at 1000 mg/kg did not induce any adverse effects on the reproductive performance of male or female rats. All the treated parent animals survived until the end of the study period with no major signs of clinical toxicity. The body weights, food consumption, male and female fertility indices, mean estrous cycle length, female fecundity index, mean gestation length, gestation index, sex ratio of pups, live birth index and lactation index, absolute and relative organ weights in males and females, as well as gross pathological and histopathology observations in parent animals, did not reveal any treatment-related adverse effects. Moreover, in comparison to the control groups, OciBest™ did not induce any treatment-related adverse effects on the offspring, including effects on body weight, hair growth and ear-opening in any sex. The only effects were significant earlier eye opening in males with 500 and 1000 mg/kg bw per day, and in females with all three doses. Tooth eruption was

significantly earlier only with the 250 mg/kg bw dose, in the females only. Since these effects were not clearly dose-dependent and did not correlate with the body weight of the pups, they were not considered by the authors to be treatment-related. Histopathological examination revealed that at dose levels of 250, 500 and 1000 mg/kg OciBest™, the number of pups that showed histopathological changes were 7, 12 and 3, respectively. The authors concluded that the no observed adverse effect level (NOAEL) was 1000 mg/kg bw.

Comment:
It is noted that five of the authors of this publication work in the company owning the trademark OciBest™.

One study on effects on **female reproduction** in rats was found. Poli and Challa (2019) evaluated the antifertility efficacy of *O. sanctum* Linn. (OS) leaf extract in 4 months old female Wistar albino rats (n = 6 per group). The rats were administered saline as vehicle or OS leaf extract at a dose of 500 mg/kg bw per day by oral gavage for 15 days. No further information was given on the OS leaf extract other than that it was prepared according to 'WHO Protocol CG-04. Preparation of alcoholic extract for bioassay and phytochemical studies' from 1983. The duration of proestrus, estrus, metestrus and diestrus phases and the total duration of the estrous cycle were not significantly affected by the OS leaf extract. The OS leaf extract did not affect the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) or prolactin. However, the testosterone levels were significantly decreased (-88.88%) and progesterone levels increased (+20.58%). The OS leaf extract did not significantly affect the estradiol levels. After treatment with the OS leaf extract, the tissue somatic index (w/w %, per 100 g bw) was increased significantly in ovary (+18.29%), uterus (+44.23%) and vagina (+18.00%). The total proteins, total carbohydrates and total lipids were increased significantly in ovary (+37.37%, +29.46% and +36.60%, respectively) and uterus (+18.73%, +42.26% and +45.28%, respectively), whereas in vagina the total protein was increased (+28.44%), and total carbohydrates (-17.47%) and total lipids (-44.44%) were reduced, by the OS leaf extract. The authors concluded that the administration of the OS leaf extract significantly increased the serum estradiol and progesterone levels leading to reduced frequency of ovulation and resulted in the **impairment of fertility**.

Comments:
The authors did not clearly demonstrate the relationship between the hormone profiles and the changes in estrous cycle, and the rats were not mated to test their fertility, thus, it is uncertain whether the observed small changes in estrous cycle may affect the reproduction. This study was not performed according to OECD guidelines for reproductive studies, such as guideline no. 415: One-Generation Reproduction Toxicity Study.

Summary of reproductive toxicity effects
In the following summary, the lowest dose per day showing an effect is noted in parentheses after the reference for each study (see also Table 9).

A high number of studies reported that administration of *O. tenuiflorum/O. sanctum* preparations significantly affected male rat reproductive organ weights and spermatogenesis (Seth et al., 1981 (100 mg/kg bw); Khanna et al., 1986 (200 mg/kg bw); Reghunandanan et al., 1995 (300 mg/kg bw); Ahmed et al., 2002a (250 mg/kg bw); Ahmed et al., 2008 (250 mg/kg bw); Leigh and Fayemi, 2008 (5 x 10^4 ml/kg bw); Ahmed et al., 2009a (250 mg/kg bw); Ahmed et al., 2009b (150 mg/kg bw); Ahmed et al., 2011 (250 mg/kg bw); Mankapure et al., 2013 (4000 mg/kg bw) and Srinivasulu and Changamma, 2017 (500 mg/kg bw)). These effects may lead to male infertility, which was either only anticipated based on the results reported or in some studies demonstrated by reduced mating and/or implantation loss of embryos (Vohora et al., 1969 (100 mg/kg bw); Batta and Santhakumari, 1970 (100 mg/kg bw); Ahmed et al., 2008 (250 mg/kg bw); Ahmed et al., 2009a (250 mg/kg bw), Ahmed et al., 2011 (250 mg/kg bw). Negative effects were also

reported on reproductive mating behaviour of male rats (Kantak and Gogate, 1992 (200 mg/kg bw). Results on male reproduction and fertility were also observed in male mice (Kasinathan et al., 1972 (465 mg leaves), Pragya et al., 2012 (250 mg/kg bw), Verma et al., 2016 (250 mg/kg bw).

Effects on estrous cycle and confirmed reduced fertility (Khanna et al., 1986 (200 mg/kg bw)) or increased reproductive organ weights and affected hormone levels (Poli and Challa, 2019 (500 mg/kg bw)), were reported in female rats. Negative effects were also reported on reproductive mating behaviour of female rats affecting their fertility as well as their estrous cycle (Sardessai et al., 1999 (80 mg/rat)).

Changes in reproductive organs and reduced fertility were also shown in male and female rabbits (Reghunandan et al., 1997) (1 g/kg bw of fresh leaves twice weekly for one month, and decreased sperm count and changes in reproductive hormone levels in male rabbits (Sethi et al., 2010) (2 g fresh leaves).

No teratogenic effects or congenital malformations in rats with doses between 100 and 4000 mg/kg bw were reported in the only three publications where this was mentioned (Vohora et al., 1969; Batta and Santhakumari, 1970; Khanna et al., 1986).

Discussion of reproductive toxicity effects

Most of these studies available on reproductive toxicity, fertility or mating behaviour, as well as teratogenic effects, were quite old and not performed according to OECD or other guidelines for such studies. The reporting was lacking in details and the language was often not clear, making it difficult to fully evaluate the reported results. The description of the doses was also often lacking sufficient detail for a quantitative exposure assessment. It was not possible to know for certain whether the organic solvents in the extracts used were fully removed before they were given to the animals, and the solvents were usually not included in the vehicle given to the control animals.

It is striking that most of these older studies, many of them using benzene or other organic solvents as extractants reported adverse effects, whereas an aqueous/methanolic extract of *O. sanctum* L., such as the commercial reparation OciBest™, did not find any treatment-related adverse effects up to 1000 mg/kg bw (Raina et al., 2018). One explanation is that the solvents could extract more of the active substance(s) in the plant compared to more polar solvents resulting in adverse effects. Another possible explanation could be that the effects were caused by the organic solvents themselves, if remnants of solvents were present in the extracts given to the experimental animals, but were not present in the vehicle given to the control animals. Benzene is an example of a solvent that may adversely affect reproduction, i.e. as fetal loss, reduced fertility and low birth weight (OSHWiki, 2023). The only study (Batta and Santhakumari, 1970) comparing various extracts found varying percentages of implantation loss with different extracts. The loss of implantation sites for benzene was 80% (dose 200 mg/kg bw), petroleum ether (both 100 and 200 mg/kg bw) 60%, ether 20%, acetone 40%, ethanol 43% (the last three in doses of 100 mg/kg bw). However, since the benzene extract, which was used in many studies, was not also tested in 100 mg/kg bw, it is not possible to quantitatively compare its effects with the more polar extracts. Some studies using aqueous extracts (Vohora et al., 1969; Leigh and Fayemi, 2008; Pragya et al., 2012), ethanolic or methanolic extracts (Srinivasulu and Changamma, 2017; Raina et al., 2018; Poli and Challa, 2019), or apparently had not used any extracts at all (Kasinathan et al., 1972; Khanna et al., 1986; Sethi et al., 2010; Mankapure et al., 2013), also observed adverse toxicity effects, indicating that the solvents are not likely to be the sole cause of the observed reproductive toxicity effects.

The study by Raina et al. (2018) was the only study reporting no effects on male or female rat reproduction with OciBest™ (up to 1000 mg/kg bw per day). It was the only reproductive toxicity study available claimed to be performed according to an OECD guideline. However, it had several

weaknesses in design and reporting and was performed by employees of the company owning the trademark OciBest™.

Several studies indicated that the observed adverse effects on spermatogenesis and fertility were reversible (Ahmed et al., 2002a; Ahmed et al., 2008; Mankapure et al., 2013; Verma et al., 2016).

Conclusions on reproductive toxicity effects of basil plants

Based on the available data, reproductive toxicity, observed as impaired spermatogenesis in males, disturbed estrous cycle and loss of embryo implantation in uterus in females, as well as changes in weight and structure of reproductive organs, changes in hormone levels, reduced sexual mating behaviour and reduced fertility of both males and females, appeared to be the most critical adverse effects from intake of holy basil. However, no teratogenic effects in the offspring were reported after exposure to basil plants. Based on these reproductive toxicity studies performed in three species and both sexes of experimental animals, in spite of many of them being old and with weaknesses, it seem reasonable to conclude that various preparations of *O. tenuiflorum/O. sanctum* may have adverse effects on reproduction in both males and females in doses in the dose range of 100-4000 mg/kg bw per day when administered during gestation or for 14-90 days to non-pregnant individuals.

Endocrine effects

The effects of *Ocimum tenuiflorum* L. leaf extract (0.5 g/kg bw per day) orally by gastric intubation for 15 days on the changes in the concentrations of serum triiodothyronine (T3), thyroxine (T4) and serum cholesterol were investigated in adult male Swiss albino mice (n = 10) (Panda and Kar, 1998). The controls were given distilled water (n = 10). T_3 and T_4 were measured with radioimmunoassay (RIA). Significant decrease was observed in serum T_4 concentration, but not in serum T_3 concentration, T3/T4 ratio or concentration of serum cholesterol. This indicated that the extract acted on the thyroid, which is the only organ where T_4 is synthesized, but not on the extrathyroidal conversion of T_4 to T_3. Significant decreases were also observed in hepatic lipid peroxidation and in the gluconeogenic activity of the hepatic glucose-6-phosphate enzyme, whereas the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were increased.

Drug interactions

Mahomoodally et al. (2018) reported three citations on herb-drug interactions on the island of Mauritius in the Indian Ocean between *Ocimum sanctum* L. and the anti-diabetic drug pioglitazone hydrochloride used as treatment of type 2 diabetes, causing severe dizziness.

There was no significant pharmacokinetic interaction between the anti-epileptic drug levetiracetam (300 mg/kg bw) and *Ocimum sanctum* hydroalcoholic extract (OSHE) (1000 mg/kg bw) in adult male Wistar rats treated for 14 days, except increased Tmax in the levetiracetam + OSHE group compared with levetiracetam alone (P = 0.009) (Sarangi et al., 2020). This indicated that OSHE may have the potential to delay the absorption of levetiracetam or hindering the drug to achieve its peak concentration at an early phase. Another study in adult Wistar rats also showed that OSHE (1000 mg/kg bw) orally for 14 days did not cause pharmacokinetic interaction with the antiepileptic drug valproate except an increase in $T_{1/2}$, which was not statistically significant (Sarangi et al., 2017).

The safety of a herbal product is not only dependent on its toxicological effects but also on the risk of its pharmacokinetic and/or pharmacodynamic interactions with other drugs, which can lead to treatments failures, significant toxicity or even to fatal events. Several plants, including *O. sanctum* L. and *O. basilicum*, and their isolated bioactive compounds, such as eugenol and ursolic acid, are now being examined for antiviral activity (Denaro et al., 2020). However, interactions of plant substances with conventional antiviral medications can interfere with the treatment of the viral

infection. Studies of the safety profile of plant extracts and their isolated compounds, alone and in combination with conventional antiviral drugs, are needed.

Phase I metabolism mediated by cytochrome P450 (CYP) enzymes represents a major route of elimination of many drugs that can compete for the same CYP enzyme. Some CYP enzymes are also involved in bioactivation of chemicals to their genotoxic and carcinogenic metabolites. Zehetner et al. (2019) summarized how essential oils (EOs) and their chemical compounds may affect the activity of CYP enzymes. Eugenol is reported to inhibit CYP1A1 and CYP1B1 activity in human cells and also to decrease CYP2E1 activity in rats. CYP2A can metabolically activate a number of carcinogens, including nitrosamines and aflatoxins, and therefore, the induction of CYP2A activity should be regarded with caution. In male Wistar rats, CYP2A1 and CYP2A2, occurring in hepatic tissue, and CYP2A3, occurring in lungs, were significantly influenced by (3*R*)-(−)-linalool. (3*S*)-(+)-Linalool showed a slight inhibition of CYP2B6 in human liver. It was found that at low concentrations of methyleugenol, CYP1A2 is the main enzyme involved in the bioactivation of methyleugenol to its carcinogenic metabolite 1′-hydroxymethyleugenol, causing liver tumours, whereas at high substrate concentrations CYP2C9 and CYP2C19 may also contribute to the bioactivation of methyleugenol in humans. The enzyme activities of CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 were not significantly affected by β-caryophyllene and β-caryophyllene oxide, whereas CYP1A2, CYP3A4 and partly CYP2B1 were significantly inhibited in rat and human liver microsomes. β-Caryophyllene and β-caryophyllene oxide showed only weak inhibition of CYP1A2 activity, but a strong inhibition of CYP3A4 activity, and the inhibition by β-caryophyllene oxide was competitive in human but non-competitive in rat liver microsomes. CYP1A2 and CYP2A6, in particular, appeared to be active in estragole hydroxylation, and also CYP2C19, CYP2D6 and CYP2E1 showed moderate activity, however, it was concluded that CYP2B6, CYP2C9 and CYP3A4 does not contribute to estragole hydroxylation in the human liver.

Because CYP1A2 is an important enzyme in the hydroxylation of estragole and the related alkenylbenzene methyleugenol, and CYP2A6 is an important enzyme in the hydroxylation of estragole and safrole, competitive effects can be expected (Zehetner et al., 2019). As these alkenylbenzenes have carcinogenic potential, herb-based exposure to them should be regarded with attention, since in herbs such as anise, basil and nutmeg, all three alkenylbenzenes are present. Furthermore, there are known polymorphisms for CYP1A2 and CYP2A6, which leads to the decreased/deleted or induced activity of these enzymes, and therefore to the decreased or increased bioactivation of estragole. Lifestyle factors like cigarette smoking and the consumption of chargrilled food and cruciferous vegetables increase the activity of CYP1A2, and therefore can increase the bioactivation, and thus, the genotoxicity, of estragole. Eucalyptol induced CYP2B1 and CYP3A2 activity in rat liver microsomes. Using human enzymes expressed in insect cells, CYP3A4 exhibited the highest activity for eucalyptol hydroxylation, whereas the rate catalysed by CYP3A5 was about one-quarter of that catalysed by CYP3A4. CYP3A4- and CYP3A5-converted metabolites of eucalyptol were detected in human urine. Eucalyptol is a very effective substrate for CYP3A enzymes both in rat and human liver. Thus, as metabolic clearance and toxic effects of different compounds might be modulated (increased or decreased) by an EO component-induced change in the activity of CYP enzymes, drug interactions with serious clinical consequences may arise when the difference between the toxic and the effective concentrations of a drug is small.

Bartonková and Dvorák (2018a) investigated the effects of 31 essential oils (EOs) of culinary herbs and spices, including EO made from flowering tops of basil (no further information available), on the transcriptional activity of pregnane X receptor (PXR), one of the transcriptional activators of xenobiotic-metabolizing enzymes, and the expression of the metabolic enzyme cytochrome P450 3A4 (CYP3A4), in human intestinal and hepatic *in vitro* cell models. All tested EOs, including basil, activated PXR dose-dependently in intestinal LS180 cells and all EOs induced CYP3A4 mRNA expression in PXR-transfected LS180 cells, primary human hepatocytes from three

male donors and wild-type hepatic progenitor HepaRG cells. EO-mediated induction of CYP3A4 mRNA expression was nullified in PXR-knock out HepaRG cells, suggesting the involvement of PXR in these effects. Basil also activated MDR1 and CYP2B6, other target genes of PXR. This showed that EOs of culinary herbs and spices, including basil, might be common activators of PXR and inducers of CYP3A4 at doses present in foods (estimated to be 35-55 µg/ml), thereby, they might have a potential for food-drug interactions.

Bartonková and Dvorák (2018b) also examined the effects of 31 EOs of culinary herbs and spices, including from flowering tops of basil (*Ocimum basilicum*) (no further information available), on the transcriptional activity of human aryl hydrocarbon receptor (AhR), a pivotal xenobiotic receptor, having also multiple other roles in human physiology. Basil was found to be AhR-inactive (neither an agonist nor an antagonist) in all tested concentrations (0.01-250 µg/ml). The compound found in basil, estragole, was not an AhR agonist (without 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) in any concentrations (0.01-200 µg/ml) and an antagonist (with TCDD) only in the highest concentration (200 µg/ml). Eugenol was identified as an AhR partial agonists (in concentrations 10-200 µg/ml) and an antagonist (in concentrations 1-200 µg/ml). Estragole induced expression of the CYP1A1 gene, a target gene for the AhR involved in metabolism of xenobiotics and chemically-induced toxicity and carcinogenesis, in human Caucasian colon adenocarcinoma cell line LS180 only in antagonist mode, whereas eugenol induced this gene both in agonist and antagonist mode.

See also the chapters on *Drug interactions* under the individual substances.

Hazards from individual substances in basil plants

Studies indicate that the plant matrix may contain also substances having a protective effects when used in food, for instance by inhibiting the metabolic enzymes necessary to bioactivate procarcinogenic substances to electrophilic intermediates reacting with DNA (Jeurissen et al., 2007a). However, food supplements are containing semi-purified substances that may be harmful to health, such as the genotoxic and carcinogenic alkylbenzenes methyleugenol and estragole. Available data are conflicting regarding whether this matrix effect could attenuate genotoxicity of such substances or not (EMA, 2014). In the following, toxicological information on some of the biologically active and potentially harmful substances in *O. tenuiflorum* L. and *O. sanctum* L. is reported.

Methyleugenol

Regulation

Because of its genotoxic and carcinogenic properties (see below), methyleugenol is prohibited in EU for use as pure substances in foodstuffs since September 2008, with exceptions for specific products (EU, 2008).

Exposure and previous risk assessments

Methyleugenol was evaluated as a flavouring agent by the EU Scientific Committee on Food and found to be genotoxic and carcinogenic (SCF, 2001a).

EMA (2005a) concluded that several studies had clearly established that the metabolism, metabolic activation and covalent binding of methyleugenol were dose-dependent and that the relative importance diminished markedly at low levels of exposure. They concluded further that rodent studies had showed that toxicological events were minimal probably in the dose range of 1-10 mg/kg bw.

The presence and accompanying risks of methyleugenol and other alkenylbenzenes in instant herbal beverages available on the Indonesian market were evaluated by Suparmi et al. (2019). Of the 114 samples, all as powders packaged in sachets, 98 were meant for adults and 16 were dedicated to children. Methyleugenol was detected in 49 out of 114 samples, at levels amounting to 2.6-443.7 µg/g. The highest level of methyleugenol was sold as a household industry food product. The estimated daily intake (EDI) resulting from drinking these preparations amounted to 0.1-51.2 µg/kg bw per day and 1.1-3.3 µg/kg bw per day of methyleugenol, respectively, for samples targeted at adults and children. A $BMDL_{10}$ value of 22.2 mg/kg bw per day for methyleugenol was defined using literature data and model averaging. MOE values were below 10000 for 46 samples (40.4% of all samples studied), indicating a priority for risk management when assuming daily lifelong consumption. Using Haber's rule (C1 X T1 = constant *k* = C1 X T2, where *k* is the toxic outcome, C is concentration of the chemicals and T is time of exposures) to correct for less than lifetime exposures, consumption of methyleugenol via these beverages would be of low concern when consumed for less than 2 weeks/year during a lifetime. This conclusion holds for herbal beverages collected by targeted sampling, and included traditional medicines, domestic processed foods, domestic supplements, foreign processed foods and food household industry, not for all herbal beverages on the Indonesian market. The study provided data that could support establishment of a maximum permitted level (MPL) for methyleugenol in herbal beverages in Indonesia.

Comment:
Whether herbal teas would be a category for which this conclusion holds is not known.

The consumer risks of jamu, Indonesian traditional herbal medicines, as powders mixed in hot water, were assessed focussing on the presence of alkenylbenzene-containing botanical ingredients by Suparmi et al. (2018). Twenty-three out of 25 samples contained alkenylbenzenes at levels ranging from 3.8 to 440 µg/kg, with methyleugenol being the most frequently detected. The estimated daily intake (EDI) resulting from jamu consumption was estimated to amount to 0.2–171 µg/kg bw per day for individual alkenylbenzenes, to 0.9–203 µg/kg bw per day when adding up all alkenylbenzenes detected, and to 0.9–551 µg/kg bw per day when expressed in methyleugenol equivalents using interim relative potency (REP) factors (methyleugenol was the reference compound with REP = 1.00). The margin of exposure (MOE) values obtained were generally <10000 indicating a priority for risk management when assuming daily consumption during a lifetime. Using Haber's rule (Suparmi et al. (2019), it was estimated that one period of two weeks consumption of these jamu would not raise a concern (MOE >10000). However, when considering use for two weeks every year during a lifetime, 5 samples still raised a concern. It was concluded that the consumption of alkenylbenzene containing jamu could be of concern especially when consumed on a daily basis for longer periods of time.

Smith et al. (2010) performed a weight of evidence (WOE) assessment and concluded that methyleugenol had a weak genotoxic activity. The putative genotoxic carcinogen was the 1'-sulphate ester metabolite. Dose-response modelling of the data gave a $BMDL_{10}$ for male rat liver adenoma or carcinoma (combined) of 7.9 mg/kg bw per day following adjustment to daily average doses. The estimated exposure based on data in the literature of methyleugenol from all food sources ranged from 10 µg/kg bw per day (average intake) to 66 µg/kg bw per day (high consumers), using the EU model diet with maximum permitted levels across all food categories. The margin of exposure (MOE) values ranged from 100 to 800 depending on the assumptions used in the exposure estimation.

In a risk assessment by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA), Davidsen et al. (2023) calculated MOE = 1200000 for methyleugenol in basil oil from *Ocimum basilicum* (with intake of 0.4 µg/person/day, i.e. that exceeds the TTC value of 0.15 µg/person/day for structural alerts for genotoxicity) based on $BMDL_{10}$. MOE values for

methyleugenol were 1900000 and 8000000 for high and low intake, based on high or low % of essential oil, which were 0.3 and 0.07 μg/person/day. The scope of this safety evaluation, indicating no safety concern, does not include added use in dietary supplements or any products other than food. The natural flavour complex (NFC) derived from basil was determined or affirmed as generally recognized as safe (GRAS) under their conditions of intended use as flavor ingredients based on an evaluation of each NFC and the constituents and congeneric groups therein. MOE values >10,000 that are based on a $BMDL_{10}$ derived from an animal study would be of low public health concern and of low priority for risk management actions (EFSA, 2005).

Absorption, distribution, metabolism and excretion (ADME)

The following summary is based on NTP (2000), SCF (2001a) and EMA (2005a). Methyleugenol was rapidly absorbed following oral administration to rats and mice, and the maximum concentration in the blood was reached after approximately 5-15 minutes. The kinetic data were consistent with rapid clearance from the blood, metabolism in the liver and excretion of the parent and various metabolites in the urine. Following administration of a single oral dose of methyleugenol (200 mg/kg bw) to rats, several urinary metabolites were identified. The major metabolic pathways included the oxidation of the allylic side chain, the formation of the hydroxyl acid via epoxidation of the double bond followed by hydroxylation of the benzene ring and *O*-demethylation. The hydroxylation of C-1' of the allylic side chain, with formation of 1'-hydroxymethyleugenol, is catalysed by CYP2E1 and most probably by CYP2C6, but not by CYP3A, CYP1A2, CYP2D1 or CYP2C11. Administration of high doses of methyleugenol to rats (at least 30 mg/kg bw per day for 25 days) caused dose-dependent auto-induction of 1'-hydroxylation of methyleugenol, mediated by various cytochrome P450 isozymes, whereas a lower dose (10 mg/kg bw per day for 5 days) did not. The rate of 1'-hydroxylation of methyleugenol *in vitro* in 13 human liver samples varied 37-fold, with the highest activities being similar to that in control rat liver microsomes. This suggested that the risk from dietary intake of methyleugenol varies markedly in the human population. Besides to the liver, methyleugenol was also distributed to the spleen, ovaries, fat and stomach after absorption. Eight-five % of the absorbed methyleugenol was eliminated within 72 h as metabolites, no unchanged methyleugenol was detected in the urine. Elimination from the bloodstream was rapid, with initial and terminal half-lives in the order of 5 and 120 minutes, respectively. Excretion of metabolites was also found in bile. There is some evidence that methyleugenol is eliminated more rapidly in males than in females after treatment of rats for 6 or 12 months. Methyleugenol was highly lipophilic, having an octanol-water partition coefficient estimated at 800, indicating that it can pass the cell membranes easily.

Jeurissen et al. (2006) performed *in vitro* studies to elucidate the human cytochrome P450 (CYP) enzymes involved in the bioactivation of methyleugenol to its proximate carcinogen 1'-hydroxymethyleugenol. Incubations with Supersomes, expressing individual P450 enzymes to a high level, revealed that CYP1A2, CYP2A6, CYP2C9, CYP2C19 and CYP2D6 were able to 1'-hydroxylate methyleugenol. An additional experiment with Gentest microsomes, expressing the same individual enzymes to roughly average liver levels, indicated that CYP1A2, CYP2C9, CYP2C19 and CYP2D6 contributed to methyleugenol 1'-hydroxylation in the human liver. A study in which correlations between methyleugenol 1'-hydroxylation in human liver microsomes from 15 individuals and the conversion of enzyme specific substrates by the same microsomes were investigated showed that CYP1A2 and CYP2C9 were important enzymes in the bioactivation of methyleugenol. This was confirmed in an inhibition study in which pooled human liver microsomes were incubated with methyleugenol in the presence and absence of enzyme specific inhibitors. Kinetic studies revealed that at physiologically relevant concentrations of methyleugenol, CYP1A2 was the most important enzyme for bioactivation of methyleugenol in the human liver showing an enzyme efficiency (kcat/Km) that is approximately 30, 50 and >50 times higher than the enzyme efficiencies of, respectively, CYP2C9, CYP2C19 and CYP2D6. Only when relatively higher methyleugenol concentrations were present did CYP2C9 and CYP2C19 contribute as well to the bioactivation of methyleugenol in the human liver.

Toxicokinetics

Al-Subeihi et al. (2011) defined a physiologically based biokinetic (PBBK) model for the alkenylbenzene methyleugenol in male rats based on *in vitro* metabolic parameters determined in relevant tissue fractions, *in silico* derived partition coefficients and physiological parameters derived from the literature. The model was used to obtain the relative extent of bioactivation and detoxification of methyleugenol at different oral doses. At low doses, formation of 3-(3,4-dimethoxyphenyl)-2-propen-1-ol and methyleugenol-2',3'-oxide, leading to detoxification, were the major metabolic pathways in the liver. At high doses, the model showed a relative increase in formation of the proximate carcinogenic metabolite 1'-hydroxymethyleugenol in the liver, which further lead to a relative increase in formation of 1'-hydroxymethyleugenol glucuronide, 1'-oxomethyleugenol and 1'-sulfooxymethyleugenol, of which the last compound was the ultimate carcinogenic metabolite of methyleugenol. These results indicated that the relative importance of different metabolic pathways of methyleugenol may vary in a dose-dependent way, leading to a relative increase in bioactivation of methyleugenol at higher doses. See Figure 1 in this publication for the suggested metabolic pathways of methyleugenol.

Al-Subeihi et al. (2012) further defined a physiologically based kinetic (PBK) model for methyleugenol in humans based on parameters derived *in vitro* and *in silico*, investigated bioactivation and detoxification of methyleugenol at different dose levels and compared the outcomes of the current model with those of the previous PBK model for methyleugenol in male rats (Al-Subeihi et al., 2011). The results showed that formation of 1′-hydroxymethyleugenol glucuronide, a major metabolic pathway in male rat liver, appeared to represent a minor metabolic pathway in human liver, whereas in human liver a significantly higher formation of 1′-oxomethyleugenol compared with male rat liver was found. Furthermore, formation of 1′-sulfooxymethyleugenol, which readily undergoes desulfonation to a reactive carbonium ion that can form DNA or protein adducts, was predicted to be the same in the liver of both humans and male rats at oral doses of 0.0034 and 300 mg/kg bw. Altogether, despite a significant difference in especially the pathways of the proximate carcinogenic metabolite 1′-hydroxymethyleugenol between humans and male rats, the influence of species differences on the ultimate overall bioactivation of methyleugenol to 1′-sulfooxymethyleugenol appeared to be negligible. Moreover, the PBK model predicted the formation of 1′-sulfooxymethyleugenol in the liver of humans and rats to be linear from doses as high as the benchmark dose (BMD_{10}) down to as low as the virtual safe dose (VSD). These kinetic data did not provide a reason to argue against linear extrapolation from the rat tumour data to the human situation. See Figure 1 in this publication for the suggested metabolic pathways of methyleugenol.

Irritation

Methyleugenol (98%) was not an eye or skin irritant (PubChem, 2020).

Mutagenicity and genotoxicity

In vitro: Methyleugenol was not mutagenic in *Salmonella typhimurium* strain TA98, TA100, TA1535 or TA1537, with or without exogenous metabolic activation (S9) (NTP, 2000; SCF, 2001a; EMA, 2005a). In cytogenetic tests with cultured Chinese hamster ovary cells, methyleugenol induced sister chromatid exchanges in the presence of S9, but not chromosomal aberrations in cultured Chinese hamster ovary cells with or without S9. Further studies confirmed the non-mutagenicity of methyleugenol in various strains of *S. typhimurium* and in the *Escherichia coli* WP2 uvrA strain with and without S9. Methyleugenol was found able to induce intra-chromosomal recombination in *Saccharomyces cerevisiae* with and without metabolic activation. Methyleugenol, 1'-hydroxymethyleugenol and 2',3'-epoxymethyleugenol induced unscheduled DNA synthesis (UDS) in cultured rat hepatocytes. The 1'-hydroxy metabolite was a stronger inducer of UDS than the parent substance. It was mutagenic also in the *Bacillus subtilis* DNA repair test.

In vivo: Methyleugenol was reported negative in a micronucleus assay in male and female mice treated by gavage with methyleugenol for 14 weeks with doses up to 1000 mg/kg bw (NTP, 2000; SCF, 2001a; EMA, 2005a). In 20/29 mouse hepatocellular carcinomas induced by methyleugenol, mutations in the β-catenin gene were observed, while such mutations were only found in 2/22 spontaneous liver tumours. The activation of the β-catenin gene, with the subsequent deregulation of the Wnt signaling pathway, was considered as an early event in chemically-induced hepatic carcinogenesis in mice. These results were considered a further indication of the genotoxic potential of methyleugenol.

Methyleugenol formed adducts with DNA and proteins in human fibroblasts V79 cells transfected with human genes expressing sulfotransferase and in the mouse liver *in vivo* (Randerath et al., 1984; Phillips et al., 1984). ^{32}P-Post-labelling showed liver DNA adducts prevalently on the N^2 of guanine and, with less extent, on the N^6 of adenine (SCF, 2001a).

Based on the available information, methyleugenol was considered to be genotoxic (NTP, 2000; SCF, 2001a; EMA, 2005a).

Acute toxicity

Methyleugenol was considered moderate toxic, with oral LD50 values from 810 or 850 to 1560 mg/kg bw reported for rats and 540 mg/kg bw for mice (SCF, 2001a; EMA, 2005a).

Subchronic toxicity

Abdo et al. (2001) performed a 14-week toxicity study where male and female F344/N rats (n = 10/sex) and B6C3F1 mice (n = 10/sex) were exposed to methyleugenol in 0.5% aqueous methylcellulose (vehicle) by gavage, five days per week, in doses of 0, 10, 30, 100, 300 or 1000 mg/kg bw per day. The controls were given the vehicle (n = 10/sex and species). Additional groups of rats and mice of each sex were dosed similarly and used for hematology and clinical chemistry studies. Methyleugenol caused reductions in body weight gain after exposure to 100 mg/kg bw per day and higher (female rat) or 300 mg/kg bw per day and higher (male rat and female and male mice), increased mortality (mice only, with the highest dose), and adverse effects in the liver (hepatocellular damage, cholestasis, altered hepatic functions, increase in liver weight) and glandular stomach (increased incidences of atrophy, necrosis, oedema, mitotic alterations and cystic glands) from 300 mg/kg bw in rats and 30 mg/kg bw in mice. A NOAEL was estimated at 30 mg/kg bw per day for rats and 10 mg/kg bw per day for mice, thus, resulting in a NOAEL for methyleugenol of 10 mg/kg bw per day.

Liver toxicity

When adult male ddY mice (n = 4-6) were treated with methyleugenol in olive oil in doses of 3.7 mmol/kg bw (equivalent to 600 mg/kg bw eugenol) by oral intubation in combination with pretreatment with an inhibitor of glutathione (GSH) synthesis, DL-buthionine sulfoximine (BSO) as 4 mmol/kg bw, given i.p. 1 h before eugenol as in Mizutani et al. (1991a) (see the Chapter *Liver toxicity* of eugenol), methyleugenol did not cause significant hepatotoxicity seen as changes in relative liver weight, liver blood volume or serum glutamic pyruvic transaminase (GPT) levels (Mizutani et al., 1991b). Thus, methylation of the free hydroxyl group in eugenol to give methyleugenol, resulted in complete loss of the hepatotoxicity, showing that the free hydroxyl group in eugenol was essential for this effect.

Carcinogenicity

Miller et al. (1983) treated pre-weanling B6C3F1 male mice with i.p. injections of a solution of methyleugenol in the solvent trioctanoin twice weekly for 12 weeks (total dose 42.4 mg/kg bw). At 13-18 months, a significant increase in percentage of mice with hepatomas was found (96% in treated vs. 41% in controls). Similar activity was found with 1'hydroxymethyleugenol (93% vs. 41%, respectively).

The critical toxic effect of methyleugenol is carcinogenicity (i.e. hepatocellular carcinomas). On the basis of the NTP study (2000), it was calculated that a lifetime dose of 0.4 μg/kg bw per day of methyleugenol will represent a lifetime cancer risk of 10^{-5} (Sanner et al., 2001; NFSA, 2012).

Methyleugenol is considered a multisite, multispecies genotoxin and carcinogen (SCF, 2001a; EMA, 2005a). Male and female F344/N rats and $B6C3F_1$ mice received methyleugenol (approximately 99% pure) in 0.5% methylcellulose by gavage for 14 weeks or 2 years (NTP, 2000; Johnson et al., 2000). In the 2-year study in rats, groups of 50 male and 50 female rats received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75 or 150 mg/kg, 5 days per week for 105 weeks; groups of 60 male and 60 female rats received the 0.5% methylcellulose vehicle only. Stop-exposure groups of 60 male and 60 female rats received 300 mg/kg in 0.5% methylcellulose by gavage for 52 weeks followed by just the 0.5% methylcellulose vehicle for the remaining 53 weeks of the study. In the 2-year study in mice, groups of 50 male and 50 female mice received methyleugenol in 0.5% methylcellulose by gavage at doses of 37, 75 or 150 mg/kg for 105 weeks. Under the conditions of these 2-year gavage studies (NTP, 2000; Johnson et al., 2000), there was clear evidence of carcinogenic activity of methyleugenol in male and female F344/N rats based on the increased incidences of liver neoplasms and neuroendocrine tumours of the glandular stomach in male and female rats, and the increased incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma and fibroma or fibrosarcoma (combined) in male rats. A marginal increase in the incidence of squamous cell neoplasms of the forestomach may have been related to methyleugenol administration in female rats. There was clear evidence of carcinogenic activity of methyleugenol in male and female $B6C3F_1$ mice based on the increased incidences of liver neoplasms. Neuroendocrine tumours of the glandular stomach in male mice were also considered related to methyleugenol administration. In male and female rats and mice, methyleugenol administration also caused significant increases in non-neoplastic lesions of the liver and glandular stomach.

Yang et al. (2020) studied whether reactive metabolites of methyleugenol could cause RNA adducts. Cultured mouse primary hepatocytes were incubated with methyleugenol followed by RNA extraction and NaOH and alkaline phosphatase-based RNA hydrolysis. Three adenosine adducts were detected in the hydrolytic mixture by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The same adenosine adducts were also detected in hepatic tissues from ME-treated male Kunming mice. Additionally, two guanosine adducts and one cytidine adduct were detected in the *in vivo* samples. The results provided evidence that the reactive metabolites of ME attacked RNA, resulting in RNA adducts.

Methyleugenol and its metabolite 1'-hydroxy-methyleugenol are shown to be carcinogenic in rodent studies. In the liver, methyleugenol is converted into 1'-hydroxy-methyleugenol by different CYP450-enzymes and further converted into an unstable sulfo-species which decays into a DNA-reactive carbocation that forms two DNA-adducts (see above). Carlsson et al. (2021) found that cell viability was reduced over time and that the cell death showed apoptotic features. The main 1'-hydroxy-methyleugenol-induced cell death pathway was mitochondrial apoptosis mediated by p53. The displayed caspase cleavage pattern indicated mitochondrial apoptosis. Induction of p53-axis via Mdm2 inhibition as well as caspase inhibition confirmed the results.

Methyleugenol was classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B) (IARC, 2013). However, in 2023, IARC classified methyleugenol as probably carcinogenic to humans (Group 2A) on the basis of sufficient evidence for cancer in experimental animals, strong mechanistic evidence in experimental systems and inadequate evidence regarding cancer in humans (Riboli et al., 2023). At the same time, isoeugenol was classified in Group 2B.

Reproductive and developmental toxicity
No information on reproductive and developmental toxicity of methyleugenol was found.

Other studies
Methyleugenol is characterized as both a weak direct γ-aminobutyric acid type A receptor ($GABA_AR$) agonist and a positive allosteric modulator (Li et al., 2020). Such receptor interactions may possibly explain the sedative and anticonvulsant effects and insecticide activities of monoterpenoids.

Vulnerable groups
A 5-fold interindividual difference in activities of CYP enzymes metabolizing methyleugenol was found in liver microsomes between 15 humans (range 0.89-4.30 nmol min^{-1} nmol $CYP450^{-1}$), indicating human variation in sensitivity toward methyleugenol (Jeurissen et al., 2006). Lifestyle factors such as smoking (induces CYP1A) and the use of barbiturates (induces CYP2C) can increase the susceptibility for adverse effects of methyleugenol.

Data to use in the risk characterization
Since methyleugenol is genotoxic and carcinogenic, its health risks should be assessed by estimating the margin of exposure (MOE) (EFSA, 2005).

Estragole

Exposure and previous risk assessments
Estragole was evaluated as a flavouring agent by the EU Scientific Committee on Food and found to be genotoxic and carcinogenic (SCF, 2001b).

EMA (2014) indicated that background exposure to estragole from foodstuffs is in the range of 0.5-5 mg per day (8.3-83.3 µg/kg bw per day, for a person of 60 kg bw) from an average food intake. In this opinion, the arguments for the relevance of the experimental carcinogenic effect in animals for human risk assessment were discussed: genotoxicity-initiated tumours in animals and the mode of action for tumour formation are probably relevant for humans, and it is probable that the toxicokinetics in humans are sufficiently similar to these processes in rodents in which carcinogenicity is observed so that extrapolation can be regarded as adequately reliable. Thus, as a genotoxic carcinogen, the exposure to estragole should be kept as low *as reasonably* achievable, according to the ALARA principle. EMA (2014) used a $BMDL_{10}$ value of 10 mg/kg bw per day (it varied between 9 and 33 mg/kg bw per day) based on hepatomas in female mice (EFSA ESCO Report, 2009) to derive an acceptable daily intake of 0.5 mg/person per day, using 50 kg bw and an uncertainty factor of 1000 for exposure of adults to estragole in herbal medicinal products in maximum 14 days.

Comment:
Using 60 kg bw, the corresponding value for this intake would be 0.6 mg/person per day.

In a risk assessment by FEMA, Davidsen et al. (2023) calculated MOE = 13000 for estragole in basil oil from *Ocimum basilicum* (with intake of 46 µg/person/day, i.e. that exceeds the TTC value of 0.15 µg/person/day for structural alerts for genotoxicity) based on $BMDL_{10}$ MOE values for estragole were 15000 and 67000 for high and low intake, based on high or low % of essential oil, which were 37 and 8 µg/person/day. The scope of this safety evaluation, indicating no safety concern, did not include added use in dietary supplements or any products other than food. The natural flavour complex (NFC) derived from basil was determined or affirmed as generally recognized as safe (GRAS) under their conditions of intended use as flavor ingredients based on an evaluation of each NFC and the constituents and congeneric groups therein.

Absorption, distribution, metabolism and excretion (ADME)

The following text is based on SCF (2001b) if not stated otherwise. Estragole belongs to the class of alk-2-enylbenzenes comprising, among others methyleugenol and eugenol. The major metabolic pathways of estragole have been established in rats and mice. At low doses, estragole mainly undergoes *O*-demethylation, of which CO_2 is the terminal metabolite (detoxification pathway), but as the dose is increased, the proportion of *O*-demethylation falls and other pathways, notably 1'-hydroxylation (bioactivation pathway), come into prominence. The formation of the metabolite 1'-hydroxyestragole involved CYP450 enzymes, and further metabolism of this metabolite into 1'-sulfoestragole involved sulfotransferases (Martins et al., 2018). Estragole is further converted to a carbocation which is the electrophilic and carcinogenic metabolite formed upon breakdown of 1′-sulfooxyestragole (Prinsloo et al., 2018). Single doses of estragole in the range of 0.05 to 50 mg/kg bw administered to female Wistar albino rats by oral intubation were largely (52-58%) excreted as CO_2. At higher doses (500 and 1000 mg/kg bw), CO_2 excretion only accounted for 28-29% of the administered dose. The metabolite 1'-hydroxyestragole excreted in the urine accounted for 1.3-5.4% of the dose in the range 0.05 to 50 mg/kg bw or for 11.4-13.7% in the dose range 500-1000 mg/kg bw. Comparable dose fractions were excreted as 1'-hydroxyestragole and CO_2 by CD-1 mice dosed i.p. with 0.05 to 50 mg/kg bw estragole. These data indicated that *O*-demethylation was more important than 1'-hydroxylation in the low dose range. Concerning human studies, it has been reported that after oral administration of estragole to two volunteers (100 µg/day for 6 months) the excretion of 1'-hydroxyestragole in the urine amounted to 0.2 and 0.4% of the administered dose. *O*-demethylation seems to be the most prevalent pathway in humans at low doses, similar to in rats and mice, yielding a phenolic derivative that can be excreted as sulfate or glucuronic acid conjugates (Martins et al., 2018). See Figure 2 in Martins et al. (2018) for a figure on metabolism of estragole, and a short summary text in EMA (2014).

Punt et al. (2008) defined a physiologically-based biokinetics (PBBK) model for estragole in male Sprague-Dawley rats based on *in vitro* metabolic parameters determined using relevant tissue fractions, *in silico* derived partition coefficients and physiological parameters derived from the literature. The model consisted of eight compartments, including liver, lung and kidney as metabolizing compartments, and additional compartments for fat, arterial blood, venous blood, rapidly perfused tissue and slowly perfused tissue. Evaluation of the model was performed by comparing the PBBK predicted dose-dependent formation of the estragole metabolites 4-allylphenol and 1′-hydroxyestragole glucuronide to levels of these metabolites reported in the literature, demonstrated to be in the same order of magnitude. With this model, the relative extent of bioactivation and detoxification of estragole at different oral doses was examined. At low doses, formation of 4-allylphenol, leading to detoxification, was observed to be the major metabolic pathway, occurring mainly in the lung and kidney. Saturation of this metabolic pathway in these organs lead to a relative increase in formation of the proximate carcinogenic metabolite 1′-hydroxyestragole, mainly in the liver. This relative increase in formation of 1′-hydroxyestragole lead to a relative increase in formation of 1′-hydroxyestragole glucuronide and 1′-sulfooxyestragole, the latter being the ultimate carcinogenic metabolite of estragole. These results indicated that the relative importance of different metabolic pathways of estragole may vary in a dose-dependent way, leading to a relative increase in bioactivation of estragole at higher doses.

The extent of bioactivation of estragole to its ultimate carcinogenic metabolite 1'-sulfooxyestragole was further explored by Punt et al. (2009), who investigated the kinetics of the metabolic reactions of both estragole and its proximate carcinogenic metabolite 1'-hydroxyestragole in humans. Based on the kinetic data obtained, a physiologically-based biokinetics (PBBK) model for estragole in humans was defined to predict the relative extent of bioactivation and detoxification at different dose levels of estragole. The results were compared with those previously predicted by a PBBK model for estragole in male rats to evaluate the

occurrence of species differences in metabolic activation. The results obtained revealed that formation of 1'-oxoestragole, which represents a minor metabolic route for 1'-hydroxyestragole in rat, was the main detoxification pathway of 1'-hydroxyestragole in humans. Due to a high level of this 1'-hydroxyestragole oxidation pathway in human liver, the predicted species differences in formation of 1'-sulfooxyestragole remained relatively low, with the predicted formation of 1'-sulfooxyestragole being twofold higher in humans compared with in male rats, even though the formation of its precursor 1'-hydroxyestragole was predicted to be fourfold higher in humans. Overall, it was concluded that in spite of significant differences in the relative extent of different metabolic pathways between humans and male rats there was a minor influence of species differences on the ultimate overall bioactivation of estragole to 1'-sulfooxyestragole.

Human cytochrome P450 enzymes (CYP) involved in the bioactivation of estragole to its proximate carcinogenic metabolite 1'-hydroxyestragole were identified by Jeurissen et al. (2007b). Incubations with Supersomes revealed that all enzymes tested, except CYP2C8, were able to 1'-hydroxylate estragole. Experiments with Gentest microsomes, expressing CYP enzymes to roughly average liver levels, indicated that CYP1A2, CYP2A6, CYP2C19, CYP2D6 and CYP2E1 might contribute to estragole 1'-hydroxylation in the human liver. Especially CYP1A2 was important based on the correlation between CYP1A2 activity and estragole 1'-hydroxylation in human liver microsomal samples and inhibition of estragole 1'-hydroxylation by the CYP1A2 inhibitor α-naphthoflavone. Kinetic studies revealed that, at physiologically relevant concentrations of estragole, CYP1A2 and CYP2A6 were the most important enzymes for bioactivation in the human liver showing enzyme efficiencies (kcat/Km) of, respectively, 59 and 341 min^{-1} mM^{-1}. Only at relatively high estragole concentrations, CYP2C19, CYP2D6 and CYP2E1 might contribute to some extent. Comparison with results from similar studies for methyleugenol and safrole revealed that competitive interactions between estragole and methyleugenol 1'-hydroxylation and between estragole and safrole 1'-hydroxylation are to be expected because of the involvement of, respectively, CYP1A2 and CYP2A6 in the bioactivation of these compounds.

Irritation

When estragole was tested as 3% in petrolatum in humans, it produced no irritation after a 48-hours closed-patch test on human subjects (PubChem, 2020). Applied full strength to intact or abraded rabbit skin for 24 hours under occlusion estragole was moderately irritating.

Mutagenicity and genotoxicity

In vitro, estragole was non-mutagenic in Ames test and in *Escherichia coli* WP2 uvrA reversion test and negative in the *Bacillus subtilis* repair test (Rec-assay) without S9 (SCF, 2001b). Highly purified 1'-hydroxyestragole was mutagenic in strain TA100 in the absence of fortified liver microsomes. Supplementation with NADPH-fortified rat liver microsomes and cytosol increased the mutagenic activity of 1'-hydroxyestragole and estragole. The electrophilic 2',3'-oxides of 1'-hydroxyestragole and estragole showed dose-dependent mutagenic activities in strain TA1535 in the absence of fortified liver microsomes. Estragole was found to be very weakly mutagenic in TA1535 strain of *S. typhimurium*, however, the addition of 3'-phosphoadenosine-5'-phosphosulphate (PAPS) to the microsomal assay markedly increased the mutagenic activity of estragole. This result suggested that estragole may be converted to DNA-binding sulphuric acid esters under these conditions. Estragole (10^{-3}-10^{-5} M) was unable to induce chromosomal aberrations in V79 Hamster cells in the presence and in the absence of S9 or in primary rat hepatocytes. In the same range of concentrations, estragole was able to induce unscheduled DNA synthesis (UDS) in primary rat hepatocytes. Estragole and its 1'-hydroxy metabolites induced UDS in cultured hepatocytes derived from male Fischer 344 rats. The 1'-hydroxy-derivatives were more potent genotoxins than their parent compounds.

In an *in vivo* liver UDS assay, rats were treated with estragole at doses of 500, 1000 and 2000 mg/kg bw (SCF, 2001b). UDS was induced only at the highest dose.

Two major DNA adducts and two minor DNA adducts were found in enzymatic digests of hepatic DNA from mice treated i.p. with 1'-hydroxyestragole (SCF, 2001b). Two of these adducts were characterised as N^2-(trans-isoestragol-3'-yl) deoxyguanosine and N^6-(trans-isoestragol-3'-yl) deoxyadenosine. Further characterization of the DNA adducts formed by electrophilic esters of 1'-hydroxyestragole *in vitro* and in mouse liver *in vivo*, included new adducts at C-8 and N-7 of guanine residues. In a previous study, it was found that estragole induced adducts to liver DNA of newborn male B6C3F1 mice treated by i.p. injection on day 1, 8, 15 and 22 after birth at doses of 0.25, 0.5, 1.0 and 3.0 μmol per mouse. The adduct level with estragole was 30.0 pmol/mg DNA.

When incubating estragole with hepatic S9-fractions from rats and humans, specific adducts with hemoglobin (N-(isoestragole-3-yl)-valine, IES-Val) and DNA (isoestragole-2′-deoxyguanosine and isoestragole-2′-deoxyadenosine) were formed (Bergau et al., 2021). IES-Val levels in human blood (n = 7) were determined during and after the daily consumption of 0.5 litre of an estragole-rich fennel tea for four weeks. A significant increase of IES-Val levels was observed during the consumption phase and followed by a continuous decrease during the washout period to background levels after 15 weeks. IES-Val may be used to monitor the internal exposure to the reactive genotoxic metabolites of estragole, 1′-sulfoxyestragole.

In the micronucleus assay, no mutagenic potential of estragole was found in HepG2 cells whereas in HepG2-CYP1A2 cells 1 μM estragole led to a 3.2 fold increase and 300 μM estragole led to a 7.1 fold increase in micronuclei counts (Schulte-Hubbert et al., 2020).

SCF (2001b) concluded that estragole was demonstrated to be genotoxic.

Acute toxicity

Oral LD50 was 1230 mg/kg bw of estragole in rats and 1250 mg/kg bw in mice (PubChem, 2020).

Liver toxicity

By investigating chemical structures of hepatotoxic substances in herbs, He et al. (2019) found that alkaloids and terpenoids were the two major groups causing hepatotoxicity. They further identified eight major structural skeletons for hepatotoxicity and 15 structural alerts for hepatotoxicity. Among the eight skeletal categories, the sub-category phenylpropene-type simple phenylpropanooids contained i.a. estragole.

Hudson et al. (2018) also summarized liver toxicity and other toxic effects of estragole in a review of the toxicity of compounds in herbal dietary supplements.

Carcinogenicity

Estragole and/or its metabolite 1'-hydroxyestragole induced hepatic tumours as well as lung adenomas and angiosarcomas in CD-1 or B6C3F1 mice either after dietary chronic exposure or after i.p. or s.c. injections prior to or after weaning, where males appeared to be more susceptible than females (SCF, 2001b). Miller et al. (1983) performed several experiments in mice and rats summarized by SCF (2001b), also including other metabolites of estragole; estragole 2'3'-oxide, which induced hepatomas, lung adenomas, benign skin papillomas and keratoacanthomas, and 1'-hydroxyestragole 2'3-oxide, which induced skin papillomas and keratoacanthomas, all in mice. In the study where adult female CD-1 mice (n = 48-49) were exposed for 12 months to 0.23% (2300 mg/kg diet) and 0.46% (4600 mg/kg diet) of estragole in the diet by Miller et al. (1983), the doses were estimated to be 150-300 mg/kg bw per day and 300-600 mg/kg bw per day, respectively, which induced hepatomas with incidence of 56% and 71%, respectively (EFSA ECSO Report, 2009).

Miller et al. (1983) also studied estragole in rats. Groups of 20 male Fischer rats were administered by s.c. injections 1'-hydroxyestragole, estragole-2',3'-oxide or 1'-hydroxyestragole 2',3'-oxide twice weekly for 10 weeks (total dose: 900 mg/kg bw). Besides three sarcomas at the injection site (by 1'-hydroxyestragole) and one sarcoma (by estragole 2',3'-oxide), one hepatic carcinoma was induced by 1'-hydroxyestragole and one by estragole 2',3'-oxide. In addition, one or two other tumours were found at other sites (both adenomas and carcinomas). No tumours were found in the controls.

A direct carcinogenicity of estragole and *in vitro* low levels of DNA adducts were found with a significant dose response up to 1000 mM, suggesting the possibility of a direct-acting mechanism of adduction, which can lead to alkali-labile sites in DNA, resulting in tails in the Comet assay and sister chromatid exchange (SCE), due to DNA strand-breaks (Gori et al., 2012).

After CYP450-catalyzed bioactivation (hydroxylation) of the allylic side chain and sulfation via sulfotransferase (SULT), estragole acts as a genotoxic hepatocarcinogen. The DNA adducts N^2-(isoestragole-3'-yl)-2'-deoxyguanosine (E-3'-N^2-dG) and N^6-(isoestragole-3'-yl)-deoxyadenosine (E-3'-N^6-dA) were found in primary rat hepatocytes after incubation with estragole (Schulte-Hubbert et al., 2018; 2019; 2020). E-3'-N^2-dG was the major adduct found. At all incubation times, formation of this DNA adduct was concentration-dependent and increased with higher incubation times. E-3'-N^6-dA adduct formation was only observed after incubation times longer than 12 h and at higher estragole concentrations.

Chemical-specific DNA adducts may be a trigger of gene mutations and therefore play a role in chemical carcinogenesis. However, DNA adduct formation may not inevitably lead to mutations. Cell proliferation plays a key role in fixing mutations induced by DNA damage. Ishii et al. (2019) examined this issue by studying the effects of the antibiotic flumequine (FL), a residue of veterinary medicinal products in foodstuffs, thought to be a non-genotoxic hepatocarcinogen, on mutagenicity in the liver of mice treated with estragole. Male B6C3F1 *gpt* delta mice (n = 4-5) were given 10 or 100 mg/kg bw per day for two weeks, thereafter 70 mg/kg bw per day for two weeks, of estragole (with >98% purity) in corn oil by gavage and simultaneously fed a diet containing 0.4% FL for 4 weeks. The high estragole dose was reduced since all FL-mice given the high dose of estragole showed severe weight loss and one mice died after two weeks. The same weight loss was not seen in the mice given high dose estragole without FL. All mice given FL also had decreased final body weight and increased relative liver weight, which were not seen in the mice given only estragole in any dose. Proliferating cell nuclear antigen (PCNA)-positive cells and cell cycle-related genes were additively increased in the livers of combined treatment groups compared with high-dose estragole or FL groups. Estragole is metabolically activated by CYP1A2, followed by SULTA1-mediated reactions forming the nucleophilic form, leading to formation of estragole (ES)-specific DNA adducts in the liver. These adducts, ES-3'-N^2-dG, ES-3'-C8-dG and ES-3'-N^6-dA, were dose-dependently increased in all estragole-treated groups and not affected by FL. Mutant frequencies (MFs) in *gpt*, indicating point mutations, after co-treatment with low-dose estragole and FL were significantly increased, although treatment with estragole alone increased MFs only in the high-dose group. The high estragole dose increased CYP1A2 mRNA, and this was further increased by FL. SULT1A1 mRNA levels were unchanged after estragole or FL treatment. Liquid chromatography with tandem-mass spectrometry analysis (LC-MS/MS) showed that FL did not affect the amount of estragole-specific DNA adducts in the livers, indicating that FL treatment did not influence metabolic pathways of estragole. Thus, enhancement of the mutagenic potential of a chemical such as estragole by chemical (FL)-induced cell proliferation may occur as a result of the combined effects of chemicals in food.

Schulte-Hubbert et al. (2020) analysed the time- and concentration-dependent levels of the DNA adducts N^2-(isoestragole-3'-yl)-2'-desoxyguanosine (E3'N^2dG) and N^6-(isoestragole-3'-yl)-desoxyadenosine (E3'N^6dA), reported to be the major adducts formed in rat liver, in rat

hepatocytes in primary culture after incubation with estragole. E3'N^{2}dG, the main adduct at all incubation times and concentrations, could be detected at estragole concentrations <0.1 μM after 24 h and <0.5 μM after 48 h. Adduct levels were highest after 6 h and showed a downward trend at later time-points, possibly due to DNA repair and/or apoptosis. While the concentration-response characteristics of adduct formation were apparently linear over the whole concentration range, strong indication for marked hypo-linearity was obtained when the modeling was based on concentrations <1 μM only. Thus, the default assumption of dose-linearity of carcinogenicity of estragole strongly based on dose-response data at high concentrations/doses is probably inadequate, at least based on this *in vitro* findings, and needs to be replaced by a thorough dose-response analysis at relevant dose levels including additional *in vivo* studies.

Potential consequences of combined exposure to estragole and safrole and or their proximate carcinogenic 1'-hydroxy metabolites were evaluated in HepG2 cells *in vitro* and *in silico* (Yang et al., 2022). The results indicated that concentration addition adequately described the cytotoxic effects and no statistically significant differences were shown in the level of the major DNA adducts. Furthermore, physiologically based kinetic models revealed that at normal dietary intake the concentration of the parent compounds and their 1'-hydroxymetabolites remained substantially below the Km values for the respective bioactivation and detoxification reactions, providing further support to the fact that the simultaneous presence of the two carcinogens, or of their proximate carcinogenic 1'-hydroxy metabolites, may not affect their DNA adduct formation. Overall, the results pointed at the absence of interactions upon combined exposure to estragole and safrole at realistic dietary intake levels.

In conclusion, estragole is a multi-site carcinogen in at least two rodent species and both sexes (SCF, 2001b; EFSA ECSO Report, 2009).

Reproductive and developmental toxicity

No information was found on reproductive and developmental toxicity of estragole.

Allergy

A maximization test with estragole as 3% in petrolatum was carried out on 25 volunteers and produced no sensitization reactions (PubChem, 2020).

Other studies

Estragole was a weak antagonist of the γ-aminobutyric acid type A receptor ($GABA_AR$) and very high doses were required to produce any change in GABA-induced currents (Li et al., 2020). Such receptor interactions may possibly explain the sedative and anticonvulsant effects and insecticide activities of monoterpenoids.

Drug interactions

Humans with poor metabolizer phenotypes in CYP2A6 might diminish the chances on bioactivation of estragole, whereas lifestyle factors increasing CYP1A2 activities, such as cigarette smoking and consumption of charbroiled food, might increase bioactivation of estragole (Jeurissen, 2007b).

Data to use in the risk characterization

Since estragole is genotoxic and carcinogenic, its health risks should be assessed by estimating the margin of exposure (MOE) (EFSA, 2005). $BMDL_{10}$ values calculated for incidence of hepatomas in female mice exposed for 12 months via diet to estragole based on data from Miller et al. (1983) varied between 9 and 33 mg/kg bw per day (EFSA ESCO Report, 2009).

Safrole

Exposure and previous risk assessments

In a risk assessment by FEMA, Davidsen et al. (2023) did not calculate MOE for safrole in basil oil from *Ocimum basilicum*, since the intake (0.02 µg/person/day) was below the TTC value of 0.15 µg/person/day for structural alerts for genotoxicity. The MOE was not calculated for safrole in basil oleoresin either, with estimated high (0.02 µg/person/day) and low (0.004 µg/person/day) intake of basil oleoresin, based on high and low % of essential oil, respectively.

Carcinogenicity

The alkenylbenzene safrole was classified by IARC as possibly carcinogenic to humans (Group 2B), based on sufficient evidence in animals (IARC, 1987). Safrole may exert its toxicity upon its conversion to 1'-hydroxy-safrole (a proximate carcinogen) due to the cytochrome P450 (CYP)-dependent hydroxylation formed in humans mainly by CYP2A6 and subsequent sulfation to form 1'-sulfooxy-safrole (the ultimate carcinogen), which is responsible for the formation of DNA adducts causing the genotoxicity (Pedroni et al., 2023).

See also the Chapter on estragole regarding competitive actions between the alkenylbenzenes and the Chapter on eugenol regarding the chemical relationship with safrole, as well as some information in the more general chapters.

Eugenol

Exposure and previous risk assessments

The presence and accompanying risks of eugenol and other alkenylbenzenes in instant herbal beverages available on the Indonesian market were evaluated by Suparmi et al. (2019). Of the 114 samples, all as powders packaged in sachets, 98 were meant for adults and 16 were dedicated to children. Eugenol was detected in 4 samples, all specified to be used by adults, which contained eugenol at levels of 21.4-101.2 µg/g. The estimated daily intake (EDI) resulting from drinking these preparations amounted to 5.0-46.9 µg/kg bw per day. The EDI for the four samples containing eugenol did not exceed the acceptable daily intake (ADI) of 0-2.5 mg/kg bw established by JECFA (2006) or the ADI of 1.0 mg/kg bw established by EFSA (2012b), thus, did not raise a concern for human health. This conclusion holds for herbal beverages collected by targeted sampling, and included traditional medicines, domestic processed foods, domestic supplements, foreign processed foods and food household industry, not for all herbal beverages on the Indonesian market.

Comment:
Whether herbal teas would be a category for which this conclusion holds is not known.

In an updated Research Institute for Fragrance Materials (RIFM) fragrance ingredient safety assessment of eugenol by Api et al. (2022c), it was stated that eugenol was not genotoxic. The calculated Margin of Exposure (MOE) for eugenol was >100 for repeated dose toxicity. Reproductive toxicity was evaluated using the Threshold of Toxicological Concern (TTC) for a Cramer Class I substance, and the exposure to eugenol was below the TTC (0.03 mg/kg per day and 1.4 mg/day). Data provided for eugenol indicated a No Expected Sensitization Induction Level (NESIL) of 5900 µg/cm^2 for skin sensitization. Eugenol was not phototoxic and was not expected to be photoallergenic.

Absorption, distribution, metabolism and excretion (ADME)

The metabolism of eugenol is very similar to other alkenylbenzenes. However, as opposed to the other alkenylbenzenes methyleugenol and estragole, eugenol has a free phenolic hydroxyl group,

which is an active group ready for conjugation with glucuronic acid or sulphate, leading to more efficient detoxification and excretion in the urine (Martins et al., 2018). Eugenol may be biotransformed to electrophilic quinone methides, thus, it can potentially form oxidative base damage and DNA adducts. Eugenol may also be oxidized to intermediate(s) that react with proteins and GSH to form adducts. In rats, eugenol preferentially induced phase II enzymes, such as glutathione *S*-transferases, rather than cytochrome P450 enzymes (phase I), but this was not conformed in humans. Mean half-live of eugenol in rats was long (14 h in plasma and 18.3 h in blood), possibly involving enterohepatic circulation, suggesting that some level of accumulation can occur after repeated oral administration. Eugenol, being highly lipophilic, seems to accumulate in the central nervous system, however, without visible toxicity.

Irritation

Eugenol may cause serious eye irritation (PubChem, 2020).

Mutagenicity and genotoxicity

IARC (1985) summarized the mutagenic and genotoxic results on eugenol. Eugenol was not mutagenic to *Salmonella typhimurium* TA1530, TA1535, TA1537, TA 1538, TA98 or TA100 in the presence of a metabolic system (S9) in several studies. However, supplementation with 3'-phosphoadenosine-5'-phosphosulphate (PAPS) and S9 was reported to result in significant, although not dose-dependent, mutagenicity to *S. typhimurium* TA 1535. Eugenol was not mutagenic to *Escherichia coli* WP2 uvrA when tested in the presence or absence of S9. Reports concerning the activity of eugenol in the *Bacillus subtilis* rec+/rec- DNA-repair assay were contradictory: both positive and negative results were reported, both in the absence of S9. β-Glucuronidase-treated urine (300 µI) of Sprague-Dawley rats given 0.5 ml eugenol by intubation was not mutagenic to *S. typhimurium* TA100 or TA98 in the presence of S9. Similarly, eugenol was not mutagenic to *S. typhimurium* TA1950, TA1951, TA1952 or TA1964 in the host-mediated assay in which male C3H/HeJ mice were given 200 mg/kg bw i.m. Eugenol (concentration not indicated) induced neither mutation nor gene conversion in *Saccharomyces cerevisiae*. Eugenol induced chromosomal aberrations in Chinese hamster ovary cells in the absence of S9. ln a second study, chromosomal aberrations were induced by eugenol in Chinese hamster ovary cells only in the presence of S9, and a small increase in the incidence of sister chromatid exchanges was also observed in the presence or absence of S9. 2',3'-Epoxyeugenol was mutagenic to *S. typhimurium* TA1535 and TA100 in the absence of S9, but not to strains TA1537, TA1538 or TA98 in the presence or absence of S9. Two metabolites of eugenol present in rat urine, 3-piperidyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone and 3-pyrrolidinyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone, were not mutagenic to *S. typhimurium* in the presence or absence of S9. Similarly, these metabolites were not mutagenic to *S. typhimurium* in the host-mediated assay in male C3H/HeJ mice at doses of 200 mg/kg.

Detailed descriptions of *in vitro* and *in vivo* studies of mutagenicity and genotoxicity of eugenol can also be found in EFSA (2009a), when eugenol and six related hydroxyallylbenzene derivatives were evaluated as flavourings, as summarised in the following. Eugenol gave consistently negative results in assays for reverse mutation in various strains of *S. typhimurium* and *E. coli*. Generally, negative results were also found for DNA repair in *B. subtilis* M45 (rec-) and H17 (rec+) cells; the one positive finding for DNA repair occurred at a concentration of 400 µg/disc, while a similar study with higher concentrations (≤100000 µg/disc) yielded negative results. In assays for unscheduled DNA synthesis in rat and mouse hepatocytes *in vitro*, no genotoxic activity was observed at concentrations ≤164.2 µg/ml of eugenol and ≤15 µg/ml of eugenyl acetate, however, one positive result was reported with eugenol in Syrian hamster embryo cells at concentrations ≤1 µg/ml. Assays in mammalian cells *in vitro* in which mutagenicity was found (forward mutation in mouse lymphoma cells, sister chromatid exchange and chromosomal aberrations) were performed at concentrations that resulted in cytotoxicity or severe cell cycle delay. Mammalian cells in culture might not have the metabolic pathways of detoxification available to counter such

toxicity. Assays for mutagenicity (micronuclei, chromosomal aberrations and mutation) and genotoxicity (unscheduled DNA synthesis and DNA fragmentation) *in vivo* generally gave negative results, even at very high doses of eugenol (≤800 mg/kg bw by i.p. injection, ≤2680 mg/kg bw orally). The two reports of micronucleus induction involved doses as high as 740 mg/kg bw given by i.p. injection and 14794 mg/kg bw given orally. The available results indicated that eugenol and other hydroxypropylbenzene derivatives are unlikely to pose a significant mutagenic or genotoxic risk to humans under the intended conditions of use as flavouring agents.

According to Cox et al. (2019), eugenol, which is not DNA-reactive, can indirectly elicit genotoxicity *in vitro*, i.e. is a "false positive", and is thought to disturb cell culture conditions or exert cytotoxicity on cells *in vitro* in a way that leads to an apparent genotoxic response. The MutaMouse primary hepatocyte (PH) gene mutation assay did not yield a significant increase in mutation frequency over the solvent control in any of the tested concentrations (5, 10, 50 and 100 µg/ml) of eugenol. The results demonstrated a significant overall concentration-response relationship ($\chi 2 = 15.0$, $P < 0.05$), however, this compound was too cytotoxic to test at higher concentrations and the effect appeared to be driven solely by the highest concentration tested. Thus, eugenol was considered equivocal in the MutaMouse PH gene mutation assay. As summarized by Cox et al. (2019), eugenol has also been considered negative in the *in vitro* gene mutation MutaMouse FE1 assay (± S9), positive in the *in vitro* mouse lymphoma TK assay (MLA) without S9, positive in the *in vitro* chromosome aberration test and positive in the *in vitro* micronucleus assay in p53-deficient hamster cells, but negative in the *in vitro* micronucleus assay in p53-functional human cells. *In vivo*, negative results were seen in hematopoietic tissues (bone marrow and blood), in the liver measured by the unscheduled DNA synthesis (UDS) assay and the *in vivo* MutaMouse assay. It was considered by the authors that cytotoxicity was the major factor influencing the positive results seen with eugenol.

Jannuzzi et al. (2022) examined cytotoxic and genotoxic effects of eugenol on ultraviolet A (UVA, 320-400 nm)-induced damage using human keratinocyte cells (HaCaT). HaCaT cells were treated with increasing concentrations of eugenol (10-500 µM) for 1 hour and irradiated with 5, 10 or 15 J/cm^2 UVA. After 24 hours, the neutral red uptake (NRU) cytotoxicity assay indicated that eugenol caused a cytotoxic effect in a dose-dependent manner in HaCaT cells and increasing doses of UVA irradiation enhanced the cytotoxic effect of eugenol. The alkaline Comet assay was carried out immediately after the UVA irradiation to measure the genotoxic potential of eugenol. Eugenol caused DNA single-strand breaks and increasing doses of previous UVA irradiation aggravated the genotoxic potential of eugenol.

Based on the available information, eugenol was considered not to be mutagenic or genotoxic (EFSA, 2009a; Cox et al., 2019).

Toxicity in dental pulp fibroblasts of primary teeth *in vitro*

Escobar-García et al. (2016) determined the eugenol concentrations at which toxicity occurred in human dental pulp fibroblasts of primary teeth. Samples of primary dental pulp tissue were taken. Tissue samples were seeded by means of explant technique and used in the 4th–5th pass. Single Cell Gel Electrophoresis (Comet), phenazine MeThoSulfate (MTS), LIVE/DEAD® Cell Viability/Toxicity and trypan blue assays for evaluation of the cytotoxicity of increasing concentrations of eugenol (0.06 to 810 µM) were performed. The results of toxicity tests showed toxic effects on dental pulp fibroblasts, even at very low concentrations of eugenol (0.06 µM). The authors concluded that all of the concentrations of eugenol that were evaluated produced high toxicity in human dental pulp fibroblasts of primary teeth. According to Barboza et al. (2018), eugenol toxicity was observed in human dental pulp fibroblasts from deciduous teeth, with DNA damage at concentrations ranging from 0.06–5.1 μM, which was not observed at higher interval concentrations of 320 to 818 μM.

Acute toxicity
Oral LD50 values for eugenol have been reported as 3000 mg/kg bw in mice and as 2680 mg/kg bw and 1930 mg/kg bw in rats (Mizutani et al., 1991a).

Subacute toxicity
A subacute oral toxicity study (14 days) of eugenol was carried out by Dhara and Tripathi (2020) in Swiss albino mice, females (n = 5) and males (n = 5), to evaluate toxicological and behavioural effects (novelty suppressed feeding, novel object recognition, tail suspension test and social interaction test) of eugenol. The mice were administered vehicle (5% dimethyl sulfoxide (DMSO)), or 7.34 or 70 mg/kg bw eugenol, by gavage daily for 14 days. For hematological and biochemical analyses, blood was collected, and histological examinations were performed on liver and kidney. There were no significant differences in total red blood cells, total white blood cells, % of hemoglobin or % of hematocrit between the control and any of the two eugenol doses. The only statistically significant differences with eugenol from controls in hematological parameters were increased number of lymphocytes with low dose males and females, decreased numbers of neutrophils in high dose males and decreased numbers of eosinophils in high dose females and males. In the biochemical parameters, the only significant differences from controls were increased alkaline phosphatase (ALP) and decreased urea in low dose males and high dose females. Thus, these differences were mostly not consistent between doses and sexes. There were no alterations in food and water intake, organ weights (liver, kidney, spleen) or body weights, with any dose among the eugenol-treated animals. No abnormal changes in gross morphology of liver or kidney were noticed with histology. The behavioural tests displayed no significant behavioural effects of eugenol.

Ribeiro-Silva et al. (2022) examined effects of eugenol (7.8 mg per day in feed) for 28 days in adult female FVB/n mice (n = 5). Eugenol induced no signs of distress or behavioural changes in the mice, or in mental status or body weight, and showed only non-significant decreases in the food and water consumption. Eugenol had no effects on the absolute or relative weight of heart, lung, kidneys, spleen or liver, or on microhematocrit and biochemical parameters. Eugenol did not increase the genetic damage index (GDI) in the alkaline Comet assay. Eugenol had no significant effects on the activity of four antioxidant enzymes. Eugenol did not significantly affect the score of inflammatory infiltrate in liver, kidneys and lungs, necrosis/apoptosis in the liver, or centrilobular hypertrophy and karyomegaly in hepatocytes.

Liver toxicity
The low concentrations of eugenol and clove extracts used topically and in herbal products have not been convincingly linked to instances of liver injury in humans, either in the form of serum enzyme elevations or clinically apparent liver injury (PubChem, 2020). In high doses, however, eugenol appears to be a direct cytotoxin and several instances of severe acute liver and kidney injury have been reported after accidental overdose of eugenol-containing herbal products, largely in children. Overdoses (10-30 ml of clove oil) have been marked by the onset of agitation, decrease in consciousness and coma arising within hours on ingestion.

Adult male ddY mice were treated with eugenol, 400 (n = 6), 600 (n = 10) or 800 mg/kg bw (n = ?), in olive oil, by oral intubation, in combination with an inhibitor of glutathione (GSH) synthesis, DL-buthionine sulfoximine (BSO) as 4 mmol/kg bw, given i.p. 1 h before eugenol (Mizutani et al., 1991a). The negative controls (n = 4) were given saline + olive oil. The dose 800 mg/kg bw of eugenol in combination with BSO resulted in significant mortality (in 6 of 7 mice) within 3 h. Also the dose of 600 mg/kg bw eugenol and BSO gave high mortality (number not stated) during 3-5 h after receiving eugenol, when not terminated at 3 h. The mice given 600 mg/kg bw eugenol and BSO developed hepatotoxicity 3 h after eugenol administration, characterized by significant increases in relative liver weight, serum glutamic pyruvic transaminase (GPT) level, hepatic congestion (ml blood in the liver) and extensive centrilobular necrosis of hepatocytes. Two of 10

mice died in the group given 600 mg/kg bw of eugenol and BSO. After the dose of 400 mg/kg bw eugenol and BSO, none of the mice died and the only indication of hepatotoxicity was significantly increased serum GPT level. Eugenol (up to 600 mg/kg) alone without BSO produced no hepatotoxicity or mortality in the mice (n = 6). When using inhibitors of CYP450-dependent monooxygenase, carbon disulfide, methoxsalen or piperonyl butoxide, each abolished the hepatotoxicity of eugenol in combination with BSO, whereas phenobarbital or BNF (acronym not explained), inducers of CYP450 enzymes, enhanced the hepatotoxicity, suggesting that the hepatotoxicity was caused by a metabolite of eugenol activated by CYP450 enzymes.

When these experiments in adult male ddY mice (n = 4-6) were repeated with eugenol and the eugenol analogues methyleugenol, dihydroeugenol and chavibetol in olive oil in doses of 3.7 mmol/kg bw (equivalent to 600 mg/kg bw eugenol) by oral intubation in combination with pretreatment with BSO as above, none of the analogues gave significant changes in relative liver weight or liver blood volume, and only dihydroeugenol and chavibetol significantly increased serum GPT levels (Mizutani et al., 1991b). Thus, methylation of the free hydroxyl group in eugenol to give methyleugenol, or masking the hydroxyl group in eugenol by methylenedioxyphenyl ring closure to give safrole, resulted in complete loss of the hepatotoxicity, showing that the free hydroxyl group was essential for this effect. In addition, it appeared that the hydroxyl group must be located at the position para to an allyl substituent to develop the toxicity, because chavibetol, a positional isomer of eugenol, was not very hepatotoxic (with only a small, but significant increase in serum GPT levels). Replacement of the allyl group in eugenol by a propyl group, forming dihydroeugenol, markedly lowered the extent of liver damage that was only evident as a markedly increased GPT level. Thus, the saturation of the allyl substituent in eugenol seemed to alter the type and possibly the underlying mechanism of the liver damage. This result showed that the double bond (between C2' and C3') in the 4-allyl substituent also has a role in hepatotoxicity caused by eugenol.

By investigating chemical structures of hepatotoxic substances in herbs, He et al. (2019) found that alkaloids and terpenoids were the two major groups causing hepatotoxicity. They further identified eight major structural skeletons for hepatotoxicity and 15 structural alerts for hepatotoxicity. Among the eight skeletal categories, the sub-category phenylpropene-type simple phenylpropanooids contained i.a. eugenol, with well-documented hepatotoxicity.

Carvalho et al. (2022b) treated male Wistar rats (n = 5/group) with 10, 20 and 40 mg/kg bw of eugenol by gavage. After 60 days of treatment, liver samples were collected and analyzed by biometric, histological, biochemical and oxidative analyses. The results showed that 10, 20 and 40 mg/kg bw of eugenol did not alter body and liver weights, serum and hepatic alanine aminotransferase (ALT) levels and catalase, glutathione-S-transferase, or total, Ca^{2+} and Mg^{2+} ATPase activities in treated animals. However, 20 and 40 mg/kg bw of eugenol reduced Na^{+}/K^{+} ATPase pump activity and blood glucose levels. They also increased hepatic glycogen content, superoxide dismutase activity, ferric reducing antioxidant power, and nitric oxide and malondialdehyde levels. In addition, 20 and 40 mg/kg bw of eugenol caused structural and functional damage to the liver tissue of eugenol-treated rats. The authors concluded that 10 mg/kg bw of eugenol was a safe dose for consumption in long-term treatment of rats. Doses higher than 20 mg/kg bw lead to hepatic damage that could impair vital liver processes.

Carcinogenicity

Classification of carcinogenicity of eugenol: 1) Evidence in humans: No adequate data. 2) Evidence in animals: Limited evidence. Overall summary evaluation of carcinogenic risk to humans is Group 3: The agent is not classifiable as to its carcinogenicity to humans (IARC, 1985), thus, eugenol is not considered a carcinogenic compound (Martins et al., 2018).

Reproductive toxicity

Poli and Challa (2019) evaluated the antifertility efficacy of eugenol on female reproduction in 4 months old female Wistar albino rats (n = 6). The rats were administered saline as vehicle or eugenol (99% pure) by i.m. injection of 0.4 ml/day (the dose as mg/kg bw per day was not stated) by oral gavage for 15 days. Eugenol increased the duration of proestrus (+28.57%), metestrus (+22.22%) and diestrus (+16.00%) phases and the total duration of the estrous cycle (+16.82%). Eugenol did not affect the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) or prolactin. However, the testosterone levels were significantly decreased (-77.77%) and progesterone levels increased (+48.34%). Eugenol significantly increased the estradiol levels (+86.53%). The tissue somatic index (w/w %, per 100 g bw) of the ovary increased significantly (+31.70%), while no such significant effects were observed on the sex accessory tissues uterus and vagina by eugenol, which also elevated total ovarian (+68.20%) and uterine (+65.87%) proteins and reduced vaginal (-17.62%) proteins. The ovarian carbohydrates were significantly decreased (-20.89%), and the uterine (+19.13%) and vaginal (+39.42%) carbohydrates were increased by eugenol. The total lipids were increased significantly in ovary (+92.15%) and uterus (+59.14%), whereas in vagina these were decreased (-14.41%). The authors concluded that the administration of eugenol significantly increased the serum estradiol and progesterone levels leading to reduced frequency of ovulation and resulted in the impairment of fertility.

Comments:

The authors did not clearly demonstrate the relationship between the hormone profiles and the changes in estrous cycle, and the rats were not mated to test their fertility, thus, it is uncertain whether the observed small changes in estrous cycle may affect the reproduction. This study was not performed according to OECD guidelines for reproductive studies, such as guideline no. 415: One-Generation Reproduction Toxicity Study.

Carvalho et al. (2022a) administered 10, 20 and 40 mg/kg bw per day of eugenol by gavage to adult male Wistar rats (n = 10/group) for 60 days. Testis, epididymis and spermatozoa were analyzed with microscopic, biochemical and functional methods. Eugenol did not alter testicular and epididymal biometry and microscopy, including daily sperm production and sperm number. However, 20 and 40 mg/kg eugenol reduced serum testosterone levels. The highest dose altered lactate and glucose concentrations in the epididymis. All the eugenol doses diminished catalase (CAT) activity and malondialdehyde levels in the testis and increased total antioxidant capacity and CAT activity in the epididymis. Epididymal sperm from rats receiving 10, 20 and 40 mg/kg eugenol presented high Ca^{2+} ATPase activity and low motility. In conclusion, eugenol at 10-40 mg/kg bw doses per day negatively impacted the competence of epididymal sperm and modified oxidative parameters in male reproductive organs.

Allergy and other effects on the immune system

Contact allergies due to fragrances are common. Percentage of positive reactions to individual components in fragrance mix (FM) I in patients that tested positive to FM I were7.3% for eugenol (Schnuch and Griem, 2018). The absolute sensitization frequency was 0.74% for eugenol.

Eugenol is among the fragrances that need to be labelled in EU in cosmetics and household products if present at >10 ppm (0.001%) in leave-on products and >100 ppm (0.01%) in rinse-off products (de Groot, 2020). Prevalence of sensitization to eugenol was in the range of 0.3%–1.3% (average 0.68%) among 26 fragrances labelled in EU in routine testing. In 2008–2011, a random sample of the general population of 3119 individuals 18 to 74 years old in Sweden, Germany, the Netherlands, Portugal and Italy was patch-tested with the 14 ingredients of FM I and FM II. The percentages of positive reactions were 0.2% for eugenol. Eugenol was considered a prohapten, i.e. a substance that can be transformed into a hapten in the skin (bioactivation), usually via enzymatic catalysis. Eugenol was also listed as a substance that could cause pigmented cosmetic dermatitis, i.e. pigmentation of the face after having facial dermatitis. In addition to contact

dermatitis from use of cosmetics, contact dermatitis was also reported for eugenol from foods, spices and beverages, pharmaceutical products, household products and dentistry materials. Eugenol is also listed among substances that were reported to cause immediate-type reactions, mostly non-immune immediate contact reactions (contact urticaria), urticaria, photosensitivity reactions, asthma and/or rhinitis and oral lichen planus. Irritant contact dermatitis was also attributed to eugenol.

When volatile compounds with emphasis on volatile allergens were assessed in selected dried medicinal plants (not basil) in the Czech Republic with solid phase microextraction coupled with gas chromatography–mass spectrometry, among the compounds detected was eugenol (Burdejova and Vitova, 2020). Among others, eugenol and linalool are considered allergens causing contact dermatitis if present in cosmetic products, and linalool exceeded the safe value recommended by legislation for cosmetic products.

Comment:
It was confirmed that herbs may be sources of volatile allergens, but it is not known whether eugenol can cause allergy after oral consumption of food products. However, the data from de Groot et al. (2020) above and the case report below by Bui et al. (2019) indicate that this is possible.

A case report by Bui et al. (2019) referred to a 68-year-old woman investigated for lingua plicata (fissured tongue) and burning mouth syndrome for 2 years. Six months prior to the onset of her symptoms, she had a new prosthesis implanted in the upper jaw. She had been using mouthwash containing eugenol. Testing was performed and the patient reacted positively only on the mouthwash and eugenol 2% pet., but not to FM I, suggesting contact allergy to eugenol. The patient also reported that she ate yogurt sprinkled with cinnamon every morning, and that she chewed cloves (*Syzygium aromaticum*) to freshen her breath during the day. The patient was told to avoid all identified exposures. At follow-up after 8 weeks, the burning sensation was significantly reduced, whereas the lingua plicata remained. Eugenol is found in spices such as cinnamon and cloves, and is commonly used in dentistry as an analgesic and antimicrobial agent. Eugenol is also a commonly used fragrance component. In this case, eugenol was found in the patient's mouthwash and also constituted a part of her daily diet, causing the contact allergy.

Another case-report by Triviño et al. (2019) reported a 77-year-old woman, non-smoker, referred to a contact dermatitis clinic for recurrent oral aphthous stomatitis, associated with burning sensation of the mouth, of 10 months duration. The patient related the clinical lesions to a dental procedure (dental prosthesis) three weeks before onset of symptoms. Positive results for eugenol were observed in a test. Allergic contact stomatitis to eugenol contained in the cement (Temp-Bond) used as fixative of the dental prosthesis was diagnosed. The patient was referred to a dentist and the cement was replaced with Temp-Bond NE (eugenol-free). Follow-up at 6 months showed a complete resolution of the lesions, as well as the disappearance of the burning sensation in the mouth of the patient. The authors also reported that eugenol can be an irritant and is a sensitizer, and that allergic contact dermatitis to eugenol mainly occurs as hand eczema in dentists and dental assistants.

Eugenol has been considered a weak skin sensitizer in the OECD test guideline no. 429 for local lymph node assay (LLNA) and a moderate skin sensitizers in the modified local lymph node assay: 5-bromo-2-deoxyuridine-flow cytometry method (LLNA: BrdU-FCM), classified as Cat 1B (Ahn et al., 2016).

The phagocyte-microbe interaction in the immune system is a defense mechanism but when excessively or inappropriately deployed can harm host tissues and participate in the development of different non-immune and immune chronic inflammatory diseases such as autoimmune

problems, allergies, some rheumatoid disorders, cancers and others (Jantan et al., 2015). Plants may induce a non-specific immunomodulation, stimulating or suppressing or acting as adjuvant, with effects on various immune cells, such as macrophages, natural killer cells and granulocytes. Nair et al. (2019) summarized immunomodulatory effects of the plant *Ocimum sanctum*. Barboza et al. (2018) also summarized studies on modulation of inflammatory responses mediated by eugenol, claiming it to have anti-inflammatory and antioxidant properties.

Comment:
Whether immunomodulatory actions of plant extracts, eugenol or other bioactive plant substances may also cause adverse effects under certain circumstances in humans is not discussed in these publications.

Effects on the intestinal microbiome

Eugenol has antimicrobial activity, and thus, may also show adverse effects on the intestinal microbiota and impact strain biodiversity (Hu et al., 2018). It was shown *in vitro* that the beneficial commensal *F. prausnitzii* was more sensitive to eugenol at similar or even lower concentrations than pathogens *E. coli*, *S. enteritidis*, *S. typhimurium*, *C. difficile* and *C. perfringens*, and at the same concentration eugenol showed strong anti-bacterial action and anti-biofilm activity against both pathogenic and probiotic microorganisms. On the other hand, in the colon of mice eugenol treatment lead to an increase in abundance of specific families within the Clostridiales order and conferred colonization resistance to the enteric pathogen *Citrobacter rodentium*, which is beneficial in strengthening the mucosal barrier and protect against invading pathogens and disease.

Comment:
Based on this limited information it appears that eugenol may have both beneficial and adverse effects on the gut microbiota.

Other studies

Eugenol has inhibitory potential on butyrylcholinesterase (BuChE) with EC50 of 0.38 mM (Burcul et al., 2020).

Drug interactions

Mizutani et al. (1991a,b) showed that eugenol may be hepatotoxic in mice with glutathione-depleted livers. Thus, *Ocimum tenuiflorum* L. should be used with caution in patient taking drugs such as paracetamol (acetaminophen) that deplete glutathione.

Data to use in the risk characterization

Eugenol was considered unlikely to pose a significant mutagenic or genotoxic risk to humans under the intended conditions of their use as flavouring agents (EFSA, 2009a). Both JECFA and EFSA has evaluated eugenol as a flavouring substance, and an ADI of 0-2.5 mg/kg bw was established by JECFA in 1982 and maintained in 2005 (JECFA, 2006). EFSA established an ADI of 1 mg/kg bw per day (EFSA, 2012b). No newer studies were found in the literature search that would change these ADI values.

Eucalyptol

Risk assessments

A provisional tolerable daily intake (TDI) of 0.2 mg/kg bw was derived from a minimum lethal dose of 60 mg/kg bw for children and applying a safety factor of 300 by the Council of Europe in 2000 (SCF, 2002).

In a Research Institute for Fragrance Materials (RIFM) fragrance ingredient safety assessment of eucalyptol by Api et al. (2022b), it was stated that eucalyptol was not genotoxic. The calculated Margin of Exposure (MOE) was >100 for the repeated dose toxicity and reproductive toxicity endpoints. The data showed that eucalyptol was a sensitizer with a No Expected Sensitization Induction Level (NESIL) of 590 μg/cm^2. Eucalyptol was not expected to be phototoxic/photoallergenic.

Human studies

The primary terpenoid constituent in the essential oil of eucalyptus is the monoterpenoid (C_{10}) eucalyptol. Several clinical trials have been performed in persons affected with respiratory illnesses. Galan et al. (2020) summarized the adverse effects detected in these clinical trials of eucalyptol. Side effects of nausea, heartburn, exanthema, stomach ache and diarrhea were reported. However, differences between placebo and eucalyptol were either not statistically significant or not tested for significance. They also reported that other rare adverse effects had been identified in the literature, including contact allergy and skin reaction, vocal cord dysfunction and asthma exacerbation (considered likely more related to eucalyptus pollen rather than an extract). According to Galan et al. (2020) short-term use (up to 6 months) of eucalyptol at doses of 100-200 mg three times per day appeared to be safe based on clinical research, whereas larger doses of undiluted eucalyptus oil may be fatal.

Absorption, distribution, metabolism and excretion (ADME)

As summarized by SCF (2002), eucalyptol undergoes oxidation *in vivo* with the formation of hydroxycineole which is excreted as a glucuronide. In rats, 2-hydroxycineole, 3-hydroxycineole and 1,8-dihydroxycineol-9-oic acid were identified as main urinary metabolites. After oral administration to brushtail possums (*Trichosurus vulpecula*), *p*-cresol, 9-hydroxycineole and cineol-9-oic acid were found in urine. Rabbits given eucalyptol by gavage excreted 2-exo- and 2-endo-hydroxycineole as well as 3-exo- and 3-endo-hydroxycineole in the urine.

Irritation

Eucalyptol may cause skin irritation (PubChem, 2020).

Mutagenicity, genotoxicity and DNA repair

As summarized by SCF (2002), eucalyptol did not show mutagenic effects in the following strains of *Salmonella typhimurium* with or without metabolic activation: TA97a, TA98, TA100, TA102, TA1535 and TA1537. In CHO cells, eucalyptol did not induce chromosome aberrations with or without metabolic activation. Sister chromatid exchanges were induced in CHO cells only in the absence of metabolic activation at doses that induced cell cycle delay (at cytotoxic doses). Sister chromatid exchanges induced by mitomycin C in Chinese hamster ovary (CHO) K-1 cells were not increased by post-treatment with eucalyptol. The rec-assay in *Bacillus subtilis* did not give evidence for DNA damage.

Genotoxic/anti-genotoxic, mutagenic/anti-mutagenic and cytotoxic effects of the monoterpene eucalyptol were examined by Nikolić et al. (2015) in bacteria and mammalian cells using alkaline Comet assay, *Escherichia coli* K12 reversion test and MTT assay *in vitro*, respectively. When applied in low doses (up to 200 mM in bacterial assay and 50 mM in Comet assay) the monoterpene protected repair proficient *E. coli* and Vero cells against UV-induced mutagenesis and 4-nitroquinoline-1-oxide (4NQO)-induced DNA strand breaks, respectively. Anti-mutagenic response was not detected in nucleotide excision repair (NER) deficient bacteria. When eucalyptol was applied in higher doses, a weak mutagenic effect was found in mismatch repair (MMR) and NER deficient *E. coli* strains, while induction of DNA strand breaks in the Comet assay was evident in human fetal lung fibroblasts MRC-5, colorectal carcinoma HT-29 and HCT 116 cells, as well as in Vero cells. Moreover, the involvement of NER, MMR and RecBCD pathways in repair of DNA lesions induced by eucalyptol was demonstrated in *E. coli*. Eucalyptol was cytotoxic to MRC-5, HT-

29 and HCT 116 cells, with IC50 values (indicating 50% viability) of 11.0, 7.5 and 4.0 mM in the three cell types, respectively. The observed effects of eucalyptol were consistent with a hormesis response, characterized by a low dose beneficial effect and a high dose adverse effect. The authors concluded that eucalyptol was a mild genotoxic agent, but in low doses it enhanced DNA repair mechanisms, including NER, recombination and MMR, and induced anti-genotoxic effect.

Comment:
No *in vivo* studies on genotoxicity of eucalyptol were available. Based on the limited available information, eucalyptol was considered not be mutagenic or genotoxic.

Acute toxicity
In mice, the LD50 was estimated to be 3.67 and 3.75 ml/kg bw for two different eucalyptus oils, while in rats, the LD50 of eucalyptol was reported to be 1560 mg/kg bw and 2480 mg/kg bw (SCF, 2002; Galan et al., 2020).

Caldas et al. (2016) investigated the preclinical toxicity (acute and repeated dose) and reproductive toxicity of eucalyptol. In an acute toxicity study of female Swiss mice (n = 5), all died within 24 hours after a single oral dose of 2000 or 1750 mg/kg bw eucalyptol emulsified in 1% Tween-80 aqueous solution (vehicle and control), whereas the mice given 1500 mg/kg bw also showed sedation and trembling, but none died during 14 days of observation. Thus, the LD50 was between 1500 and 1750 mg/kg bw. Significant increased food and water consumption and body weight were also observed with eucalyptol versus controls after 14 days.

Subacute toxicity
In a NTP study, groups of 6 male and 6 female Fischer 344 rats received eucalyptol for 28 days either by stomach tube on 5 days/week at doses of 150, 300, 600 and 1200 mg/kg bw or in encapsulated form with the diet at concentrations of 3750, 7500, 15000 and 30000 mg/kg, equivalent to 381-3342 mg/kg bw per day for the male rats and to 353-3516 mg/kg bw per day for the female rats. At dose levels of 600 mg/kg bw and higher, dose-related decrease of body weight gain and absence of a normal degree of hepatic centrilobular cytoplasmic vacuolization were observed in male rats. In addition, other dose-related lesions in the liver, kidneys and parotid salivary glands were found at all dose levels in male rats fed encapsulated eucalyptol (Wolff et al., 1987a (NTP)). Thus, the NOAEL appeared to be 420 mg/kg bw per day based on the data from males (300 mg/kg bw recalculated for exposure 7 days a week instead of 5 days a week).

Groups of 10 male Wistar rats were given 0, 500 or 1000 mg/kg bw per day of eucalyptol (99% purity) by gavage for 28 days. Statistically significant decreases in the terminal body weight and increased relative liver and kidney weights were found in both dose groups, whereas the relative brain weight was increased only in the highest dose group. No macroscopic changes were seen. Only brain, liver and kidneys were examined histopathologically, showing no changes in the brain and minor focal infiltration of mononuclear cells in the liver among all groups. In kidneys, a dose-related accumulation of eosinophilic protein droplets containing α2u-globulin in the cytoplasma of proximal tubular epithelial cells was induced (Kristiansen and Madsen, 1995). Based on this experiment, a NOAEL could not be established.

Comment:
The α2u-globulin nephropathy in the kidneys of male rats is not considered relevant for humans.

In a NTP study, groups of 6 male and 6 female B6C3F1 mice were fed eucalyptol for 28 days either by stomach tube on 5 days/week at doses of 150, 300, 600 and 1200 mg/kg bw or in encapsulated form at concentrations of 3750, 7500, 15000 and 30000 mg/kg, equivalent to 600-5607 mg/kg bw per day for males and 705-6777 mg/kg bw per day for females. The liver weight/body weight ratio in males was increased at all but the lowest dose given in encapsulated form as was the brain

weight/body weight ratio in females at the top dose level. Microscopic examination revealed a minimal hypertrophy of centrilobular hepatocytes in animals of both sexes fed the encapsulated compound, especially at the two highest dose levels (Wolff et al., 1987b (NTP)). Thus, NOAEL appeared to be 840 mg/kg bw per day based on the data from males (600 mg/kg bw recalculated for exposure 7 days a week instead of 5 days a week).

Chronic toxicity/carcinogenicity

Eucalyptol was tested as one of the constituent of toothpaste in an oral long-term study with specific pathogen-free male CFLP mice (Swiss albino mice of the ICI strain) (Roe et al., 1979). Groups of 52 mice were given 8 or 32 mg eucalyptol/kg bw per day in 1 ml toothpaste base/kg bw per day by gavage 6 days/week from 10 weeks of age for 80 weeks followed by an observation period between 16 and 24 weeks depending on survival. The negative control group (n = 52) was either untreated or received a toothpaste base which lacked eucalyptol (vehicle control, n = 260). No treatment-related effects on survival, body weight, food consumption, behaviour, incidence of clinical signs, weight of adrenals, kidneys, liver, lungs or spleen, or on the microscopic appearance of brain, lungs, liver and kidneys and on the tumour incidence, were observed. Based on this experiment, a NOAEL of 37.3 mg/kg bw per day was established (corrected for exposure only 6 days/week).

In the repeated oral dose toxicity study, male and females Wistar rats (n = 10/sex) were given 0, 100, 500 or 1000 mg/kg eucalyptol emulsified in 1% Tween-80 aqueous solution (vehicle and control) by gavage daily for 50 days (Caldas et al., 2016). None of the doses gave any signs of toxicity, such as piloerection, changes in locomotor activity or deaths, but the two highest doses gave diarrhea in the first week and decreased body weight gain days 7-50. All doses affected the water and food intake in both sexes. The significant differences in hematological parameters were increase in the mean corpuscular volume with high dose in males, decrease in mean corpuscular hemoglobin concentration with middle and high dose, increase in platelets with the middle and high dose and decrease in mean platelet volume in all doses, in males. No significant hematological changes were seen in females. The significant differences in biochemical parameters were decrease in alkaline phosphatase (ALP) levels with the low dose in males and increased blood urea nitrogen in females with the middle and high dose. The only significant differences in organ weights were decrease in absolute weight of the lungs and spleen with the middle and high dose, respectively, in males, and increase in absolute and relative weight of the liver in females treated with the high dose. Histopathological analysis showed weak changes in the lungs (eosinophilic and lymphocytic infiltrate), liver (lymphocytic infiltrate) and kidneys (increase in glomerular space) in both sexes and in uterus (eosinophilic and lymphocytic infiltrate) in females. The results of the hematological and biochemical parameters indicated that the repeated dose treatment showed occasional alterations in rats of both sexes, however, the effects were not consistent among the sexes and were within the physiological ranges described for the species. Since the lower dose of eucalyptol (100 mg/kg bw) induced reduction of water consumption in both sexes and mean platelet volume and ALP levels in males, it was not possible to establish a NOAEL for chronic toxicity.

Reproductive toxicity

In the reproductive toxicity study by Caldas et al. (2016), eucalyptol (0, 250, 500 or 1000 mg/kg bw) was given to pregnant Wistar rats (n = 7-10) daily by gavage for 7 days during the preimplantation period (days 1-6 of pregnancy) or during the organogenesis period (days 7-14 of pregnancy). No deaths or changes in food and water intake were observed in the dams. All three doses produced a reduction in body weight in pregnant rats treated during the preimplantation or organogenesis periods, and the highest dose also when treated during the whole pregnancy (days 1-20). The highest dose induced a reduction in the mass of fetuses (pre-implantation) and a few dead fetuses (both periods) of pregnant rats. However, dead fetuses were also observed in the control group. There was also a reduction in the number of corpora lutea in females treated

with only the low dose during the organogenesis. The reproductive study indicated that eucalyptol may have maternal (reduced body weight gain) and possibly fetal toxicity (may be caused by the maternal decrease in weight gain). It was not possible to establish a NOAEL from this study.

dos Santos et al. (2021) investigated whether 1,8-cineole (eucalyptol) affected fertility and fetal development during the organogenic period in mice. Pregnant female rats (strain not specified) were given 134.6 mg/kg bw of eucalyptol by gavage from gestation day 6 to 15, a concentration proportional to in EO solutions. No signs of systemic toxicity, hemorrhage, abortion, differences in water and feed consumption, histopathological changes or differences in reproductive rate (number of pups per litter, post-implantation loss, birth rate etc.), were observed compared with the vehicle control (n = 12). Relative weight of spleen and liver vs. body weight was significantly increased. However, the treatment interfered with fetal development, seen as skeletal development delays, and the eucalyptol-treated rat fetuses (n = 10) had 95.1% skeletal abnormalities (poor ossification of the head, thoracic limbs and femurs) vs. 43.3% in controls.

Neurotoxicity

de Figueiredo et al. (2019) characterized the central effects of the *Hyptis martiusii* leaf essential oil (OEHM) and eucalyptol, the major sample compound (25.93%), using behavioural animal models. Female Swiss mice (n = 9) were given one i.p. injection of eucalyptol (50 mg/kg bw) and subjected to various tests (for most of them 30 minutes after the injection): open field, elevated cross maze, rotarod, sodium pentobarbital- or ethyl ether-induced sleep time, pentylenetetrazole-induced convulsions, haloperidol-induced catalepsy and ketamine-induced hyperkinesia. The results showed that eucalyptol reduced animal motility in the open field test, increased pentobarbital- and ethyl ether-induced sleep time, as well as death latency in the pentylenetetrazole-induced convulsion model. However, the tested compounds were devoid of anxiolytic-like and myorelaxant activity. In addition, eucalyptol potentiated haloperidol-induced catalepsy and reduced ketamine-induced hyperkinesia. Taken together, the results suggested OEHM has important hypnotic-sedative and antipsychotic-like effects, which appear to be due to the monoterpene eucalyptol, possibly through modulation of dopaminergic and glutamatergic systems.

Endocrine effects

Fouyet et al. (2022) examined placental toxicity and the potential endocrine-disrupting effects of 1,8-cineol (eucalyptol). It did not affect the viability of the JEG-3 human trophoblast cell line. It induced lower secretions of estradiol and human placental lactogen (hPL) than controls, but did not affect progesterone or human hyperglycosylated chorionic gonadotropin (h-hCG) levels, and did not activate the P2X7 cell death receptor.

Allergy and other effects on the immune system

In addition to contact dermatitis from use of cosmetics, contact dermatitis was also reported for eucalyptol from essential oils and pharmaceutical products (de Groot, 2020). Irritant contact mucositis was also attributed to eucalyptol (de Groot, 2020).

When volatile compounds with emphasis on volatile allergens were assessed in selected dried medicinal plants (not basil) in the Czech Republic with solid phase microextraction coupled with gas chromatography–mass spectrometry, among the compounds detected was eucalyptol (Burdejova and Vitova, 2020).

Comment:

It was confirmed that herbs may be sources of volatile allergens, however, it is not known whether eucalyptol can cause allergy after oral consumption of food products.

Eucalyptol has been shown to modulate the production of cytokines, both by reducing pro-inflammatory cytokines and/or activating anti-inflammatory cytokines, reducing inflammation, probably via NF-κB (Quintans et al., 2019).

Comment:
Whether immunomodulatory effects of eucalyptol on cytokines may also be adverse is not known.

Data to use in the risk characterization

Based on the limited available information, eucalyptol was considered not be mutagenic or genotoxic.

The available data were not sufficient to derive an ADI, however, the available animal data did not indicate a cause of concern associated with the daily intake from food, including hard candies, estimated from the small amount of information available (SCF, 2002). The case reports on acute toxicity in humans refer to the ingestion of eucalyptus oil and not to eucalyptol as such. They do not provide information for adequate estimates of toxic dose levels for eucalyptol (SCF, 2002; Bhowal and Gopal, 2015). A provisional TDI of 0.2 mg/kg bw was derived from a minimum lethal dose of 60 mg/kg bw for children and applying a safety factor of 300 by Council of Europe in 2000 (SCF, 2002).

Based on short-term use (up to 6 months) of eucalyptol at doses of 100-200 mg three times per day appears to be safe based on clinical research (Galan et al., 2020). Based on 200 mg x 3 = 600 mg/day, divided by 60 kg bw, is 10 mg/kg bw per day. Using an uncertainty factor of 10 for interindividual susceptibility in humans and a factor of 3 for short-term exposure, in total 30, gives a tentative safe level of 0.3 mg/kg bw per day.

In the subacute (28 day) oral toxicity studies in male rats and male mice performed by NTP, NOAEL values appeared to be 420 and 840 mg/kg bw per day, respectively (Wolff et al., 1987a,b (NTP)). Based on the lowest NOAEL value of 420 mg/kg bw per day and using an uncertainty factor of 300 gives a tentative safe level of 1.4 mg/kg bw per day.

Eucalyptol was tested as one of the constituent of toothpaste in an oral long-term (80 weeks followed by 16 to 24 weeks observation period) in mice (Roe et al., 1979). Based on this experiment, a NOAEL of 37.3 mg/kg bw per day was established. Using an uncertainty factor of 300, this gives a tentative safe level of 0.1 mg/kg bw per day.

Thus, the available data indicated that a tentative safe level of eucalyptol appear to be in the range of 0.1-1.4 mg/kg bw per day.

β-Caryophyllene

Risk assessments

In a Research Institute for Fragrance Materials (RIFM) ingredient safety assessment of β-caryophyllene by Api et al. (2022a), it was stated that β-caryophyllene was not genotoxic. The calculated Margin of Exposure (MOE) was >100 for the repeated dose toxicity and fertility endpoints. The developmental toxicity endpoints were evaluated using the Threshold of Toxicological Concern (TTC) for a Cramer Class I material, and the exposure to β-caryophyllene was below the TTC (0.03 mg/kg per day and 1.4 mg/day, respectively). Data showed that there were no safety concerns for β-caryophyllene for skin sensitization under the current declared levels of use. β-Caryophyllene was not expected to be phototoxic/photoallergenic.

Human studies

Alizadeh et al. (2022) studied the effect of β-caryophyllene on food addiction and its related behaviors in a randomized, double-blind, placebo-controlled trial. β-Caryophyllene is a dietary cannabinoid type 2 (CB2) receptor agonist. Women with obesity and food addiction, diagnosed by the Yale Food Addiction Scale Score (YFAS-S) ≥3, were randomly allocated to receive a β-caryophyllene softgel (n = 26) (100 mg daily with a meal) or placebo (n = 26) for 8 weeks. The compliance rate was high with 92% and 95% of softgels consumed in the intervention and placebo groups, respectively. Regarding side effects, two subjects in the β-caryophyllene group reported intestinal discomfort (nausea) and one subject reported headache, and they were excluded from the study. Serious adverse effects were not reported. No side effects were detected in the placebo group.

Comment:

For a person with 60 kg bw, the intake was 1.7 mg/kg bw per day.

***In silico* studies**

In computational analysis, using quantitative structure-activity relationships (QSAR), probabilistic reasoning, machine learning and human expert rule-based systems for hepatobiliary safety signals, adverse effects of β-caryophyllene, such as bile duct disorder, hepatotoxicity, liver necrosis and liver weight gain, were predicted in extract from chasteberry (Wang et al., 2011). For caryophyllene (no specification), jaundice and gall bladder disorder were predicted in extract from red clover.

Comment:

Basil plants were not included in this publication.

Toxicokinetics

He et al. (2018) reported pharmacokinetic profiles of β-caryophyllene alcohol (BCPA) (with purity >98.0%), an active metabolite of β-caryophyllene, in both sexes of Sprague-Dawley rats and beagle dogs (both n = 3/sex for both species). The rats were given 5, 10 and 20 mg/kg bw doses by i.v. administration (in 5% glucose containing 30% ethanol) or 100 mg/kg bw by i.g. administration (in 0.5% sodium carboxymethylcellulose and a PEG400 formulation). The dogs were given 20 mg/kg bw BCPA solution i.v. or 100 mg/kg BCPA solution or PEG400 formulation i.g. After i.v. administration, BCPA exhibited moderate volumes of distribution (*Vz*) ranging from 5.63 to 8.97 l/kg in rats and low *Vz* (2.89 ± 1.12 l/kg) in dogs. Systemic plasma clearance was high in both species, resulting in a short elimination half-life ranging from 29.6 to 48.3 minutes. In rats, the i.v. pharmacokinetics (C_{max} and $AUC_{(0\text{-infinite})}$) were dose-dependent in the range 5-20 mg/kg bw. The measured oral bioavailability was low in rats for BCPA solution (1.17 ± 0.78%), suspension (1.21 ± 0.33%) and PEG400 formulation (6.22 ± 2.63%). The bioavailability was lower in dogs for BCPA solution (0.12 ± 0.05%) and PEG400 formulation (0.25 ± 0.07%), indicating significant species difference. Contribution of phase II metabolism on the first-pass effects of BCPA was examined by enzymatic hydrolysis of BCPA glucuronide conjugates in rats given 40 mg/kg bw i.g. and in dogs given 100 mg/kg bw i.g. of BCPA solution. Treatment of plasma samples with β-glucuronidase increased the systematic exposure of BCPA as assessed from $AUC_{(0\text{-infinite})}$ by 24.7- or 2.62-fold in rats and dogs, respectively, suggesting glucuronidation was a significant metabolic pathway for BCPA, possibly due to first-pass metabolism.

Irritation

β-Caryophyllene caused irritation in intact or abraded rabbit skin, but tested at a concentration of 4% in petrolatum, it was not irritating in a 48 h closed-patch test in 25 human subjects (Opdyke, 1973).

Mutagenicity and genotoxicity

β-Caryophyllene (JECFA Flavouring no. 01.007 1324) was found to be non-mutagenic in multiple *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, TA1537 and TA1538 up to 150000 µg/plate, and did not induce sister chromatid exchange in Chinese hamster ovary (CHO) K-1 cells up to 204.4 µg/ml or unscheduled DNA synthesis in rat hepatocytes up to 10000 µg/ml (EFSA, 2009b). It was also negative in concentrations of 1-100 µg/ml in the *in vitro* micronucleus test in human peripheral lymphocytes (Di Sotto et al., 2010).

Nararak et al. (2020) investigated phototoxic and genotoxic effects of β-caryophyllene oxide in Balb/c 3T3 mouse fibroblasts (3T3-L1) and Chinese hamster ovary cell line (CHO-K1), respectively. The results demonstrated that β-caryophyllene oxide did not show any phototoxic potential (photo-irritation factor (PIF) = 0.38, PIF <2 predicts no phototoxicity) nor was there any significant genotoxic response as indicated by no increase in micronucleated cells with or without metabolic activation.

EFSA (2015) concluded that β-caryophyllene was not mutagenic or genotoxic.

Acute, subacute and subchronic toxicity

The acute, subacute and subchronic toxicity studies on β-caryophyllene are summarized in Table 10 and described in detail below the table.

Table 10. Acute, subacute and subchronic toxicity of β-caryophyllene.

Study	Species/strain/sex	Effects	Substance	Exposure	NOAEL/ Comments
Acute toxicity					
Opdyke (1973)	Rats	Acute oral LD50 was >5 g/kg bw	β-Caryophyllene (no further information)		
Kaur et al. (2018)	Adult Wistar rats, males and females (n = 5/sex)	No adverse effects were observed	β-Caryophyllene (no further information)	Single oral administration of 2000 mg/kg bw, observed for 14 days	Performed according to OECD guidelines nos. 420 and 423. NOAEL was 2000 mg/kg bw for both sexes.
Oliveiro et al. (2018)	Adult Swiss mice, females (n = 6)	No adverse effects observed with either dose	β-Caryophyllene (purity ≥98.5%), emulsified in 0.05% Tween 80 and dissolved in 0.9% saline (vehicle)	Single administration of 300 or 2000 mg/kg bw by oral gavage, observed for 14 days	Performed according to OECD guideline no. 423. NOAEL was 2000 mg/kg bw.
Al-Taee et al., 2019	Adult Wistar albino rats, males (n = 15)	No adverse effects were observed	β-Caryophyllene diluted in olive oil	One dose of 100 mg/kg bw given i.p. daily for five days, terminated day 6	NOAEL was 100 mg/kg bw per day.
Subacute toxicity					
Schmitt et al. (2016)	Adult Wistar rats, males and females (n = 5/sex)	No adverse effects were observed	β-Caryophyllene oil with approximately 77% of β-caryophyllene, 1.28% eugenol and eugenol derivatives and 21.72% of other	700 mg/kg bw, given by oral gavage daily for 28 days	NOAEL was 700 mg/kg bw per day for both sexes.

			essential oils (all as wt/wt %)		
Kaur et al. (2018)	Adult Wistar rats, males and females (n = 5/sex)	No adverse effects were observed with either dose	β-Caryophyllene (no further information)	300, 600 and 900 mg/kg bw per day given orally? daily for 28 days	Performed according to an OECD guideline (no. not stated). NOAEL was 900 mg/kg bw per day for both sexes.
Oliveira et al. (2018)	Adult Swiss mice, females (n = 7)	No adverse effects observed with either dose	β-Caryophyllene (purity ≥98.5%), emulsified in 0.05% Tween 80 and dissolved in 0.9% saline (vehicle)	300 and 2000 mg/kg bw given by gavage daily for 28 days	Performed according to OECD guideline no. 407. NOAEL was 2000 mg/kg bw per day.
Subchronic toxicity					
Schmitt et al. (2016)	Adult Wistar rats, males and females (n = 10/sex), including a 21-day recovery period in control and high-dose groups (both n = 5/sex)	No adverse effects were observed with any dose	β-Caryophyllene oil with approximately 77% of β-caryophyllene, 1.28% eugenol and eugenol derivatives and 21.72% of other essential oils (all as wt/wt %)	150, 450 and 700 mg/kg bw day given by oral gavage daily for 90 days	Performed according to OECD guideline no. 408 and GLP. NOAEL was 700 mg/kg bw per day for both sexes. The corresponding author D. Schmitt was contracted to assist in the authorship of the publication, and R. Levy and B. Carroll were current employees of Primus Pharmaceuticals Inc., the sponsor of the toxicity study and

					manufacturer of β-caryophyllene.
Bastaki et al. (2020), evaluated also by **EFSA (2014b)**	Adult CRL Sprague-Dawley CD IGS rats, males and females (n = 10/sex)	Toxicological findings in hematology, coagulation and clinical chemistry in males, liver pathology and infiltration of erythrocytes within sinuses of mesenteric lymph nodes in both sexes and effects in female kidneys that were not explained by the species-specific effects (α2u-globulin) seen in males	β-Caryophyllene (purity 88.91%) given in the diet	Mean intake of β-caryophyllene based on body weight and food consumption data was 0, 222, 456 and 1367 mg/kg bw per day for males and 0, 263, 1033 and 4278 mg/kg bw per day for females, given in the diet for 90 days	Performed according to OECD guideline no. 408 and GLP. EFSA (2014b) concluded on this study: NOAEL was 222 mg/kg bw per day for males and 263 mg/kg bw per day for females of β-caryophyllene. The study was conducted at a contract research organization product safety lab under the sponsorship of the International Organization of the Flavor Industry (IOFI).

Acute toxicity

The acute oral LD50 for β-caryophyllene in rats was reported as >5 g/kg bw (Opdyke, 1973).

Kaur et al. (2018) performed an acute oral toxicity test of 2000 mg/kg bw of β-caryophyllene (no further information) in adult male and female Wistar rats (n = 5/sex) according to the relevant OECD guidelines nos. 420 and 423, and this dose was found to be non-toxic after the 14-day observation period.

An acute toxicity study of β-caryophyllene (purity ≥98.5%) emulsified in 0.05% Tween 80 and dissolved in 0.9% saline (vehicle)) in adult female Swiss mice was conducted according to OECD guideline no. 423 by Oliveiro et al. (2018). Doses of 300 and 2000 mg/kg bw were given once orally (both doses, n = 6). Controls were given vehicle (n = 6). The mice were observed for 14 days. There was absence of adverse clinical signs and mortality in all mice. In addition, no significant changes in body weight, food and water intake, oxidative stress biomarkers, hematological and biochemical parameters were observed when compared to the controls. Thus, the oral administration of 300 or 2000 mg/kg bw per day induced no acute toxic effects.

β-Caryophyllene in olive oil (100 mg/kg bw) was administered i.p. daily for five days to male Wistar albino rats (n = 15), which were terminated the sixth day (Al-Taee et al., 2019). No significant effects of β-caryophyllene were seen on their body weight, levels of serum creatinine kinase-MB, malondialdehyde, superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), tumour necrosis factor (TNF)-α, interleukin (IL)-6, interleukin (IL)-1β, inducible nitric oxide synthase (iNOS) or cyclooxygenase (COX)-2, or on NF-κB activation or expression of γ-H2AX, Bax, Bcl12, p53 or active caspase-3. The muscle fibres in their heart showed no degeneration or inflammatory cells.

Subacute toxicity

Schmitt et al. (2016) also included a 28-day interim sacrifice (subacute study) with the control and 700 mg/kg bw groups (both n = 5/sex) in a subchronic (90 day) toxicity study (see below) of β-caryophyllene oil by oral gavage in adult male and female Wistar rats. The β-caryophyllene oil contained approximately 77% of β-caryophyllene, 1.28% eugenol and eugenol derivatives and 21.72% of other essential oils (all as wt/wt %). The oil was well tolerated and did not produce any significant toxic effects.

Kaur et al. (2018) performed a repeated dose (28 days) (oral?) toxicity study of 300, 600 and 900 mg/kg bw per day of β-caryophyllene in adult male and female Wistar rats (n = 5/sex) according to an OECD guideline (no. not stated). Mortality was not observed in any dose and the body weights were not significantly affected. No changes in biochemical or hematological parameters that were considered treatment-related or toxicologically relevant, and no abnormal changes in organ weights, were observed. No abnormalities were detected by histopathology of liver, spleen, heart, kidney, pancreas and lung after 900 mg/kg bw. The food intake was not affected by the 900 mg/kg bw dose.

Oliveira et al. (2018) studied repeated dose (28 days) oral toxicity of β-caryophyllene emulsified in 0.05% Tween 80 dissolved in 0.9% saline (vehicle) in adult female Swiss mice and analysed changes in body weight, food intake, water intake, hematological and biochemical parameters, organ weight after necropsy, oxidative stress markers and histopathology of various tissues. The repeated oral dose (300 (n = 7) and 2000 (n = 7) mg/kg bw) toxicity study was performed according to OECD guideline no. 407. The control group was given the vehicle (n = 7). After 28 days of daily treatment, there was absence of adverse clinical signs and mortality in all mice. In addition, no significant changes in body weight, food and water intake, oxidative stress biomarkers, hematological and biochemical parameters were observed when compared to the control group. The authors concluded

that β-caryophyllene could be considered a compound with toxicity at doses above 2000 mg/kg bw.

Oral administration of 300 mg/kg bw of β-caryophyllene daily for 4 weeks to hypercholesterolemic adult male Wistar rats (n = 6-7), previously given a high cholesterol and fat diet for two weeks, induced hepatomegaly, as significantly increased relative liver weight (g/100 g bw), whereas 30 and 100 mg/kg bw did not (Harb et al., 2018).

Comment:
This study was not considered relevant for risk assessment in healthy persons.

Subchronic toxicity

Schmitt et al. (2016) performed a subchronic (90 day) toxicity study of β-caryophyllene oil by oral gavage in adult male and female Wistar rats in the doses 0, 150, 450 and 700 mg/kg bw day (n = 10), including a 21-day recovery period in the control and high-dose groups (both n = 5). The study was conducted in accordance with OECD guideline no. 408 and good laboratory practice (GLP). The β-caryophyllene oil contained approximately 77% of β-caryophyllene, 1.28% eugenol and eugenol derivatives and 21.72% of other essential oils (all as wt/wt %). The oil was well tolerated and did not produce any significant toxic effects, shown by the absence of major treatment-related changes in the general condition and appearance of the rats, neurobehavioral end points, growth, feed and water intake, ophthalmoscopic examinations, routine hematology and clinical chemistry parameters, urinalysis and necropsy findings. Sporadic differences observed between treated and control groups were not considered adverse or specifically related to β-caryophyllene oil because the differences were not biologically or clinically significant, were of a small magnitude, were within the normal range for the clinical pathology parameters and/or were not accompanied with pathological findings, according to the authors. They concluded that the no observed adverse effect level (NOAEL) was the highest dose level of 700 mg/kg bw per day for both male and female rats.

Comment:
The authors declared that the corresponding author D. Schmitt was contracted to assist in the authorship of the publication, and that R. Levy and B. Carroll were current employees of Primus Pharmaceuticals Inc., the sponsor of the toxicity study and manufacturer of β-caryophyllene.

Two independent subchronic 90-day GLP-compliant studies were conducted according to OECD guideline no. 408 in CRL Sprague-Dawley CD IGS rats (7-8 weeks) of both sexes (n = 10/sex) with β-caryophyllene (CAS no. 87-44-5, JECFA No. 1324, FEMA No. 2252 and FL No. 01.007) (purity 88.91%) or β-caryophyllene epoxide (purity 96.4%) by Bastaki et al. (2020). *Based on the information in the publication, it appears that the second substance used here called β-caryophyllene epoxide is what is often called β-caryophyllene oxide, based on the CAS no. 1139-30-6, JECFA No. 1575, FEMA No. 4085, FL No. 16.043).* Dietary concentrations of β-caryophyllene were 0, 3500, 7000 and 21000 ppm (mg/kg) for males (target doses of 0, 250, 500 and 1500 mg/kg bw per day) and 0, 3500, 14000 and 56000 ppm for females (target doses 0, 250, 1000 and 4000 mg/kg bw per day). Dietary concentrations of β-caryophyllene epoxide were 0, 1750, 10500 and 21000 ppm (target doses of 0, 125, 750 and 1500 mg/kg bw per day for both sexes). Based on body weight and food consumption data from the study (days 0-91), the mean intake of β-caryophyllene was 0, 222, 456 and 1367 mg/kg bw per day for males and 0, 263, 1033 and 4278 mg/kg bw per day for females. The mean intake of β-caryophyllene epoxide was 0, 108.8, 672.2 and 1397.9 mg/kg bw per day for males and 0, 136.9, 799.8 and 1660.4 mg/kg bw per day for females. There were no

deaths or clinical toxicity attributed to either substance. Statistically significant, dose-dependent reductions in body weight, body weight gain, food consumption and food efficiency at the highest dietary concentrations of β-caryophyllene, but not of β-caryophyllene epoxide, were attributed to palatability issues. Neither β-caryophyllene nor β-caryophyllene epoxide influenced estrous cyclicity or sperm parameters (sperm mobility, epididymal sperm count, homogenization-resistant sperm count and percent abnormal sperm). Macroscopic and microscopic findings were related to changes in the kidneys of male rats, consistent with α2u-globulin nephropathy. Microscopic findings were similar for both β-caryophyllene and β-caryophyllene epoxide and included dose-dependent increases in incidence and severity of nephropathy and tubular cytoplasmic droplets in kidneys of all treated males and correlated with enlarged kidneys and increased kidney weights. The intensity of the nephropathy ranged from minimal to slight with concentration-dependent trend. The second changes observed were in the liver of male and female rats. In both studies, dose-dependent increases at the middle and high intake levels in incidence and severity of centrilobular to midzonal-distributed hepatocellular hypertrophy in both sexes were considered related to the test substances. The incidence was higher in females in the β-caryophyllene study, but similar in males and females in the β-caryophyllene epoxide study. These changes correlated with increased absolute and relative organ weights. In the absence of degenerative histopathological abnormalities, the hepatic changes were considered indicative of adaptive response to the increased metabolic load in the middle and high doses, which was not expected to occur in humans at the levels consumed as flavourings in food according to the authors. In addition, minimal to slight infiltration of erythrocytes within sinuses of mesenteric lymph nodes in both sexes was considered to be related to test substances, but was considered a non-adverse subclinical change by the pathologist in the absence of bleeding in the gastrointestinal tract. Other changes were not dose-dependent, small in magnitude, within the range of historical control values for this strain, sex and age of rats and/or had no correlating histopathology. Since the kidney findings were a species- and sex-specific effect, the NOAEL in each study was based on hepatocyte hypertrophy at the two highest dietary concentrations and were determined to be 222 mg/kg bw per day for β-caryophyllene and 109 mg/kg bw per day for β-caryophyllene epoxide by the study authors.

Comments:
The α2u-globulin nephropathy in the kidneys of male rats is not considered relevant for humans. These studies were conducted at a contract research organization product safety lab under the sponsorship of the International Organization of the Flavor Industry (IOFI).

In the publication by Bastaki et al. (2020), it is stated that upon review of these studies, European Food Safety Authority (EFSA) considered both the **kidney** and **liver effects** to be species-specific (EFSA, 2014b). Hepatocyte hypertrophy reported in the β-caryophyllene epoxide study was considered to be non-adverse evidence of a metabolic adaptation mechanism to high test substance levels. However, this interpretation was not fully endorsed for β-caryophyllene, pending confirmation by measurements of relevant hepatic enzymes. Regardless of the interpretation of hepatocyte hypertrophy, EFSA agreed with the NOAEL derivation for β-caryophyllene and β-caryophyllene epoxide equal to the lowest dietary level of each substance, albeit based on different interpretation of the findings. The NOAEL values would then be 222 and 263 mg/kg bw of β-caryophyllene for males and females, respectively. The NOAEL values would then be 109 and 137 mg/kg bw of β-caryophyllene epoxide for males and females, respectively. The stated basis for the NOAEL for β-caryophyllene was the reported toxicological findings in hematology, coagulation and clinical chemistry in males, along with the liver pathology and the mesenteric lymph node findings in both sexes and effects in female kidneys that were not explained by the species-specific effects seen in males. The stated basis for the NOAEL for β-caryophyllene epoxide

was the reported erythrocyte infiltration into the sinusoids of the mesenteric lymph nodes, which could not be dismissed. However, erythrocyte infiltration into the sinusoids of the mesenteric lymph nodes was only observed at the highest intake level of β-caryophyllene epoxide, and on this basis alone the NOAEL would be equal to the middle dietary level (10500 ppm) in this study (equivalent to 672 mg/kg bw per day in males).

Reproductive toxicity

Endpoints of male and female rat reproductive function were also included in both studies by Bastaki et al. (2020). The administration of either β-caryophyllene or β-caryophyllene epoxide in the diet did not influence estrous cycle pattern in females, based on mean estrous cycle length and the number of cycles assessed in two intervals during the study (weeks 6-7 or 12-13). No effects on male reproductive parameters were reported from intake of either substance based on sperm morphology, epididymal sperm count, homogenization-resistant spermatid count and motility measurements. Thus, in this study, β-caryophyllene or β-caryophyllene epoxide did not appear to induce adverse effects on male or female reproduction in doses up to 1367 and 4278 mg/kg bw per day of β-caryophyllene and up to 1397.9 and 1660.4 mg/kg bw per day of β-caryophyllene epoxide, in male and female rats, respectively.

Allergy

β-Caryophyllene caused no sensitization reactions when tested at a concentration of 4% in petrolatum in a maximization test carried out on 25 volunteers (Opdyke, 1973).

In addition to contact dermatitis from use of cosmetics, contact dermatitis of β-caryophyllene was also reported from occupational exposure to essential oils (de Groot, 2020).

When volatile compounds with emphasis on volatile allergens were assessed in selected dried medicinal plants (not basil) in the Czech Republic with solid phase microextraction coupled with gas chromatography–mass spectrometry, among the compounds detected was β-caryophyllene (Burdejova and Vitova, 2020).

Comment:

It was confirmed that herbs may be sources of volatile allergens, however, it is not known whether β-caryophyllene can cause allergy after oral consumption of food products.

Drug interactions

Evaluation of possible interactions with enzymes of drug metabolism is an important part of studies on safety of biologically active compounds. Nguyen et al. (2017) tested the inhibitory effects β-caryophyllene and its derivative β-caryophyllene oxide (BCPO) and its isomer α-humulene against enzymes involved in metabolism and xenobiotic detoxification in subcellular hepatic fractions of Wistar rats and humans. *It is stated in the publication that in BCPO, the olefin of β-caryophyllene has become an epoxide, but since no CAS no. is given, it is not clear exactly which substance was used in this study.* Xenobiotics undergo metabolic biotransformations, followed by degradation and subsequent elimination. An inhibitory effect exerted by these substances on drug metabolizing enzymes may increase considerably the levels of drugs in the organism, with prolonged duration of action and potentially increased toxicity and severe side effects. Thus, the doses must be reduced for drugs taken at the same time as enzymatic inhibitors. All three substances, with BCPO as the most efficient, significantly and strongly inhibited the cytochrome P450 enzymes CYP3A (mainly)/2B (partly) with a non-competitive mechanism in both species, with slightly lower effect in humans vs. in rats. The isomer CYP3A4 specifically was inhibited weakly by β-

caryophyllene in humans. CYP3A metabolizes about 50% of all drugs, such as ciclosporin, paclitaxel and statins, as well as sexual hormones (testosterone). The extent of the inhibition of CYP1A2 was strongest with BCPO, and was greater in rat microsomes (with a non-competitive mechanism) than in human microsomes (with a competitive mechanism). Only BCPO could be regarded as an inhibitor in humans, however, with a low potency. Finally, none of the three terpenes significantly inhibited CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 or CYP2E1 when tested in humans, or the carbonyl reductase (CBR-1), NADPH-quinone oxireductase (NQO1), aldo-keto-reductases (AKR1A og AKR1C), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT) or glutathione *S*-transferase (GST), in either humans or rats.

Lnenicková et al. (2018) studied effects of β-caryophyllene oxide (BCPO) (CAS no. not stated) on expression of xenobiotic-metabolizing enzymes in liver and small intestine of mice *in vivo*. Adult male NMRI mice were given a single dose of 50 mg/kg bw BCPO in sunflower oil by gavage and the controls were given the vehicle sunflower oil. The mice were terminated after 6 or 24 h (both n = 6). Liver and small intestine were obtained, and subcellular cytosol and microsomal fractions were prepared for studies of activity of phase I (cytochrome P450 enzymes CYP1A1, CYP1A2, CYP2B, CYP2C9, CYP3A and the carbonyl reducing enzymes carbonyl reductase (CBR) 1/3, aldo-keto-reductase (AKR) 1A1 and AKR1C), and phase II (UDP-glucuronosyl transferase (UGT), glutathione *S*-transferase (GST) and sulfotransferase (SULT)) metabolic enzymes using specific substrates. BCPO markedly increased CYP2B, CYP3A and CYP2C activity. CYP2B mRNA was increased in the liver, and CYP2B, CYP3A13 and CYP3A25 mRNA were increased in the small intestine. Liver also showed elevated activity of AKR1C and CRB after the treatment. BCPO decreased NADPH-quinone oxidoreductase 1 (NQO1) activity in small intestine. None of the three phase II enzymes were affected by BCPO. Induction of CYP enzymes may affect bioavailability and efficacy of concurrently or subsequently taken drugs, thus, serious herb-drug interactions may be expected.

Spicáková et al. (2019) investigated interactions of the sesquiterpene β-caryophyllene oxide (CAS no. not stated), a metabolite of β-caryophyllene, with CYP3A4, an important enzyme in drug metabolism in humans, by *in vitro* and *in silico* studies. The results indicated a possibility for different mode of interaction within the active site of CYP3A4. Further docking experiments showed β-caryophyllene oxide to bind to the CYP3A4 active site and cause a significant decrease of binding affinity of the substrates tested, which corresponded well to the inhibition studies. However, according to the authors, the inhibition observed does most probably not pose a real harm to microsomal drug metabolism as the levels of sesquiterpenes in plasma do not usually exceed the micromolar range when these compounds are used as spices or flavouring additives. Hence, the interaction of drugs metabolized by CYP3A4 with sesquiterpenes was considered less probable.

Other studies

β-Caryophyllene belongs to the cannabinoid family, which are ligands of the cannabinoid receptors. Cannabinoid receptors CB1-R and CB2-R are metabotropic receptors that are G protein (protein binding GTP)-coupled receptors, involved in the regulation of neurotransmitters responsible for maintaining energetic balance, in metabolism and in the immune response (Francomano et al., 2019). β-Caryophyllene is a selective phytocannabinoid agonist of type 2 receptors (CB2-R), and is not psychogenic due to the absence of an affinity to cannabinoid receptor type 1 (CB1) (Gertsch et al., 2008; Francomano et al., 2019).

Data to use in the risk characterization

The data available indicated that β-caryophyllene was not mutagenic or genotoxic (EFSA, 2015), and no data indicating carcinogenic effects were found. Thus, the substances can be assessed as a non-genotoxic, non-carcinogenic substance. However, no TDI value has been established.

Using the lowest NOAEL value from the subchronic studies (Table 10), which was 222 mg/kg bw per day for male rats in the study by Bastaki et al. (2020), as evaluated by EFSA (2014b), and an uncertainty factor of 300, a tentative health-based guidance value (HBGV) for β-caryophyllene could be estimated at 0.74 mg/kg bw per day.

The randomized, double-blind, placebo-controlled trial by Alizadeh et al. (2022), reported that no serious adverse effects were observed after exposure to 100 mg per day of β-caryophyllene softgel (1.7 mg/kg bw per day) for 8 weeks.

Ursolic acid

Human studies

According to Woźniak et al. (2015), ursolic acid has been through three phase I trials in China administered i.v. as liposomes, due to poor water solubility and low bioavailability, to evaluate its safety and adverse effects in patients as a pharmaceutical against cancer. Ursolic acid liposomes showed tolerable toxicity and adverse effects, only one of 108 patients reported third grade adverse activity. The most frequent complaints were nausea, diarrhea and skin problems. The common conclusion based on all studies was the necessity of the continuation of research during phase II tests.

Comment:
The relevance of the ursolic acid liposomes administered i.v. for use in food supplements is not known.

A phase I, open-label, single-centre dose-escalation study of ursolic acid liposomes (freeze-dried powder in 5% glucose, containing 3 mg of active drug) as a pharmaceutical was carried out in China (Wang et al., 2013). In total, 63 persons were included; 4 patients with advanced solid tumours and 35 healthy volunteers in the toxicity study (each cohort of at least three persons) given a single dose (11, 22, 37, 56, 74, 98 and 130 mg/m^2), and 24 healthy volunteers in the pharmacokinetic study (each cohort of eight persons) given a single dose (37, 74 and 98 mg/m^2), as a 4-hour i.v. infusion. A maximum tolerated dose (MTD) of 98 mg/m^2 was derived and the dose-limiting toxicity (DLT) was liver toxicity and diarrhea. Other adverse events included grade 1 nausea, grade 2 abdominal distention, grade 1 microscopic hematuria, grade 2 elevated serum sodium, grade 1 vascular stimulation and grade 1 skin rash. The ursolic acid liposomes showed a linear pharmacokinetics with dose.

Comment:
The relevance of the ursolic acid in the form of liposomes that were administered i.v. for use in food supplements is not known.

Absorption, distribution, metabolism and excretion (ADME)

Ursolic acid is almost insoluble in water and has difficulty in crossing biological membranes and limited penetration through gastrointestinal mucosa, and thus, low oral bioavailability (Jinhua, 2019). Various delivery systems have therefore been invented, such as nano-emulsions, mesoporous silica nano-particles, solid lipid nanoparticles, liposomes,

niossomal gels and solid dispersions, which change the pharmacokinetic properties of ursolic acid. Ursolic acid was absorbed by the gastrointestinal tract mainly by passive diffusion. After oral administration, ursolic acid was distributed in lungs > spleen > liver > cerebrum > heart > kidneys. Ursolic acid was rapidly eliminated by metabolism, mainly by CYP3A4 and CYP2C9 enzymes. Only a small part of ursolic acid was eliminated by excretion in the kidneys.

Genotoxicity

In vitro: Ursolic acid did not induce DNA damage in the human hepatoma cell line (HepG2) at 0-25 µM in the alkaline Comet assay (Ramos et al., 2008) and induced no DNA damage (strand breaks and oxidized purines) in CaCo-2 cells treated with 5 or 10 µM ursolic acid for 24 h in the alkaline Comet assay (Ramos et al., 2010). Ursolic acid induced no genotoxic effects in human lymphocytes and Chinese hamster fibroblast (V79) cells in doses of 5, 10, 25, 50 and 100 µM in the alkaline Comet assay or in the micronucleus test Bacanli et al., 2017).

In vivo: The frequencies of bone marrow micronuclei in mice (n = 5) after 1.2, 2.3 and 4.6 mg/kg bw of an ursolic acid extract were 1.8, 2 and 2, respectively (Lu et al., 2009). The chromosomal aberration rates in bone marrow of mice (n = 5) treated with the same doses of the extract were 1.0%, 1.2% and 1.2%, respectively. For both genotoxicity tests, the results were not significantly different from the negative control, but were significantly different from a positive control, confirming the sensitivity of the tests.

Comment:

Based on the limited available information, ursolic acid was not considered to be genotoxic.

Acute toxicity

An acute oral toxicity test conducted in mice according to the Horn's method identified the LD50 value of an ursolic acid extract to be 9.26 g/kg bw (Lu et al., 2009).

Mishra et al. (2021) evaluated acute toxicity of ursolic acid according to the OECD guideline 423 limit test in five adult female Swiss albino mice and found no mortality during two weeks of observation after a single oral dose of 2000 mg/kg bw. Thus, the oral LD50 was >2000 mg/kg bw.

Subacute toxicity

Mishra et al. (2021) evaluated 28-day subacute oral toxicity of ursolic acid according to the OECD guideline 407 in Wistar rats of both sexes (n = 10, 5/sex). After exposure to 1, 5 or 10 mg/kg bw ursolic acid, it was found dose-dependent elevation of serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase (ALP), and elevation of neutrophil count and urea with 10 mg/kg bw. White blood cells, lymphocyte, platelet and hemoglobin counts were found to be low. The eosinophil count in male rats was normal whereas females treated with 10 mg/kg bw showed decreased eosinophil count. Histopathological examinations of body organs revealed alterations in the architecture of the liver, kidney and spleen with 10 mg/kg bw. No effects were seen with the two lowest doses, indicating a NOAEL of 5 mg/kg bw of ursolic acid. However, all the changes were recoverable as evident in a satellite group, indicating reversible effects.

Subchronic toxicity

Geerlofs et al. (2022) performed a repeated dose (90-day) oral toxicity study of ursolic acid in Han-Wistar rats of both sexes. A solution was made by dissolving ursolic acid in a mixture of 0.1% Tween 80 and 0.5% hydroxypropyl methylcellulose in Milli-Q Water. The control

group received the vehicle and the test groups received doses of 100, 300 or 1000 mg/kg bw per day via oral gavage. Ursolic acid did not cause any deaths, abnormal body weights or abnormal pathology at any test doses. No toxicological changes were observed in behaviour, neurotoxicity, coagulation, haematology or clinical chemistry that were related to the administration of ursolic acid. The NOAEL in this study was likely to be higher than 1000 mg/kg bw per day (the highest dose tested).

Reproductive and developmental toxicity

Akbarsha et al. (1998) examined the effects of daily treatment of ursolic acid (5 mg/kg bw i.p. for 15 days) dissolved in a minimum amount of ethanol and diluted in 0.9% saline (vehicle and control) to adult male Wistar albino rats (n = 10). They reported that this resulted in severe disruption of spermatogenesis. The most diagnostic change in the seminiferous epithelium was the opening of the intercellular bridges between the male germ cell clones, resulting in the formation of symplasts (multinucleated cells created either by the fusion of cells into one cytoplasmic mass or by the division of the nucleus of a single cell), comparable to cytochalasin D. The cells in the symplasts do not develop into spermatozoa. Symplasts were exfoliated from the Sertoli cell. The Leydig cells showed hypertrophy. The seminal vesicles were not much affected. Cauda epididymidal sperm motility was impaired and several sperm exhibited abnormalities; they were agglutinated tail-to-tail, with adherent cytoplasmic droplets and detached heads. Among the epididymal epithelial cell types, the clear cells of the caput as well as the cauda appeared to be increased in abundance and were rounded-up. The results indicated that regarding male reproduction, caution is required in using ursolic acid as a curative/protective agent.

During spermatogenesis, spermatozoa lose much of their cytoplasm from a residual body that is phagocytosed by the Sertoli cells. Spermatozoa leaving the testis contain a small droplet of cytoplasm, called the cytoplasmic droplet, which they release during transit through the epididymis before reaching the cauda epididymidis. The cytoplasmic droplet shows P450 aromatase activity, which plays a role in synthesis of estrogen from androgen. In the study by Akbarsha et al. (2000), adult male Wistar albino rats were administered ursolic acid (purity 90%), dissolved in a minimum quantity of ethanol and diluted in 0.9% NaCl, as i.p. injections of 25 mg/kg bw per day for 48 days. The controls were given vehicle. Segments of the epididymis were subjected to histopathological and ultrastructural analyses. It was found that 95% of the spermatozoa residing in the lumen of the cauda epididymidis retained the cytoplasmic droplet after treatment with ursolic acid, which was significantly different from the control group, where none of the spermatozoa retained this droplet. The motility of the spermatozoa released from the cauda epididymidis was significantly impaired after ursolic acid treatment compared with the controls.

Srinivasulu and Changamma (2017) studied the effect of ursolic acid (5 mg/kg bw per day for 20 days, purity not stated) on spermatogenesis in adult male Wistar rats (n = 6). The administration route was not stated, but was likely oral. The controls were given distilled water (1 ml/day) orally for 20 days (n = 6). Ursolic acid did not affect the body weight. Ursolic acid reduced the weight (presumably absolute weight) of paired testes (-12.98%), paired epididymis (-25.00%) and paired seminal vesicles (-11.29%) and prostate gland (-9.52%), however, only paired epididymis and seminal vesicles were significantly reduced at $P < 0.01$. Ursolic acid caused negligible reductions in the sperm count (-0.3%), sperm motility (-0.94%) and sperm viability (-3.1%) at this dose. Ursolic acid did not significant affect hormone levels (follicle-stimulating hormone (FSH) (+1.84%), luteinizing hormone (LH) (+6.5%) and testosterone (-5.05%). Thus, except for the reduction in weight of epididymis and seminal vesicles, no adverse effects of ursolic acid were found, i.e. on sperm count, motility and viability, or on the levels of FSH, LH or testosterone.

Geerlofs et al. (2020) studied effects of ursolic acid on fetal development, adult reproductive system and organs. Ursolic acid was dissolved in a 0.5% hydroxypropyl methylcellulose and 0.1% Tween 80 in Milli-Q Water solution. Doses of 100, 300 or 1000 mg/kg bw per day of ursolic acid or vehicle were administered orally in two experiments: for 15 days to adult male and female Han Wistar rats (n = 5 of each sex/group), and to pregnant female Sprague-Dawley rats on days 6-20 of gestation. In total, 30 rat offspring were used per group, combined from a preliminary and a second experiment. None of the treatments caused deaths or resulted in abnormal relative to body weight reproductive organ weights or body weight, and no differences in gross pathology in any reproductive or major other organs, at any doses in males or females. There were no significant toxicological changes in either dams nor fetuses in terms of body weight, organ weights, food consumption, gross pathology, sex organs, maternal or fetal performances, or in malformation or ossification of the fetuses. The NOAEL was 1000 mg/kg bw per day (the highest dose tested) in both experiments.

Neurotoxicity

Ursolic acid showed antidepressant-like effects in adult male Swiss mice (n = 7-10) in two predictive tests of antidepressant property, the tail suspension test (TST) (with 0.01 and 0.1 mg/kg, p.o.) and the forced swimming test (FST) (10 mg/kg, p.o.) (Machado et al., 2012). Ursolic acid did not cause significant alterations in the locomotor and exploratory activities. Using receptor agonists, the results also indicated that the antidepressant-like effect of ursolic acid in the TST was likely mediated by an interaction with the dopaminergic system, through the activation of dopamine D1 and D2 receptors.

Effects on the immune system

In review publications on therapeutic effects of ursolic acid and its putative mechanisms of action by Kashyap et al. (2016) and Bacanli et al. (2018), some undesirable pro-inflammatory effects of ursolic acid *in vitro* and *in vivo* were summarized. Studies in macrophages suggested increased expression of inducible nitric oxide synthase (iNOS) and tumour necrosis factor (TNF)-α mRNA via NF-kβ activation, increased release of macrophage migration inhibitory factor (MIF) was observed by activating extracellular signal regulated kinase (ERK)-2, and significant increased expression of cyclooxygenase (COX)-1, COX-2 and TNF-α was seen in mouse skin, as well as upregulated pro-inflammatory mediator interleukin-1β (IL-1β) in ursolic acid-treated macrophages and colonic mucosa of ICR mice.

Drug interactions

Ursolic acid is a competitive inhibitor of UDP-glucuronosyltransferase 1A3, binds to and inhibit CYP2C19 and CYP3A4 enzymes, and it inhibits the uptake of the lipid lowering drug rosuvastatin and hence can lead to therapeutic failure (Suroowan et al., 2021).

Other studies

Feng et al. (2020) treated 3T3-L1 adipocytes, female diet-induced obese (DIO) mice (n = 5/group) and male lean mice (n = 6-7/group), strain C57BL/6J, with ursolic acid or vehicle. The results showed that ursolic acid increased the expression of interleukins and chemokines, but not tumour necrosis factor (TNF)-α, in both adipocytes and adipose tissue. Interleukin (IL)-6 and monocyte chemokine protein (MDP)-1 levels in the cell culture medium and mouse serum were induced by ursolic acid treatment. Cd14 expression level and number of CD14+ monocytes were higher in ursolic acid-treated adipose tissue than in controls. Glucose tolerance test was impaired by ursolic acid treatment in DIO mice. Mechanistically, ursolic acid induced the expression of Toll-like receptor 4 (Tlr4) and the

phosphorylation levels of extracellular regulated protein kinases (ERK) and nuclear factor kappa B (NFκB) in adipocytes. In conclusion, short-term ursolic acid administration might impair adipose tissue insulin sensitivity by recruiting CD14+ monocytes.

Data to use in the risk characterization

Based on the limited available information, ursolic acid was not considered to be genotoxic. There were not sufficient data to establish a TDI value.

A subchronic oral 90-day study of Geerlofs et al. (2022) in rats indicated a NOAEL of >1000 mg/kg bw. A reproductive toxicity study by Geerlofs et al. (2020) also indicated a NOAEL of >1000 mg/kg bw based on effects on both sexes of rats. Using an UF of 300, a tentative safe level for ursolic acid would be 3.3 mg/kg bw per day.

Risk characterization

Plant material

Plant material used in tea

It was assumed by NIPH that the plant material of holy basil used in the teas given as examples of such products sold on the Norwegian market by NFSA, was dried. However, no further information was given for these products on which part(s) of the plants were used (only the leaves or the whole plant), where the plants were grown etc., that would affect the content of active substances in the plant material.

According to information from NFSA, the content of dried material of holy basil in products on the Norwegian market may be 0.6-2.0 g per tea bag. For teas, it was assumed that the hazard data on effects of **aqueous extracts** would be most comparable and relevant.

Based on the available information, **reproductive toxicity** appears to be the most critical adverse effects from intake of holy basil. Four original studies gave information on reproductive toxicity in experimental animals after exposure to aqueous extracts of holy basil (Vohora et al. (1969); Panda and Kar (1998); Leigh and Fayemi (2008); Verma et al. (2016)) (Table 9). Only three of these studies gave quantitative data in dose per mg/kg bw. Vohora et al. (1969) reported that no implantation sites were detected in the uterus of female rats exposed to 100 mg/kg bw on gestational day (GD) 1-4 (in 3 of 5 rats) or exposed to 200 mg/kg bw on GD 1-7 (in 2 of 5 rats). Panda and Kar (1998) reported significantly increased relative weights of all the sexual organs in male mice given 500 mg/kg bw per day for 15 days. Verma et al. (2016) reported significant decreased sperm counts and motility of spermatozoa, and significant increased mortality of spermatozoa, after 10-50 days of treatment, and decreased weight of reproductive organs after 20-50 days of treatment, in male mice given 250 mg/kg bw of the aqueous extract. Since this last study observed clearly adverse effects on male reproduction at the lowest dose, it was used further in the risk characterization.

Based on a body weight of 60 kg and intake of one cup of tea with one tea bag with holy basil per day, the exposure to the active substances in holy basil for an adult person could be 10-33 mg/kg bw per day from the tea, assuming that all of the active substances are extracted by and ends up in the hot water as a worst-case scenario due to lack of more specific data (Table 1). If two or three cups of tea were drunk per day, the estimated exposure would be 20-66 or 30-99 mg/kg bw per day, respectively (Table 1).

For non-genotoxic substances, a 100-fold uncertainty factor (UF) composed of a factor 10 for variation in susceptibility between species and a factor 10 for interindividual variation in susceptibility between humans, is routinely applied to the no observed adverse effect level (NOAEL) from an animal study to derive a health-based guidance value (HBGV) for humans, such as acceptable daily intake (ADI), used for intentionally added substances, or tolerable daily intake (TDI), used for unintentionally substances, i.e. contaminants (EFSA, 2005).

If assuming that the plant material as such is not genotoxic and employing the usual UF of 100, a HBGV can be estimated as 100 mg/kg bw/100 = 1 mg/kg bw per day. Thus, even intake of one cup of tea with holy basil may be a risk for embryo implantation loss in pregnant women. For effects on male reproduction, 250 mg/kg bw/100 = 2.5 mg/kg bw. Thus, even intake of one cup of tea with holy basil may be a risk for impaired reproduction in males.

Alternatively, margin of exposure (MOE) can be estimated from the daily dose giving an adverse effect in experimental animals divided by the human exposure (EFSA, 2005). MOE values are usually considered safe in risk assessments of non-genotoxic substances with a threshold of effect if they are above 100 (EFSA, 2005). If estimating MOE from the lowest observed adverse effect level (LOAEL) divided by the human exposure, 100 mg/kg bw divided by 10-33 mg/kg bw (for one cup of tea), will give a MOE of only 3-10. Since LOAEL is used instead of NOAEL, the MOE values could be 1000 instead of 100, or even 3000, if using an additional factor of 3 since the exposure in the animal experiment was not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 10-33 mg/kg bw, MOE is 8-25, thus, also too low. Estimations based on more than one cup of tea per day, will make the MOE values even lower.

These calculations may be overestimating risk, if not all of the active substances causing the adverse effects are extracted by and ends up in the hot water, or humans are less sensitive than rats and mice to these reproductive toxicity effects. It is not known which substances in the plant material that are responsible for the adverse reproductive effects (see the limited information available in the chapters on Reproductive toxicity for the individual substances in holy basil), and thus, toxicokinetics differences between rats or mice and humans for these substances cannot be taken into consideration.

The studies used in the estimations above are old, with less than optimal reporting and not performed according to OECD guidelines for reproductive studies. From the literature search, a study on reproductive toxicity effects in rats performed according to OECD guideline no. 415 was found with the commercial product OciBest™, extracted with methanol and water from the whole plant *O. sanctum* Linn. (Raina et al., 2018). The authors stated that the NOAEL level was 1000 mg/kg bw, which would indicate a safe level of 1000 mg/kg bw/100 = 10 mg/kg bw, a level that could be reached by one cup of tea. However, the relevance of this extraction method compared with only water and the use of the whole plant versus only leaves, which is most often used, is unknown. In addition, five of the authors of this publication work in the company owning the trademark OciBest™, clearly having a conflict of interest.

Considering all the uncertainties and taking all the published publications on reproductive toxicity into account, precaution is warranted regarding intake of teas with holy basil for pregnant women and individuals of both sexes wanting to become parents.

Plant material in food supplements

It was stated from NFSA that for two examples of food supplements sold on the Norwegian market, the plant material was dried, however, no further information was given for these products on which part(s) of the plants were used (only the leaves or the whole plant), where the plants were grown etc., that would affect the content of active substances in the plant material. It was also stated that extracts of plant material could be used in food supplements, however, no further information on which extract(s), amount of extract(s) or other processing, was given.

According to information from NFSA, the recommended daily doses of food supplements (dried plant) varied from 60-360 mg in one product and from 800-1800 mg in another product.

Based on a body weight of 60 kg, and assuming intake of the recommended daily doses of food supplements, the exposure to the active substances in holy basil for an adult person could be 1-30 mg/kg bw per day of the dried plant from these two food supplements (Table 1).

For food supplements containing dried basil plant material, with lack of more specific information, it was assumed that similar scenarios as for aqueous extracts of the plant material as in teas above could be used for oral intake of dried plant material.

For non-genotoxic substances, and using a 100-fold uncertainty factor composed of a factor 10 for variation in susceptibility between species and a factor 10 for interindividual variation in susceptibility between humans, a HBGV for humans can be estimated as 100 mg/kg bw/100 = 1 mg/kg bw per day. Thus, daily intake of food supplements with holy basil in the recommended doses may be a risk for embryo implantation loss in pregnant women. For effects on male reproduction, 250 mg/kg bw/100 = 2.5 mg/kg bw. Thus, daily intake of food supplements with holy basil may be a risk for impaired reproduction in males.

Alternatively, if estimating MOE from the LOAEL divided by the human exposure, 100 mg/kg bw divided by 1-30 mg/kg bw of food supplements, will give a MOE of only 3.3-100. Since LOAEL is used instead of NOAEL, the MOE values could be 1000 instead of 100, or even 3000, for including an additional factor of 3 since the exposure in the animal experiment is not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 1-30 mg/kg bw, MOE is 8-250, thus, also too low.

Considering all the uncertainties and taking all the published publications on reproductive toxicity into account, precaution is warranted regarding intake of food supplements with holy basil for pregnant women and individuals of both sexes wanting to become parents.

Preparations with basil plant material in general

If taking a more general view independent of type of preparation of the basil plant, based on the reproductive toxicity studies available using various preparations of *O. tenuiflorum* L./*O. sanctum* L. performed in three species and both sexes of experimental animals, in spite of many of the studies being old and with weaknesses, it seems reasonable to conclude that they may have adverse effects on reproduction in both males and females in doses in the dose range of 100-4000 mg/kg bw per day when administered during gestation or for 14-90 days to non-pregnant individuals. Using an UF of 100, this indicates that a general safe level for all types of preparations of these basil plants may be below 1 mg/kg bw per day.

The estimations performed are very imprecise and have large uncertainty. For descriptions of further information needed to perform more accurate quantitative risk assessments of preparations of basil plant material, see the Chapter *Uncertainties*.

Individual substances

Methyleugenol

Exposure to methyleugenol was estimated to be 0.17-0.57, 0.34-1.14 and 0.52-1.70 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.02-0.10 and 0.23-0.52 mg/kg bw for the two food supplements, respectively.

Since methyleugenol is genotoxic and carcinogenic, the risk from intake of methyleugenol in tea and food supplements should be assessed by calculating the margin of exposure (MOE), as recommended by EFSA (2005). No BMDL values calculated for methyleugenol were found. However, Sanner et al. (2001) calculated a T25 value for methyleugenol of 53.6 mg/kg bw per day, apparently based on hepatocellular carcinomas in male F344/N rats from the two-year carcinogenicity study by NTP (2000), which was used to calculate MOE. When the dose in animals is calculated as T25, MOE should be >25000 for the exposure levels to be considered safe (EFSA, 2005).

Table 11. Estimated margin of exposure (MOE) for methyleugenol in tea or food supplements on the Norwegian market (based on a T25 of 53.6 mg/kg bw).

Holy basil product	**1 cup of tea**	**2 cups of tea**	**3 cups of tea**	**Food supplement 1**	**Food supplement 2**
Tea bag	94-315	47-158	32-103	-	-
Food supplement	-	-	-	536-2680	103-233

For intake of one, two or three cups of tea per day, there is a risk of adverse effects of methyleugenol based on a T25 <25000.

For intake of both food supplements, there is a risk of adverse effects of methyleugenol based on a T25 <25000.

Mahony et al. (2020) derived an adjusted oral TTC value of 10 µg of plant material on a dry weight basis/person per day (0.17 µg/kg bw per day for a person of 60 kg bw) for assessment of potentially genotoxic substances in botanicals.

Based on exceedance of this TTC value, there is a risk of adverse effects of methyleugenol for intake of one, two or three cups of tea per day and for intake of one of the food supplements.

Estragole

Exposure to estragole was estimated to be 0.05-0.17, 0.10-0.33 and 0.15-0.50 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.005-0.03 and 0.07-0.15 mg/kg bw for the two food supplements, respectively.

Since estragole is considered genotoxic and carcinogenic, the margin of exposure (MOE) should be calculated, and should be at least 10000 when based on a $BMDL_{10}$ value (EFSA,

2005). $BMDL_{10}$ values calculated for incidence of hepatomas in female mice exposed for 12 months via diet to estragole based on data from Miller et al. (1983), varied between 9 and 33 mg/kg bw per day (EFSA ESCO Report, 2009).

Table 12. Estimated margin of exposure (MOE) for estragole in tea or food supplements on the Norwegian market (based on range of $BMDL_{10}$ from 9-33 mg/kg bw).

Holy basil product	**1 cup of tea**	**2 cups of tea**	**3 cups of tea**	**Food supplement 1**	**Food supplement 2**
Tea bag[a]	53-180	27-90	18-60	-	-
Tea bag[b]	194-660	100-330	66-220	-	-
Food supplement[a]	-	-	-	300-1800	60-129
Food supplement[b]	-	-	-	1100-6600	220-471

Based on range of $BMDL_{10}$ from 9[a]-33[b] mg/kg bw).

For intake of one, two or three cups of tea per day, there is a risk of adverse effects of estragole based on a MOE <10000 using the whole range of calculated $BMDL_{10}$ values.

For intake of both food supplements, there is a risk of adverse effects of estragole based on a MOE <10000 using the whole range of calculated $BMDL_{10}$ values.

Alternatively, based on exceedance of the suggested TTC value for genotoxic plant materials of 0.17 µg/kg bw per day for a person of 60 kg bw derived by Mahony et al. (2020), there is a risk of adverse effects of estragole from intake of one, two or three cups of tea per day for the tea product with the highest content only and from intake of none of the food supplements.

Eugenol

Exposure to eugenol was estimated to be 0.12-0.41, 0.25-0.82 and 0.37-1.23 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.01-0.07 and 0.17-0.37 mg/kg bw for the two food supplements, respectively.

Eugenol was considered unlikely to pose a significant mutagenic or genotoxic risk to humans under the intended conditions of their use as flavouring agents (EFSA, 2009a). Both JECFA and EFSA have evaluated eugenol as a flavouring substance, and an ADI of 0-2.5 mg/kg bw was established by JECFA in 1982 and maintained in 2005 (JECFA, 2006). EFSA established an ADI of 1 mg/kg bw per day (EFSA, 2012b). No newer studies were found in the literature search that would change these ADI values.

Intake of one or two cups of tea with eugenol per day did not exceed any of the ADI values. However, if using the highest level reported in tea and an intake of three cups of tea per day, the ADI established by EFSA (2012b) will be exceeded, but not the ADI established by JECFA (2006). For intake of eugenol calculated from the two food supplements, none of the ADI values were exceeded.

Eucalyptol

Exposure to eucalyptol was estimated to be 0.05-0.17, 0.10-0.33 and 0.15-0.50 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.005-0.03 and 0.07-0.15 mg/kg bw for the two food supplements, respectively.

The available data were not sufficient to establish a TDI for eucalyptol. However, a tentative safe level appeared to be in the range of 0.1-1.4 mg/kg bw per day based on the available data.

One, two or three cups of tea with the highest basil content exceeded the lower end of this range, and so did the highest intake of one of the two food supplements. Also the intake of two or three cups of tea with the lowest basil content was at or exceeded the lower end of this range. None of the exposures to tea or food supplements exceeded the highest end of this range.

β-Caryophyllene

Exposure to β-caryophyllene was estimated to be 0.005-0.02, 0.01-0.03 and 0.01-0.05 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.0005-0.003 and 0.006-0.01 mg/kg bw for the two food supplements, respectively.

The data available indicated that β-caryophyllene was not mutagenic or genotoxic (EFSA, 2015), and no data indicating carcinogenic effects were found. Thus, the substances can be assessed as a non-genotoxic, non-carcinogenic substance. However, no TDI value has been established.

Using the lowest NOAEL value from the subchronic studies (Table 10), which was 222 mg/kg bw per day for male rats in the study by Bastaki et al. (2020), as evaluated by EFSA (2014b), and an uncertainty factor of 300, including an additional factor of 3 since the exposure in the animal experiment was not chronic, a tentative health-based guidance value (HBGV) for β-caryophyllene could be estimated at 0.74 mg/kg bw per day.

Based on this tentative HBGV value for β-caryophyllene, there was not a risk of adverse effects of β-caryophyllene from intake of one, two or three cups of tea per day or from intake of any of the two food supplements.

The same conclusions are reached based on the randomized, double-blind, placebo-controlled trial by Alizadeh et al. (2022), in which no serious adverse effects were observed after exposure to 100 mg per day of β-caryophyllene softgel (1,7 mg/kg bw per day) for 8 weeks.

Ursolic acid

Exposure to ursolic acid was estimated to be 0.20-0.66, 0.40-1.32 and 0.60-1.98 mg/kg bw per day for one, two or three cups of tea, respectively, and 0.02-0.12 and 0.27-0.60 mg/kg bw for the two food supplements, respectively.

Based on the limited data found, ursolic acid is apparently not genotoxic. There were not sufficient data to establish a TDI value. However, a subchronic oral 90-day study of Geerlofs et al. (2022) in rats indicated a NOAEL of >1000 mg/kg bw. A reproductive toxicity study by Geerlofs et al. (2020) also indicated a NOAEL of >1000 mg/kg bw based on effects on both sexes of rats. Using an UF of 300, a tentative safe level for ursolic acid would be 3.3 mg/kg bw per day.

Based on the two available studies above, there was not a risk of adverse effects of ursolic acid from intake of one, two or three cups of tea per day or from intake of any of the two food supplements.

Answers to the terms of reference

1. To assess what is the limit for a safe daily or weekly intake of the green parts of holy basil (*Ocimum tenuiflorum* L.) (except the seeds), also including the substances methyleugenol and estragole.

Many factors are causing large variations in both composition (which substances) and concentrations (levels of each substance) in the plants *O. tenuiflorum* L./*O. sanctum* L. Without knowledge of the exact composition and levels of active and potentially adverse substances in such plant material, a safe exposure level for such plant material in general cannot be established with any certainty.

Based on the available data, reproductive toxicity, observed as impaired spermatogenesis in males, disturbed estrous cycle and loss of embryo implantation in uterus in females, as well as changes in weight and structure of reproductive organs, changes in hormone levels, reduced sexual mating behaviour and reduced fertility of both males and females, appeared to be the most critical adverse effects from intake of intake of *O. tenuiflorum/O. sanctum*. However, no teratogenic effects in the offspring were reported after exposure to basil plants. Based on these reproductive toxicity studies performed in three species and both sexes of experimental animals, in spite of many of them being old and with weaknesses, it seem reasonable to conclude that various preparations of *O. tenuiflorum/O. sanctum* may have adverse effects on reproduction in both males and females in doses in the dose range of 100-4000 mg/kg bw per day when administered during gestation or for 14-90 days to non-pregnant individuals. Using an uncertainty factor of 100, this indicate that a general safe level for all types of preparations of these basil plants may be below 1 mg/kg bw per day.

This estimation is very imprecise and has large uncertainty. For descriptions of further information needed to perform more accurate quantitative risk assessments of preparations of basil plant material, see the Chapter *Uncertainties*.

Some data indicate that the adverse effects of some substances such as the genotoxic and carcinogenic alkenylbenzenes present in plant material from *O. tenuiflorum/O. sanctum* may be counteracted by other plant constituents, thus, lowering risk of adverse effects (see the Chapter *Hazard from individual substances in basil plants*). However, the impact of such matrix effects cannot be taken into consideration quantitatively in this risk assessment without further data.

For genotoxic and carcinogenic substances such as estragole and methyleugenol, a safe level cannot be established as such, but can be estimated by calculating the MOE values, i.e. the ratio between the dose showing an adverse effect in experimental animals and the human exposure. Depending on whether the dose in animals is calculated as $BLMD_{10}$ or as T25, MOE should be >10000 and >25000, respectively, for the exposure levels to be considered safe (EFSA, 2005).

2. To establish a safe exposure level for the green parts of holy basil from herbal teas, food supplements and used as spice, if possible.

Without knowledge of the exact composition and levels of active and potentially adverse substances in the plant material from *O. tenuiflorum* L./*O. sanctum* L. used in teas or food

supplements, a safe exposure level for such products cannot be established with any certainty.

However, based on the available data on adverse effects of holy basil, attempts have been made to assess the risk associated with intake of basil plant material as such and of some of the individual substances from teas and food supplements sold on the Norwegian market.

Based on the available information, **reproductive toxicity** appears to be the most critical adverse effects from intake of holy basil. Vohora et al. (1969) reported that no embryo implantation sites were detected in the uterus of female rats exposed to 100 mg/kg bw on gestational day (GD) 1-4 (in 3 of 5 rats) or exposed to 200 mg/kg bw on GD 1-7 (in 2 of 5 rats). Verma et al. (2016) reported significant decreased sperm counts and motility of spermatozoa, and significant increased mortality of spermatozoa, as well as reduced weight of reproductive organs, in male mice given 250 mg/kg bw of the aqueous extract for 10-50 days. These reproductive toxicity studies in animals given aqueous extracts of *O. sanctum* L. were used to estimate human risk from intake of teas and food supplements with holy basil.

Herbal teas

For teas, it was assumed that the hazard data on effects of aqueous extracts would be most comparable and relevant.

Plant material

If assuming that the plant material as such is not genotoxic and employing the usual uncertainty factor (UF) of 100, a HBGV in humans can be estimated as 100 mg/kg bw/100 = 1 mg/kg bw per day. Thus, intake of one cup of tea with holy basil in the given amounts may be a risk for pregnant women. For effects on male reproduction, 250 mg/kg bw/100 = 2.5 mg/kg bw. Thus, daily intake of one cup of tea with holy basil may be a risk for impaired reproduction in males.

Alternatively, margin of exposure (MOE) can be estimated from the daily dose giving an adverse effect in experimental animals divided by the human exposure (EFSA, 2005). MOE values are usually considered safe in risk assessments of non-genotoxic substances with a threshold of effect if they are above 100 (EFSA, 2005). If estimating MOE from the lowest observed effects level (LOAEL) divided by the human exposure, 100 mg/kg bw divided by 10-33 mg/kg bw (for one cup of tea), will give a MOE of only 3-10. Since LOAEL is used instead of NOAEL, the MOE values could be 1000 instead of 100, or even 3000, if including an additional factor of 3 since the exposure in the animal experiment was not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 10-33 mg/kg bw, MOE is 8-25, thus, also too low. Estimations based on more than one cup of tea per day, will make the MOE values even lower.

Considering all the uncertainties and taking all the published publications on reproductive toxicity into account, precaution is warranted regarding intake of teas with holy basil for pregnant women and for both men and women wanting to become parents.

Individual substances

For intake of one, two or three cups of tea per day, there is a risk of adverse effects of **methyleugenol** based on a T25 <25000. Further, if assessed based on exceedance of a threshold of toxicological concern (TTC) value suggested by Mahony et al. (2020) for potentially genotoxic substances in botanicals of 10 µg of plant material on a dry weight

basis/person per day (0.17 µg/kg bw per day for a person of 60 kg bw), there is a risk of adverse effects of methyleugenol for intake of one, two or three cups of tea per day.

For intake of one, two or three cups of tea per day, there is a risk of adverse effects of **estragole** based on a MOE <10000 using the whole range of calculated $BMDL_{10}$ values. Further, based on exceedance of the suggested TTC value of 0.17 µg/kg bw per day, there is a risk of adverse effects of estragole from intake of one, two or three cups of tea per day for the tea product with the highest content only.

Intake of one or two cups of tea with **eugenol** per day did not exceed any of the ADI values of 2.5 or 1 mg/kg bw per day established by JECFA (2006) or EFSA (2012b), respectively. However, if using the highest level reported in tea and an intake of three cups of tea per day, the ADI established by EFSA (2012b) will be exceeded, but not the ADI established by JECFA (2006).

The available data were not sufficient to establish a TDI for **eucalyptol**. However, a tentative safe level appeared to be in the range of 0.1-1.4 mg/kg bw per day based on the available data. One, two or three cups of tea with the highest basil content exceeded the lower end of this range. Also the intake of two or three cups of tea with the lowest basil content was at or exceeded the lower end of this range. None of the exposures to tea exceeded the highest end of this range.

Based on a tentative HBGV value for **β-caryophyllene** of 0.74 mg/kg bw per day based on a subchronic study by Bastaki et al (2020), as evaluated by EFSA (2014b), there was not a risk of adverse effects of β-caryophyllene from intake of one, two or three cups of tea per day. The same conclusion was reached based on the randomized, double-blind, placebo-controlled trial by Alizadeh et al. (2022).

There were not sufficient data to establish a TDI value for **ursolic acid**. However, based on a tentative safe level for ursolic acid of 3.3 mg/kg bw per day based on a subchronic oral 90-day study (Geerlofs et al., (2022) and a reproductive toxicity study (Geerlofs et al., 2020) in rats, there was not a risk of adverse effects of ursolic acid from intake of one, two or three cups of tea per day.

Food supplements

Plant material

For food supplements containing dried basil plant material, in lack of more specific information, it was assumed that scenarios for aqueous extracts could be used for oral intake of dried plant material.

If assuming that the plant material as such is not genotoxic and employing the usual UF of 100, a HBGV for humans can be estimated as 100 mg/kg bw/100 = 1 mg/kg bw per day. Thus, daily intake of food supplements with holy basil in the recommended doses may be a risk for pregnant women. For effects on male reproduction, 250 mg/kg bw/100 = 2.5 mg/kg bw. Thus, daily intake of food supplements with holy basil may be a risk for impaired reproduction in males.

Alternatively, if estimating MOE from the LOAEL divided by the human exposure, 100 mg/kg bw divided by 1-30 mg/kg bw of food supplements, will give a MOE of only 3.3-100. Since LOAEL is used instead of NOAEL, the MOE values could be 1000 instead of 100, or even 3000, for including an additional factor of 3 since the exposure in the animal

experiment is not chronic, i.e. life-long. Similarly, for 250 mg/kg bw divided by 1-30 mg/kg bw, MOE is 8-250, thus, also too low.

Considering all the uncertainties and taking all the published publications on reproductive toxicity into account, precaution is warranted regarding intake of food supplements with holy basil for pregnant women and for both men and women wanting to become parents.

Individual substances

For intake of both food supplements, there is a risk of adverse effects of **methyleugenol** based on a T25 <25000. Further, if assessed based on exceedance of a threshold of toxicological concern (TTC) value suggested by Mahony et al. (2020) for potentially genotoxic substances in botanicals of 10 µg of plant material on a dry weight basis/person per day (0.17 µg/kg bw per day for a person of 60 kg bw), there is a risk of adverse effects of methyleugenol for intake of one of the food supplements.

For intake of both food supplements, there is a risk of adverse effects of **estragole** based on a MOE <10000 using the whole range of calculated $BMDL_{10}$ values. However, based on exceedance of the suggested TTC value of 0.17 µg/kg bw per day, there is a not a risk of adverse effects of estragole from intake of any of the food supplements.

For intake of **eugenol** calculated from the two food supplements, none of the ADI values were exceeded (2.5 and 1 mg/kg bw per day for JECFA (2006) and EFSA (2012b), respectively.

The available data were not sufficient to establish a TDI for **eucalyptol**. However, a tentative safe level appeared to be in the range of 0.1-1.4 mg/kg bw per day based on the available data. The highest intake of one of the two food supplements with the highest basil content exceeded the lower end of this range. None of the exposures to food supplements exceeded the highest end of this range.

Based on a tentative HBGV value for **β-caryophyllene** of 0.74 mg/kg bw per day based on a subchronic study by Bastaki et al (2020), as evaluated by EFSA (2014b), there was not a risk of adverse effects of β-caryophyllene from intake of the two food supplements. The same conclusion was reached based on the randomized, double-blind, placebo-controlled trial by Alizadeh et al. (2022).

There were not sufficient data to establish a TDI value for **ursolic acid**. However, based on a tentative safe level for ursolic acid of 3.3 mg/kg bw per day based on a subchronic oral 90-day study (Geerlofs et al., (2022) and a reproductive toxicity study (Geerlofs et al., 2020) in rats, there was not a risk of adverse effects of ursolic acid from intake of any of the two food supplements.

As spice in food

No exposure data was found for use of *O. tenuiflorum* as spice in food that could be used for a quantitative risk assessment (EFSA, 2014a).

In this assessment, no new data occurred in the literature search indicating that the use as a culinary herb or spice in food may cause adverse effects.

3. To evaluate if there are any vulnerable groups.

Persons taking drugs and medications, having certain genotypes of metabolic enzymes or certain lifestyles

Some persons may be more vulnerable to the adverse effects of the basil plants than others because some of the substances in these plants interact with drugs and medications or interfere with their metabolism. Xenobiotics undergo metabolic biotransformations, followed by degradation and subsequent elimination. An inhibitory effect exerted by these substances on drug metabolizing enzymes may increase considerably the levels of drugs and medications in humans, with prolonged duration of action and potentially increased toxicity and severe side effects. Thus, the doses must be reduced for drugs taken at the same time as enzymatic inhibitors affecting these metabolic enzymes.

Herb-drug interactions were shown between *Ocimum sanctum* L. and the anti-diabetic drug pioglitazone hydrochloride causing severe dizziness, and as delayed absorption of the anti-epileptic drug levetiracetam. Phase I metabolism mediated by cytochrome P450 (CYP) enzymes represents a major route of metabolism of many drugs that can compete for the same CYP enzyme, ultimately also affecting their elimination. Some CYP enzymes are also involved in bioactivation of chemicals to their genotoxic and carcinogenic metabolites. Essential oils and many of the individual substances in basil plants may affect the activity of the CYP enzymes. β-Caryophyllene and β-caryophyllene oxide may inhibit CYP3A4 which metabolizes about 50% of all drugs, such as ciclosporin, paclitaxel and statins, as well as sexual hormones, such as testosterone. Caryophyllene oxide also markedly increased CYP2B, CYP3A and CYP2C activity. It was demonstrated a five-fold interindividual difference in activities of CYP enzymes metabolizing methyleugenol in humans, thus, indicating variations in human sensitivity toward methyleugenol. Lifestyle factors such as smoking (induces CYP1A) and the use of barbiturates (induces CYP2C) can increase the susceptibility for adverse effects of methyleugenol. Humans with poor metabolizer phenotypes in the metabolic enzyme CYP2A6 might diminish the chances on bioactivation of estragole, whereas lifestyle factors increasing CYP1A2 activities such as cigarette smoking and consumption of charbroiled food might increase those chances for estragole. Transcriptional activity of pregnane X receptor (PXR), one of the transcriptional activators of xenobiotic-metabolizing enzymes, and of human aryl hydrocarbon receptor (AhR), a pivotal xenobiotic receptor also having multiple other roles in human physiology, may be affected by basil plants. Eugenol may be hepatotoxic in mice with glutathione-depleted livers, thus, basil plants should be used with caution in patient taking drugs such as paracetamol (acetaminophen) that depletes glutathione. Interactions of plant substances administered together with conventional antiviral medications can have negative effects on treatment of the viral infection. Ursolic acid is a competitive inhibitor of UDP-glucuronosyltransferase 1A3, binds to and inhibit CYP2C19 and CYP3A4 enzymes, and it inhibits the uptake of the lipid lowering drug rosuvastatin and hence can lead to therapeutic failure.

However, whether such interactions with drugs or metabolic enzymes will happen or not is depending on the plasma concentration of the plant substances, in addition to the individual variants (polymorphisms) of the metabolic enzymes.

Pregnant and lactating women and their respective fetus and child

Most of the reproductive toxicity studies were old, not performed according to guidelines and not adequately reported. There were conflicting reports on the embryotoxicity of *O. tenuiflorum* and related basil plants, however, five studies reported loss of embryo implantation in doses of 100 or 250 mg/kg bw per day. One study reported that the fetuses

of rat dams treated with 134.6 mg/kg bw eucalyptol during gestation had increased skeletal developmental delays, such as poor ossification of the head, thoracic limbs and femurs, versus controls.

Due to the uncertainty regarding the lowest dose able to cause such adverse effects until higher quality studies performed after OECD guidelines with clearly defined and characterized plant material become available, the use of teas and food supplement preparations from holy basil plants should be avoided during pregnancy. Since there are no studies on children specifically, the intake of these products is also better avoided during lactation or by children in general.

Allergy and sensitisation

Contact dermatitis was reported for eugenol from foods, spices and beverages, for eucalyptol from essential oils and pharmaceutical products. Irritant contact mucositis was also attributed to eucalyptol. Contact dermatitis was reported for β-caryophyllene from occupational exposure to essential oils. Inflammatory effects of ursolic acid *in vitro* and *in vivo* were reported.

Conclusions

Based on the available information, reproductive toxicity appeared to be the most critical adverse effects from intake of holy basil (*O. tenuiflorum* L. and *O. sanctum* L.). Considering all the uncertainties and taking all the published publications on reproductive toxicity into account, precaution is warranted to limit the intake of teas or food supplements with holy basil for pregnant women and for both men and women wanting to become parents. Since there are no studies on children specifically, the intake of these products is also better avoided during lactation or by children in general.

Higher quality studies performed by independent contract laboratories after OECD guidelines with clearly defined and characterized plant material are needed in order to establish with more certainty the lowest dose able to cause such adverse effects, and thus, a safe intake level of holy basil (*O. tenuiflorum* L./*O. sanctum* L.).

Data gaps

Exposure

For commercial products (teas and food supplements) on the market, the following information is needed in order to estimate exposure:

- Background information of the basil plant used, i.e. species, variety, chemotype, growing conditions etc. (see the Chapter *Factors affecting the composition of essential oils in basil plants*).
- Detailed description of all steps used in producing the commercial preparations, including which part(s) of the plant used, type and amount of extract used and the extraction procedure or other details on processing, in a way that make it possible to quantitate intake of the plant material and its individual components in the exposure assessment.
- Whether essential oils from the basil plants are also used in teas or food supplements.
- Analytical assessment of the content and concentrations of potentially harmful substances in the plant material, at least of the genotoxic and carcinogenic methyleugenol and estragole.

- Analytical assessment of content of heavy metals, radionuclides, pesticides and solvent residues**,** and testing of microbial quality of the plant material.
- More accurate information on human intake, e.g. on number of cups of tea containing this plant drunk per day and on the recommended intake of the food supplements.

Hazard characterization

- Good quality studies performed by independent contract laboratories in accordance with the relevant OECD guidelines and good laboratory practice (GLP) of well-characterized preparations of the plant in experimental animals (rats, mice, rabbits). Such studies should encompass all relevant toxicological endpoints, especially reproductive toxicity, cancer and liver toxicity.
- Toxicokinetic data for comparison of potential differences between experimental animals and humans.
- More information about whether specific age groups (children, elderly) or population groups (patients, drug users) are more sensitive than others.

Risk characterization

- Because of the lack of good quality and quantitative information of exposure and hazard as stated above, only very tentative estimations of risk can be made for the commercial products (teas and food supplements) on the Norwegian market.

Uncertainties

There are uncertainties related to the following issues:

- Whether the few examples of content of holy basil in teas and food supplements on the Norwegian market as given by NFSA are representative of the exposure to such products in Norway.
- Various numbers for content of the individual substances are given in the publications, and are dependent on variety, location, methods for preparations etc., thus, it is uncertain how representative the numbers used in the calculations of exposure are for the actual products sold in Norway.
- How relevant the data on adverse effects observed in experimental animals with various extracts of plant material are for risk assessments of other preparations of the plant, such as the dried leaves used in teas and food supplements.

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Appendices

Appendix 1

Search 1: Holy basil - the plant and the substances it contains - content and concentrations

Contact person: Inger-Lise Steffensen
Search: Bente Foss

Comment: The references are collected in an Endnote library together with the results from search 2

Number of hits: 245

Databases: **Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to April 23, 2020>**

Date: 24.04.2020

Number of hits: 28

#	Searches	Results
1	ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thai-basil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	1689
2	estragol$.tw,kf.	313
3	(estragol$ or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or metyleugenol or methyl-eugenol or "methyl eugenol" or metyl-eugenol or "metyl eugenol").tw,kf.	545
4	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	4858
5	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	9
6	(Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf	2136
7	("1,8 cineol?" or "1,8-cineol?").tw,kf.	1368

8	eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	2315
9	2 or 3 or 4 or 5 or 6 or 7 or 8	10255
10	(Concentration? or occurrence? or occurence? or content? or composition? or analysis or analyses or amount? or Level or extract or (Weight adj2 percent)).tw,kf.	8547095
11	1 and 9 and 10	167
12	limit 11 to (yr="2018 -Current" and (danish or english or norwegian or swedish))	28

Database: **Embase 1974 to 20bru20 April 23**

Date: 24.04.2020

Number of hits: 48

#	Searches	Results
1	basil/ or ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thai-basil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kw.	3746
2	Estragole/ or (estragol? or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or methyl-eugenol or "methyl eugenol" or metyleugenol or metyl-eugenol or "metyl eugenol").tw,kw.	1415
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kw.	6758
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	12
5	Ursolic acid/ or (Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kw.	4207
6	Cineole/ or ("1,8 cineol?" or "1,8-cineol?").tw,kw.	5079

7	Eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kw.	6505
8	2 or 3 or 4 or 5 or 6 or 7	17737
9	(Concentration? or occurrence? or occurence? or content? or composition? or analysis or analyses or amount? or Level or extract or (Weight adj2 percent)).tw,kw.	10865518
10	1 and 8 and 9	380
11	limit 10 to (yr="2018 -Current" and (Danish or English or Norwegian or Swedish))	48

Database: **Web of Science**

Date: 24.04.2019

Number of hits: 107

11	(#10 AND #9 AND #1) *AND* **LANGUAGE:** (English OR Danish OR Multiple Languages OR Norwegian OR Swedish) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2018-2020*	107
10	**TOPIC:** (Concentration or occurrence$ or occurence$ or content$ or composition$ or analysis or analyses or amount$ or Level or extract or (Weight NEAR/1 percent)) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	16 843 951
9	#8 OR #7 OR #6 OR #5 OR #4 OR #3 OR #2 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	17 779
8	#8 OR #7 OR #6 OR #5 OR #4 OR #3 OR #2 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	893
7	**TOPIC:** (eucalyptol* or eucaluptol* or beta-caryophyllen* or betacaryophyllen* or Alfa-caryophyllen* or Alfacaryophyllen*) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	3 292
6	**TOPIC:** (Ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineol$ OR "1,8 cineol$" OR "1,8-cineol$") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	7 458

5	**TOPIC:** ("97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	36
4	**TOPIC:** (eugenol$ or "eugenic acid" or "4-allyl-2-methoxybenzene") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	6 449
3	**TOPIC:** (methyleugenol or "methyl-eugenol" or "methyl eugenol" or metyleugenol or "metyl-eugenol" or "metyl eugenol") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1 042
2	**TOPIC:** (Estragol* OR methylchavicol or metylchavicol or "methyl-chavicol" or "metyl-chavicol" or "methyl chavicol" or "metyl chavicol") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	936
1	**TOPIC:** (basil$ OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil*" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR tulsi) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	5 737

Database: **Scopus**

Date: 24.04.2020

Number of hits: 126

TITLE-ABS-KEY (*basil* OR *"ocimum basilicum"* OR *"ocimum sanctum"* OR *"hellig basilikum"* OR *"holy basil*"* OR *"sacred basil"* OR *"thai basil*"* OR *"thai-basil*"* OR *"Ocimum tenuiflorum L"* OR *tulsi*)) AND ((TITLE-ABS-KEY (*estragol** OR *methylchavicol* OR *metylchavicol* OR *"methyl-chavicol"* OR *"metyl-chavicol"* OR *"methyl chavicol"* OR *"metyl chavicol"*)) OR (TITLE-ABS-KEY (*methyleugenol* OR *"methyl-eugenol"* OR *"methyl eugenol"* OR *metyleugenol* OR *"metyl-eugenol"* OR *"metyl eugenol"*)) OR (TITLE-ABS-KEY (*eugenol* OR *"eugenic acid"* OR *"4-allyl-2-methoxybenzene"*)) OR (TITLE-ABS-KEY (*"97-53-0"* OR *"93-15-2"* OR *"140-67-0"* OR *"77-52-1"* OR *"470-82-6"*)) OR (TITLE-ABS-KEY (*ursolsyre* OR *"ursolic acid"* OR *"3beta-hydroxy-urs-12-en-28-oic acid"* OR *cineole* OR *"1,8 cineol*"* OR *"1,8-cineol*"*)) OR (TITLE-ABS-KEY (b*eta-caryophyllen** OR *betacaryophyllen** OR *alfa-caryophyllen** OR *alfacaryophyllen**)) OR (TITLE-ABS-KEY (*eucalyptol** OR *eucaluptol**))) AND (TITLE-ABS-KEY (*concentration* OR *occurrence** OR *occurence** OR *content** OR *compositi on** OR *analysis* OR *analyses* OR *amount** OR *level* OR *extract* OR (*weight* W/1 *percent*))) AND (LIMIT-TO (PUBYEAR , *2020*) OR LIMIT-	126

TO (PUBYEAR , *2019*) OR LIMIT-TO (PUBYEAR , *2018*)) AND (LIMIT-TO (LANGUAGE , *"English"*))

Database: **Crop protection compendium**

Date: 24.04.2020

Number of hits: 33

(basil OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR tulsi) AND (estragol* OR methylchavicol OR metylchavicol OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR (methyleugenol OR "methyl-eugenol" OR "methyl eugenol" OR metyleugenol OR "metyl-eugenol" OR "metyl eugenol" OR eugenol OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineole OR "1,8 cineol*" OR "1,8-cineol*" OR (beta-caryophyllen* OR betacaryophyllen* OR alfa-caryophyllen* OR alfacaryophyllen* OR eucalyptol* OR eucaluptol*) AND yr:[2018 TO 2020]Item Types: Abstract, CABI Book Chapter Info, CABI Book Chapter Info, CABI Hosted Full Text, Library Refinements: Language="English"	33

Database: **Toxline via Pubmed**

Date: 24.04.2020

Number of hits: 13

tox[subset] AND ((("ocimum basilicum"[MeSH Terms] OR ("ocimum"[All Fields] AND "basilicum"[All Fields]) OR "ocimum basilicum"[All Fields] OR "basil"[All Fields]) OR "ocimum basilicum"[All Fields] OR "ocimum sanctum"[All Fields] OR hellig[All Fields] OR "holy basil*"[All Fields] OR (sacred[All Fields] AND ("ocimum basilicum"[MeSH Terms] OR ("ocimum"[All Fields] AND "basilicum"[All Fields]) OR "ocimum basilicum"[All Fields] OR "basil"[All Fields])) OR "thai basil*"[All Fields] OR "thai-basil*"[All Fields] OR "Ocimum tenuiflorum L"[All Fields] OR tulsi[All Fields]) OR ((estragol[All Fields] OR estragol1[All Fields] OR ("estragole"[Supplementary Concept] OR "estragole"[All Fields])) OR methylchavicol[All Fields] OR methylchavicol[All Fields] OR "methyl-chavicol"[All Fields] OR "methyl chavicol"[All Fields] OR (metyl[All Fields] AND ("4-allylphenol"[Supplementary Concept] OR "4-allylphenol"[All Fields] OR "chavicol"[All Fields])) OR ("methyleugenol"[Supplementary Concept] OR "methyleugenol"[All Fields]) OR "methyl-eugenol"[All Fields]	13

OR "methyl eugenol"[All Fields] OR ("methyleugenol"[Supplementary Concept] OR "methyleugenol"[All Fields]) OR (metyl[All Fields] AND ("eugenol"[MeSH Terms] OR "eugenol"[All Fields])) OR ("eugenol"[MeSH Terms] OR "eugenol"[All Fields]) OR (("eugenics"[MeSH Terms] OR "eugenics"[All Fields] OR "eugenic"[All Fields]) AND ("acids"[MeSH Terms] OR "acids"[All Fields] OR "acid"[All Fields])) OR "97-53-0"[All Fields] OR "93-15-2"[All Fields] OR "140-67-0"[All Fields] OR "77-52-1"[All Fields] OR "470-82-6"[All Fields] OR ursolsaure[All Fields] OR "ursolic acid"[All Fields] OR (3beta-hydroxy-urs-12-en-28-oic[All Fields] AND ("acids"[MeSH Terms] OR "acids"[All Fields] OR "acid"[All Fields])) OR ("eucalyptol"[MeSH Terms] OR "eucalyptol"[All Fields] OR "cineole"[All Fields]) OR "1,8 cineol*"[All Fields] OR "1,8-cineol*"[All Fields] OR (beta caryophyllen[All Fields] OR ("caryophyllene"[Supplementary Concept] OR "caryophyllene"[All Fields] OR "beta caryophyllene"[All Fields]) OR beta caryophyllenes[All Fields] OR beta caryophyllenic[All Fields]) OR betacaryophyllene[All Fields] OR (("eucalyptol"[MeSH Terms] OR "eucalyptol"[All Fields]) OR eucalyptole[All Fields] OR eucalyptols[All Fields] OR eucalyptolus[All Fields] OR eucalyptolyma[All Fields]))) AND ("2018/01/01"[PDAT] : "2020/04/24"[PDAT]) AND (English[lang] OR Danish[lang] OR Norwegian[lang] OR Swedish[lang])

Search 2: Holy basil - the plant and the substances it contains - toxicity and adverse health effects

Contact person:	Inger-Lise Steffensen
Search:	Bente Foss
Peer review:	Trude Anine Muggerud
Comment:	The references are in the same Endnote library as search no. 1
Number of hits before duplicate check:	5539 (2786 after duplicate check and included hits from search no. 1)

Database: **Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to May 01, 2020>**

Date: 04.05.2020

No. of hits: 754

#	Searches	Results
1	ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thaibasil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	1694
2	(estragol$ or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or metyleugenol or methyl-eugenol or "methyl eugenol" or metyl-eugenol or "metyl eugenol").tw,kf.	785
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	4862
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	9
5	(Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf.	2140
6	("1,8 cineol?" or "1,8-cineol?").tw,kf.	1371
7	eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	2316
8	or/1-7	11743

9	risk/ or risk assessment/ or risk factors/	1109747
10	(risk* or safety or adverse or "side effect?" or sideeffect? or hazard* or harm* or negative or toxicity or toxic or association? or associate? or relationship or connection? or pertaining or induction?).tw,kf.	8411927
11	Reproductive Health/ or reproductive medicine/ or reproduction/ or Reproductive Techniques, Assisted/ or infertility/ or (reproductive or puberty or pregnancy or infertility or semen quality or placenta or anogenital distance or hypospadia or cryptorchidism).tw,kf.	685762
12	Endocrine System/ or Endocrine Glands/ or Endocrine System Diseases/ or Hormones/ or Gonadal Hormones/ or Placental Hormones/ or Pituitary Hormones/ or Growth Hormone/ or Thyroid Hormones/ or Gastrointestinal Hormones/ or Sex Hormone-Binding Globulin/ or Adrenocorticotropic Hormone/ or Adrenal Cortex Hormones/ or (Endocrine system or hypothyroidism or hyperthyroidism or adrenal or hormone? or corticotropin* or corticosteroid*).tw,kf.	813059
13	"Allergy and Immunology"/ or Hypersensitivity/ or "Rhinitis, Allergic, Seasonal"/ or "Food Hypersensitivity"/ or "Drug Hypersensitivity"/ or "Shellfish Hypersensitivity"/ or "Immune System"/ or "Immune System Diseases"/ or (Allergy or Hypersensitiv* or respiratory allergy or gastrointestinal allergy or multiple chemical sensitivity or allergic hypersensitivity disease or contact allergy or Immune system or autoimmune disease or cytokines or white cells or innate immune system or adaptive immune system).tw,kf.	547059
14	or/9-13	9789877
15	8 and 14	3502
16	limit 15 to (yr="2018-Current" and (danish or english or norwegian or swedish))	754

Database: Embase 1974 to 2020 May 01

Date: 04.05.2020

No. of hits:1059

#	Searches	Results
1	basil/ or ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thaibasil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kw.	3754
2	Estragole/ or (estragol? or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or methyl-eugenol or "methyl eugenol" or metyleugenol or metyl-eugenol or "metyl eugenol").tw,kw.	1417
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kw.	6774
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	12
5	Ursolic acid/ or (Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kw.	4222
6	Cineole/ or ("1,8 cineol?" or "1,8-cineol?").tw,kw.	5085
7	Eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kw.	6512
8	or/1-7	21024
9	risk/ or risk assessment/ or risk factor/ or exp side effect/ or adverse event/ or toxicity/ or acute toxicity/ or exp health hazard/ or hazard assessment/	2854948
10	(risk* or safety or adverse or "side effect?" or sideeffect? or hazard* or harm* or negative or toxicity or toxic or association? or associate? or relationship or connection? or pertaining or induction?).tw,kw.	11279100
11	reproductive health/ or reproduction/ or infertility/ or infertility therapy/ or (reproductive health/ or reproduction/ or infertility/ or infertility therapy/) or (reproductive or puberty or pregnancy or infertility or semen quality or placenta or anogenital distance or hypospadia or cryptorchidism).tw,kw.	844826
12	endocrine system/ or endocrine gland/ or endocrine disease/ or hormone/ or sex hormone/ or placental peptide hormone/ or hypophysis hormone/ or growth hormone/ or thyroid hormone/ or gastrointestinal hormone/ or sex	1193775

	hormone binding globulin/ or corticotropin/ or corticosteroid/ or (Endocrine system or hypothyroidism or hyperthyroidism or adrenal or hormone? or corticotropin* or corticosteroid*).tw,kw.	
13	Immunology/ or allergy/ or hypersensitivity/ or pollen allergy/ or food allergy/ or drug hypersensitivity/ or shellfish allergy/ or immune system/ or immunopathology/ or (Allergy or Hypersensitiv* or respiratory allergy or gastrointestinal allergy or multiple chemical sensitivity or allergic hypersensitivity disease or contact allergy or Immune system or autoimmune disease or cytokines or white cells or innate immune system or adaptive immune system).tw,kw.	1076899
14	or/9-13	13392391
15	8 and 14	6586
16	limit 15 to (yr="2018 -Current" and (Danish or English or Norwegian or Swedish))	1247
17	limit 16 to (conference abstracts or Embase)	1059

Database: Web of Science

Date: 04.05.2020

No. of hits:1498

14 (#13 AND #8) *AND* **LANGUAGE:** (English OR Danish OR Norwegian OR Swedish) **1 498**

Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=2018-2020

13 #12 OR #11 OR #10 OR #9 **12 210 844**

Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years

12 **TOPIC:** (("Allergy" or "Hypersensitiv*" or ("rhinitis NEAR/1 allerg*") or "pollen allergy" or "food hypersensitivity" or "Drug Hypersensitivity" or "Shellfish Hypersensitivity" or "Immune System" or "Immune System Disease$" or "respiratory allergy" or "gastrointestinal allergy" or "multiple chemical sensitivity" or "allergic hypersensitivity disease$" or "contact allergy" or "autoimmune disease$" or "cytokines" or "white cell$" or "innate immune system$" or "adaptive immune system$")) **554 111**

Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years

11	**TOPIC:** (("Endocrine System" or "Endocrine Gland$" or "Endocrine System Disease$" or "Hormone$" or "Gonadal Hormone$" or "Placental Hormone$" or "Pituitary Hormone$" or "Growth Hormone$" or "Thyroid Hormone$" or "Gastrointestinal Hormone$" or "Sex Hormone-Binding Globulin" or "Adrenocorticotropic Hormon$" or "Adrenal Cortex Hormone$" or "hypothyroidism" or "hyperthyroidism" or "adrenal" or "hormone$" or "corticotropin*" or "corticosteroid*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**685 847**
10	**TOPIC:** (("Reproductive Health" or "reproductive medicine" or "reproduction" or "infertility" or "infertility therapy" or "reproductive" or "puberty" or "pregnancy" or "infertility" or "semen quality" or "placenta" or "anogenital distance" or "hypospadia" or "cryptorchidism")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**795 245**
9	**TOPIC:** (("risk*" or "safety" or "adverse" or "side effect$" or "sideeffect$" or "hazard*" or "harm*" or "negative" or "toxicity" or "toxic" or "association$" or "associate$" or "relationship" or "connection$" or "pertaining" or "induction$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**11 205 320**
8	#7 OR #6 OR #5 OR #4 OR #3 OR #2 OR #1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**22 953**
7	**TOPIC:** (("eucalyptol*" or "eucaluptol*" or "beta-caryophyllen*" or "betacaryophyllen*" or "Alfa-caryophyllen*" or "Alfacaryophyllen*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**4 156**
6	**TOPIC:** ((Ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineol$ OR "1,8 cineol$" OR "1,8-cineol$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**7 472**
5	**TOPIC:** (("97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**36**
4	**TOPIC:** (("eugenol$" or "eugenic acid" or "4-allyl-2-methoxybenzene")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**6 474**
3	**TOPIC:** (("methyleugenol" or "methyl-eugenol" or "methyl eugenol" or "metyleugenol" or "metyl-eugenol" or "metyl eugenol")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**1 044**
2	**TOPIC:** (("Estragol*" OR "methylchavicol" or "metylchavicol" or "methyl-chavicol" or "metyl-chavicol" or "methyl chavicol" or "metyl chavicol")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**938**

1	**TOPIC:** (("basil$" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil*" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR "tulsi")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	**5 750**

Database: Scopus

Date: 04.05.2020

No. of hits:1266

((TITLE-ABS-KEY (*basil* OR *"ocimum basilicum"* OR *"ocimum sanctum"* OR *"hellig basilikum"* OR *"holy basil*"* OR *"sacred basil"* OR *"thai basil*"* OR *"thai-basil*"* OR *"Ocimum tenuiflorum L"* OR *tulsi*)) OR ((TITLE-ABS-KEY (*estragol** OR *methylchavicol* OR *metylchavicol* OR *"methyl-chavicol"* OR *"metyl-chavicol"* OR *"methyl chavicol"* OR *"metyl chavicol"*)) OR (TITLE-ABS-KEY (*methyleugenol* OR *"methyl-eugenol"* OR *"methyl eugenol"* OR *metyleugenol* OR *"metyl-eugenol"* OR *"metyl eugenol"*)) OR (TITLE-ABS-KEY (*eugenol* OR *"eugenic acid"* OR *"4-allyl-2-methoxybenzene"*)) OR (TITLE-ABS-KEY (*"97-53-0"* OR *"93-15-2"* OR *"140-67-0"* OR *"77-52-1"* OR *"470-82-6"*)) OR (TITLE-ABS-KEY (*ursolsyre* OR *"ursolic acid"* OR *"3beta-hydroxy-urs-12-en-28-oic acid"* OR *cineole* OR *"1,8 cineol*"* OR *"1,8-cineol*"*)) OR (TITLE-ABS-KEY (*beta-caryophyllen** OR *betacaryophyllen** OR *alfa-caryophyllen** OR *alfacaryophyllen**)) OR (TITLE-ABS-KEY (*eucalyptol** OR *eucaluptol**)))) AND ((TITLE-ABS-KEY (*"risk*"* OR *"safety"* OR *"adverse"* OR *"side effect*"* OR *"sideeffect*"* OR *"hazard*"* OR *"harm*"* OR *"negative"* OR *"toxicity"* OR *"toxi c"* OR *"association*"* OR *"associate*"* OR *"relationship"* OR *"connection*"* OR *"pertaining"* OR *"induction*"*)) OR (TITLE-ABS-KEY (*"Reproductive Health"* OR *"reproductive medicine"* OR *"reproduction"* OR *"infertility"* OR *"infertility therapy"* OR *"reproductive"* OR *"puberty"* OR *"pregnancy"* OR *"infertility"* OR *"semen quality"* OR *"placenta"*)) OR (TITLE-ABS-KEY (*"anogenital distance"* OR *"hypospadia"* OR *"cryptorchidism"*)) OR (TITLE-ABS-KEY (*"Endocrine System*"* OR *"Endocrine Gland*"* OR *"Endocrine System Disease*"* OR *"Hormone*"* OR *"Gonadal Hormone*"* OR *"Placental Hormone*"* OR *"Pituitary Hormone*"* OR *"Growth Hormone*"* OR *"Thyroid Hormone*"* OR *"Gastrointestinal Hormone*"*)) OR (TITLE-ABS-KEY (*"Sex Hormone-Binding Globulin"* OR *"Adrenocorticotropic Hormon*"* OR *"Adrenal Cortex Hormone*"* OR *"hypothyroidism"* OR *"hyperthyroidism"* OR *"adrenal"* OR *"hormone*"* OR *"corticotropin*"* OR *"corticosteroid*"*)) OR (TITLE-ABS-KEY (*"Allergy"* OR *"Hypersensitiv*"* OR (*"rhinitis W/1 allerg*"*) OR *"pollen allergy"* OR *"food hypersensitivity"* OR *"Drug Hypersensitivity"* OR *"Shellfish Hypersensitivity"* OR *"Immune System"* OR *"Immune System Disease*"* OR *"respiratory allergy"*)) OR (TITLE-ABS-KEY (*"gastrointestinal allergy"* OR *"multiple chemical sensitivity"* OR *"allergic hypersensitivity disease*"* OR *"contact allergy"* OR *"autoimmune disease*"* OR *"cytokines"* OR *"white cell*"* OR *"innate immune system*"* OR *"adaptive immune system*"*))) AND (LIMIT-

TO (SUBJAREA , *"BIOC"*) OR LIMIT-TO (SUBJAREA , *"PHAR"*) OR LIMIT-TO (SUBJAREA , *"MEDI"*) OR LIMIT-TO (SUBJAREA , *"IMMU"*)) AND (LIMIT-TO (PUBYEAR , *2020*) OR LIMIT-TO (PUBYEAR , *2019*) OR LIMIT-TO (PUBYEAR , *2018*)) AND (LIMIT-TO (LANGUAGE , *"English"*) OR LIMIT-TO (LANGUAGE , *"Danish"*) OR LIMIT-TO (LANGUAGE , *"Swedish"*))

Database: Toxline via Pubmed

Date: 04.05.2020

No. of hits:720

((tox [subset] AND (basil OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR tulsi) OR (estragol* OR methylchavicol OR metylchavicol OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR (methyleugenol OR "methyl-eugenol" OR "methyl eugenol" OR metyleugenol OR "metyl-eugenol" OR "metyl eugenol" OR eugenol OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineole OR "1,8 cineol*" OR "1,8-cineol*" OR (beta-caryophyllen* OR betacaryophyllen* OR alfa-caryophyllen* OR alfacaryophyllen* OR eucalyptol* OR eucaluptol*)) AND ([subset] AND "risk*" OR "safety" OR "adverse" OR "side effect*" OR "sideeffect*" OR "hazard*" OR "harm*" OR "negative" OR "toxicity" OR "toxic" OR "association*" OR "associate*" OR "relationship" OR "connection*" OR "pertaining" OR "induction*" OR "Reproductive Health" OR "reproductive medicine" OR "reproduction" OR "infertility" OR "infertility therapy" OR "reproductive" OR "puberty" OR "pregnancy" OR "infertility" OR "semen quality" OR "placenta" OR "anogenital distance" OR "hypospadia" OR "cryptorchidism" OR "Endocrine System*" OR "Endocrine Gland*" OR "Endocrine System Disease*" OR "Hormone*" OR "Gonadal Hormone*" OR "Placental Hormone*" OR "Pituitary Hormone*" OR "Growth Hormone*" OR "Thyroid Hormone*" OR "Gastrointestinal Hormone*" OR "Sex Hormone-Binding Globulin" OR "Adrenocorticotropic Hormon*" OR "Adrenal Cortex Hormone*" OR "hypothyroidism" OR "hyperthyroidism" OR "adrenal" OR "hormone*" OR "corticotropin*" OR "corticosteroid*" OR "Allergy" OR "Hypersensitiv*" OR "rhinitis" OR "pollen allergy" OR "food hypersensitivity" OR "Drug Hypersensitivity" OR "Shellfish Hypersensitivity" OR "Immune System" OR "Immune System Disease*" OR "respiratory allergy" OR "gastrointestinal allergy" OR "multiple chemical sensitivity" OR "allergic hypersensitivity disease*" OR "contact allergy" OR "autoimmune disease*" OR "cytokines" OR "white cell*" OR "innate immune system*" OR "adaptive immune system*" OR "Allergy" OR "Hypersensitiv*" OR "rhinitis" OR "pollen allergy" OR "food hypersensitivity" OR "Drug Hypersensitivity" OR "Shellfish Hypersensitivity" OR "Immune System" OR "respiratory allergy" OR "gastrointestinal allergy" OR "multiple chemical sensitivity" OR "allergic hypersensitivity disease*" OR "contact allergy" OR "autoimmune disease*" OR "cytokines" OR "white cell*" OR "innate immune system*" OR "adaptive immune system*")
Filters: Publication date from 2018/01/01 to 2020/12/31

Database: Crop protection compendium

Date: 04.05.2020

No. of hits:10 + 232

Comment: This database cannot manage large and extensive searches, therefore, the search was divided into two smaller searches.

Search 1 – 10 hits

(("basil" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thaibasil*" OR "Ocimum tenuiflorum L" OR "tulsi ") OR ("estragol*" OR "methylchavicol" OR "metylchavicol" OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR "methyleugenol" OR "methyl-eugenol" OR "methyl eugenol" OR "metyleugenol" OR "metyl-eugenol" OR "metyl eugenol" OR "eugenol" OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR "ursolsyre" OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR "cineole" OR "1,8 cineol*" OR "1,8-cineol*" OR "beta-caryophyllen*" OR "betacaryophyllen*" OR "alfa-caryophyllen*" OR "alfacaryophyllen*" OR "eucalyptol*" OR "eucaluptol*")) AND ("Allergy" OR "Hypersensitiv*" OR "rhinitis" OR "pollen allergy" OR "food hypersensitivity" OR "Drug Hypersensitivity" OR "Shellfish Hypersensitivity" OR "Immune System" OR "respiratory allergy" OR "gastrointestinal allergy" OR "multiple chemical sensitivity" OR "allergic hypersensitivity disease*" OR "contact allergy" OR "autoimmune disease*" OR "cytokines" OR "white cell*" OR "innate immune system*" OR "adaptive immune system*")) AND yr:[2018 TO 2020]

Search 2 – 232 hits

"Endocrine System*" OR "Endocrine Gland*" OR "Gonadal Hormone*" OR "Placental Hormone*" OR "Pituitary Hormone*" OR "Growth Hormone*" OR "Thyroid Hormone*" OR "Gastrointestinal Hormone*" OR "Sex Hormone-Binding Globulin" OR "Adrenocorticotropic Hormon*" OR "Adrenal Cortex Hormone*" OR "hyp(("basil" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thaibasil*" OR "Ocimum tenuiflorum L" OR "tulsi ") OR ("estragol*" OR "methylchavicol" OR "metylchavicol" OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR "methyleugenol" OR "methyl-eugenol" OR "methyl eugenol" OR "metyleugenol" OR "metyl-eugenol" OR "metyl eugenol" OR "eugenol" OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR "ursolsyre" OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR "cineole" OR "1,8 cineol*" OR "1,8-cineol*" OR "beta-caryophyllen*" OR "betacaryophyllen*" OR "alfa-caryophyllen*" OR "alfacaryophyllen*" OR "eucalyptol*" OR "eucaluptol*")) AND (("risk*" OR "safety" OR "adverse" OR "side effect*" OR "sideeffect*" OR "hazard*"OR "harm*" OR "negative" OR "toxicity" OR "toxic" OR "association*" OR "associate*" OR "relationship" OR "connection*" OR "pertaining" OR "induction*" OR "Reproductive Health" OR "reproductive medicine" OR "reproduction" OR "infertility" OR "reproductive" OR "puberty" OR "pregnancy" OR "semen quality" OR "placenta" OR "anogenital distance" OR "hypospadia" OR "cryptorchidism" OR othyroidism" OR "hyperthyroidism" OR "adrenal" OR"cortic

Appendix 2

Updated Search 1: Holy basil - the plant and the substances it contains - content and concentrations

Contact person: Inger Lise Steffensen
Performed the search: Bente Foss

Comments: The references from the updated searches no. 1 and 2 are combined in the same Endnote library. These updated searches did not include Toxnet or Scopus, since Toxnet no longer exists separately and the articles are included in Medline, and FHI's subscription on Scopus was terminated from 01.01.2023.
Total number of hits: 526

Database: **Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to June 29, 2023>**
Date: 30.06.2023
Number of hits: 96

			Comments
1	ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thai-basil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	2465	
2	estragol$.tw,kf.	417	
3	(estragol$ or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or metyleugenol or methyl-eugenol or "methyl eugenol" or metyl-eugenol or "metyl eugenol").tw,kf.	1046	
4	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	6059	
5	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	12	
6	(Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf.	2796	
7	("1,8 cineol?" or "1,8-cineol?").tw,kf.	1847	
8	eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	3220	
9	2 or 3 or 4 or 5 or 6 or 7 or 8	13376	
10	(Concentration? or occurrence? or occurence? or content? or composition? or analysis or analyses or amount? or Level or extract or (Weight adj2 percent)).tw,kf.	10659846	
11	1 and 9 and 10	260	

12	limit 11 to (danish or english or multilingual or norwegian or swedish)	257	
13	(2021* or 2022* or 2023*).ed,ep,yr,dp,dt.	4801061	Searches publications from 2021, 2022 and 2023
14	(202004* or 202005* or 202006* or 202007* or 202008* or 202009* or 202010* or 202011* or 202012*).ep,ed,dt.	1722818	Searches publications from April 2020 to end of December 2020
15	12 and (13 or 14)	96	The hits are limited to the period April 2020 to 2023

Database: **Embase 1974 to 2023 June 30**
Date: 03.07.2023
Number of hits: 139

1	basil/ or ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thai-basil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	5249	
2	Estragole/ or (estragol? or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or methyl-eugenol or "methyl eugenol" or metyleugenol or metyl-eugenol or "metyl eugenol").tw,kf.	1819	
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	8787	
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	16	
5	Ursolic acid/ or (Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf.	5829	
6	Cineole/ or ("1,8 cineol?" or "1,8-cineol?").tw,kf.	6558	
7	eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	8527	
8	2 or 3 or 4 or 5 or 6 or 7	23372	
9	(Concentration? or occurrence? or occurence? or content? or composition? or analysis or analyses or amount? or Level or extract or (Weight adj2 percent)).tw,kf.	13752661	
10	1 and 8 and 9	515	
11	limit 10 to (danish or english or norwegian or swedish)	502	
12	(202004* or 202005* or 202006* or 202007* or 202008* or 202009* or 202010* or 202011* or 202012*).dd,dc.	1524371	Searches publications from 2021, 2022 and 2023
13	(2021* or 2022* or 2023*).yr,dd,dp,dc.	5595626	Searches publications from April

			2020 to end of December 2020
14	11 and (12 or 13)	139	The hits are limited to the period April 2020 to 2023

Database: **Web of Science**
Date: 30.06.2023
Number of hits: 284

			Comments
10	(#10 AND #9 AND #1) *AND* **LANGUAGE:** (English OR Danish OR Multiple Languages OR Norwegian OR Swedish) *Indexes=SCI-EXPANDED, SSCI, A&HCI,* ***ESCI Timespan: 2020-05-01 to 2023-06-30***	284	Time limitations marked in bold from May 2020 to end of June 2023
9	**TOPIC:** (Concentration or occurrence$ or occurence$ or content$ or composition$ or analysis or analyses or amount$ or Level or extract or (Weight NEAR/1 percent)) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	21 532 362	
8	#8 OR #7 OR #6 OR #5 OR #4 OR #3 OR #2 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	23 918	
7	**TOPIC:** (eucalyptol* or eucaluptol* or beta-caryophyllen* or betacaryophyllen* or Alfa-caryophyllen* or Alfacaryophyllen*) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	5 715	
6	**TOPIC:** (Ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineol$ OR "1,8 cineol$" OR "1,8-cineol$") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	9 706	
5	**TOPIC:** ("97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	47	
4	**TOPIC:** (eugenol$ or "eugenic acid" or "4-allyl-2-methoxybenzene") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	8 879	

3	**TOPIC:** (methyleugenol or "methyl-eugenol" or "methyl eugenol" or metyleugenol or "metyl-eugenol" or "metyl eugenol") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1 361	
2	**TOPIC:** (Estragol* OR methylchavicol or metylchavicol or "methyl-chavicol" or "metyl-chavicol" or "methyl chavicol" or "metyl chavicol") *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1 252	
1	**TOPIC:** (basil$ OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil*" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR tulsi) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	7 941	

Database: **Crop protection compendium**
Date: 30.06.2023
Number of hits: 7

(basil OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR tulsi) AND (estragol* OR methylchavicol OR metylchavicol OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR methyleugenol OR "methyl-eugenol" OR "methyl eugenol" OR metyleugenol OR "metyl-eugenol" OR "metyl eugenol" OR eugenol OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineole OR "1,8 cineol*" OR "1,8-cineol*" OR beta-caryophyllen* OR betacaryophyllen* OR alfa-caryophyllen* OR alfacaryophyllen* OR eucalyptol* OR eucaluptol*) AND **yr:[04/01/2020 TO 06/30/2023]** Item Types: Abstract, CABI Book Chapter Info, CABI Book Chapter Info, CABI Hosted Full Text, Library Refinements: Language="English"	7	Time limitations marked in bold (2020-2023)

Updated Search 2: Holy basil - the plant and the substances it contains - toxicity and adverse health effects

Contact person: Inger-Lise Steffensen
Performed the search: Bente Foss

Comments: The references from the updated searches no. 1 and 2 are combined in the same Endnote library. These updated searches did not include Toxnet or Scopus, since Toxnet no longer exists separately and the articles are included in Medline, and FHI's subscription on Scopus was terminated from 01.01.2023. There have been changes in Embase codes and in the updated search …tw,kf is used instead of tw,kw.

Total number of hits: 7579 (after duplicate check: 5144) for both searches 1 and 2

Database: **Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to June 29, 2023>**
Date: 30.06.2023
Number of hits: 1666

			Comments
1	ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thaibasil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	2464	
2	(estragol$ or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or metyleugenol or methyl-eugenol or "methyl eugenol" or metyl-eugenol or "metyl eugenol").tw,kf.	1046	
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	6057	
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	12	
5	(Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf.	2796	
6	("1,8 cineol?" or "1,8-cineol?").tw,kf.	1846	
7	eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	3219	
8	or/1-7	15504	
9	risk/ or risk assessment/ or risk factors/	1285077	
10	(risk* or safety or adverse or "side effect?" or sideeffect? or hazard* or harm* or negative or toxicity or toxic or association? or associate? or relationship or connection? or pertaining or induction?).tw,kf.	10543575	

11	Reproductive Health/ or reproductive medicine/ or reproduction/ or Reproductive Techniques, Assisted/ or infertility/ or (reproductive or puberty or pregnancy or infertility or semen quality or placenta or anogenital distance or hypospadia or cryptorchidism).tw,kf.	816625	
12	Endocrine System/ or Endocrine Glands/ or Endocrine System Diseases/ or Hormones/ or Gonadal Hormones/ or Placental Hormones/ or Pituitary Hormones/ or Growth Hormone/ or Thyroid Hormones/ or Gastrointestinal Hormones/ or Sex Hormone-Binding Globulin/ or Adrenocorticotropic Hormone/ or Adrenal Cortex Hormones/ or (Endocrine system or hypothyroidism or hyperthyroidism or adrenal or hormone? or corticotropin* or corticosteroid*).tw,kf.	909553	
13	"Allergy and Immunology"/ or Hypersensitivity/ or "Rhinitis, Allergic, Seasonal"/ or "Food Hypersensitivity"/ or "Drug Hypersensitivity"/ or "Shellfish Hypersensitivity"/ or "Immune System"/ or "Immune System Diseases"/ or (Allergy or Hypersensitiv* or respiratory allergy or gastrointestinal allergy or multiple chemical sensitivity or allergic hypersensitivity disease or contact allergy or Immune system or autoimmune disease or cytokines or white cells or innate immune system or adaptive immune system).tw,kf.	663106	
14	or/9-13	12068676	
15	8 and 14	5019	
16	limit 15 to (danish or english or norwegian or swedish)	4915	
17	(202005* or 202006* or 202007* or 202008* or 202009* or 202010* or 202011* or 202012*).ep,ed,dt.	1549012	Limited to publications from May 2020 to December 2020
18	(2021* or 2022* or 2023*).ed,ep,yr,dp,dt.	4792108	Searches publications from 2021, 2022 and 2023
19	16 and (17 or 18)	1666	The hits are limited to the period May 2020 to 2023

Database: **Embase 1974 to 2023 June 30**
Date: 03.07.2023
Number of hits: 2515

			Comments

1	basil/ or ocimum basilicum/ or ocimum sanctum/ or ("hellig basilikum" or "holy basil*" or "sacred basil*" or "thai basil*" or "thaibasil*" or "Ocimum sanctum L" or "Ocimum basilicum" or "Ocimum tenuiflorum L" or tulsi or basil?).tw,kf.	5248	
2	Estragole/ or (estragol? or methylchavicol or metylchavicol or methyl-chavicol or metyl-chavicol or "methyl chavicol" or "metyl chavicol" or methyleugenol or methyl-eugenol or "methyl eugenol" or metyleugenol or metyl-eugenol or "metyl eugenol").tw,kf.	1820	
3	eugenol/ or (eugenol? or "eugenic acid" or "4-allyl-2-methoxybenzene").tw,kf.	8786	
4	(97-53-0 or 93-15-2 or 140-67-0 or 77-52-1 or 470-82-6).mp.	16	
5	Ursolic acid/ or (Ursolsyre or "ursolic acid" or 3beta-hydroxy-urs-12-en-28-oic acid).tw,kf.	5830	
6	Cineole/ or ("1,8 cineol?" or "1,8-cineol?").tw,kf.	6563	
7	Eucalyptol/ or (eucalyptol? or eucaluptol? or beta-caryophyllen? or betacaryophyllen? or Alfa-caryophyllen? or Alfacaryophyllen?).tw,kf.	8536	
8	or/1-7	27915	
9	risk/ or risk assessment/ or risk factor/ or exp side effect/ or adverse event/ or toxicity/ or acute toxicity/ or exp health hazard/ or hazard assessment/	3568555	
10	(risk* or safety or adverse or "side effect?" or sideeffect? or hazard* or harm* or negative or toxicity or toxic or association? or associate? or relationship or connection? or pertaining or induction?).tw,kf.	14401373	
11	reproductive health/ or reproduction/ or infertility/ or infertility therapy/ or (reproductive health/ or reproduction/ or infertility/ or infertility therapy/) or (reproductive or puberty or pregnancy or infertility or semen quality or placenta or anogenital distance or hypospadia or cryptorchidism).tw,kf.	1047423	
12	endocrine system/ or endocrine gland/ or endocrine disease/ or hormone/ or sex hormone/ or placental peptide hormone/ or hypophysis hormone/ or growth hormone/ or thyroid hormone/ or gastrointestinal hormone/ or sex hormone binding globulin/ or corticotropin/ or corticosteroid/ or (Endocrine system or hypothyroidism or hyperthyroidism or adrenal or hormone? or corticotropin* or corticosteroid*).tw,kf.	1385127	
13	Immunology/ or allergy/ or hypersensitivity/ or pollen allergy/ or food allergy/ or drug hypersensitivity/ or shellfish allergy/ or immune system/ or immunopathology/ or (Allergy or Hypersensitiv* or respiratory allergy or gastrointestinal allergy or multiple chemical sensitivity or allergic hypersensitivity disease or contact allergy or Immune system or autoimmune	1284957	

	disease or cytokines or white cells or innate immune system or adaptive immune system).tw,kf.		
14	or/9-13	16822914	
15	8 and 14	9658	
16	limit 15 to (danish or english or norwegian or swedish)	9353	
17	(202005* or 202006* or 202007* or 202008* or 202009* or 202010* or 202011* or 202012*).dd,dc.	1380542	Limited to publications from May 2020 to December 2020
18	(2021* or 2022* or 2023*).yr,dd,dp,dc.	5598626	Searches publications from 2021, 2022 and 2023
19	15 and (17 or 18)	3101	The hits are limited to the period May 2020 to 2023
20	limit 19 to (conference abstracts or embase)	2515	

Database: **Web of Science**
Date: 30.06.2023
Number of hits: 3203

			Comments
14	(#13 AND #8) *AND* **LANGUAGE:** (English OR Danish OR Norwegian OR Swedish) *Indexes=SCI-EXPANDED, SSCI, A&HCI,* ***ESCI Timespan= Timespan: 2020-05-01 to 2023-06-30***	3203	Time limitations marked in bold from May 2020 to end of June 2023
13	#12 OR #11 OR #10 OR #9 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	15627339	
12	**TOPIC:** (("Allergy" or "Hypersensitiv*" or ("rhinitis NEAR/1 allerg*") or "pollen allergy" or "food hypersensitivity" or "Drug Hypersensitivity" or "Shellfish Hypersensitivity" or "Immune System" or "Immune System Disease$" or "respiratory allergy" or "gastrointestinal allergy" or "multiple chemical sensitivity" or "allergic hypersensitivity disease$" or "contact allergy" or "autoimmune disease$" or "cytokines" or "white cell$" or "innate immune system$" or "adaptive immune system$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	689930	
11	**TOPIC:** (("Endocrine System" or "Endocrine Gland$" or "Endocrine System Disease$" or "Hormone$" or "Gonadal	796247	

	Hormone$" or "Placental Hormone$" or "Pituitary Hormone$" or "Growth Hormone$" or "Thyroid Hormone$" or "Gastrointestinal Hormone$" or "Sex Hormone-Binding Globulin" or "Adrenocorticotropic Hormone$" or "Adrenal Cortex Hormone$" or "hypothyroidism" or "hyperthyroidism" or "adrenal" or "hormone$" or "corticotropin*" or "corticosteroid*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*		
10	**TOPIC:** (("Reproductive Health" or "reproductive medicine" or "reproduction" or "infertility" or "infertility therapy" or "reproductive" or "puberty" or "pregnancy" or "infertility" or "semen quality" or "placenta" or "anogenital distance" or "hypospadia" or "cryptorchidism")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	979873	
9	**TOPIC:** (("risk*" or "safety" or "adverse" or "side effect$" or "sideeffect$" or "hazard*" or "harm*" or "negative" or "toxicity" or "toxic" or "association$" or "associate$" or "relationship" or "connection$" or "pertaining" or "induction$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	14455798	
8	#7 OR #6 OR #5 OR #4 OR #3 OR #2 OR #1 *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	30902	
7	**TOPIC:** (("eucalyptol*" or "eucaluptol*" or "beta-caryophyllen*" or "betacaryophyllen*" or "Alfa-caryophyllen*" or "Alfacaryophyllen*")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	5706	
6	**TOPIC:** ((Ursolsyre OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR cineol$ OR "1,8 cineol$" OR "1,8-cineol$")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	9694	
5	**TOPIC:** (("97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	47	
4	**TOPIC:** (("eugenol$" or "eugenic acid" or "4-allyl-2-methoxybenzene")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	8862	
3	**TOPIC:** (("methyleugenol" or "methyl-eugenol" or "methyl eugenol" or "metyleugenol" or "metyl-eugenol" or "metyl eugenol")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1359	

2	**TOPIC:** (("Estragol*" OR "methylchavicol" or "metylchavicol" or "methyl-chavicol" or "metyl-chavicol" or "methyl chavicol" or "metyl chavicol")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	1251	
1	**TOPIC:** (("basil$" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil*" OR "thai basil*" OR "thai-basil*" OR "Ocimum tenuiflorum L" OR "tulsi")) *Indexes=SCI-EXPANDED, SSCI, A&HCI, ESCI Timespan=All years*	7935	

Database: **Crop protection compendium**
Date: 30.06.2023
Number of hits: 17 + 178 = 195
Comment: Crop protection compendium does not work well with large and extensive searches. The search string is therefore divided in two searches.

Search 1		**Comments**
"query": { AllField:((("basil" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thaibasil*" OR "Ocimum tenuiflorum L" OR "tulsi ") OR ("estragol*" OR "methylchavicol" OR "metylchavicol" OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR "methyleugenol" OR "methyl-eugenol" OR "methyl eugenol" OR "metyleugenol" OR "metyl-eugenol" OR "metyl eugenol" OR "eugenol" OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR "ursolsyre" OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR "cineole" OR "1,8 cineol*" OR "1,8-cineol*" OR "beta-caryophyllen*" OR "betacaryophyllen*" OR "alfa-caryophyllen*" OR "alfacaryophyllen*" OR "eucalyptol*" OR "eucaluptol*"))) AND AllField:(("Allergy" OR "Hypersensitiv*" OR "rhinitis" OR "pollen allergy" OR "food hypersensitivity" OR "Drug Hypersensitivity" OR "Shellfish Hypersensitivity" OR "Immune System" OR "respiratory allergy" OR "gastrointestinal allergy" OR "multiple chemical sensitivity" OR "allergic hypersensitivity disease*" OR "contact allergy" OR "autoimmune disease*" OR "cytokines" OR "white cell*" OR "innate immune system*" OR "adaptive immune system*")) } "filter": { **E-Publication Date: (05/01/2020 TO 06/30/2023)** }	17	Time limitations marked in bold from May 2020 to end of June 2023

Search 2		**Comments**
"query": { AllField:(("basil" OR "ocimum basilicum" OR "ocimum sanctum" OR "hellig basilikum" OR "holy basil*" OR "sacred basil" OR "thai basil*" OR "thaibasil*" OR "Ocimum tenuiflorum L" OR "tulsi ") OR ("estragol*" OR	178	Time limitations marked in bold from

"methylchavicol" OR "metylchavicol" OR "methyl-chavicol" OR "metyl-chavicol" OR "methyl chavicol" OR "metyl chavicol" OR "methyleugenol" OR "methyl-eugenol" OR "methyl eugenol" OR "metyleugenol" OR "metyl-eugenol" OR "metyl eugenol" OR "eugenol" OR "eugenic acid" OR "4-allyl-2-methoxybenzene" OR "97-53-0" OR "93-15-2" OR "140-67-0" OR "77-52-1" OR "470-82-6" OR "ursolsyre" OR "ursolic acid" OR "3beta-hydroxy-urs-12-en-28-oic acid" OR "cineole" OR "1,8 cineol*" OR "1,8-cineol*" OR "beta-caryophyllen*" OR "betacaryophyllen*" OR "alfa-caryophyllen*" OR "alfacaryophyllen*" OR "eucalyptol*" OR "eucaluptol*")) AND AllField:(("risk*" OR "safety" OR "adverse" OR "side effect*" OR "sideeffect*" OR "hazard*"OR "harm*" OR "negative" OR "toxicity" OR "toxic" OR "association*" OR "associate*" OR "relationship" OR "connection*" OR "pertaining" OR "induction*" OR "Reproductive Health" OR "reproductive medicine" OR "reproduction" OR "infertility" OR "reproductive" OR "puberty" OR "pregnancy" OR "semen quality" OR "placenta" OR "anogenital distance" OR "hypospadia" OR "cryptorchidism" OR "Endocrine System*" OR "Endocrine Gland*" OR "Gonadal Hormone*" OR "Placental Hormone*" OR "Pituitary Hormone*" OR "Growth Hormone*" OR "Thyroid Hormone*" OR "Gastrointestinal Hormone*" OR "Sex Hormone-Binding Globulin" OR "Adrenocorticotropic Hormon*" OR "Adrenal Cortex Hormone*" OR "hypothyroidism" OR "hyperthyroidism" OR "adrenal" OR"corticotropin*" OR "corticosteroid*")) } "filter": { **E-Publication Date: (05/01/2020 TO 06/30/2023)** }		May 2020 to end of June 2023

Abbreviations and acronyms

ADI	–	acceptable daily intake
ADME	–	absorption, distribution, metabolism, excretion
AhR	–	aryl hydrocarbon receptor
AKR	–	aldo-keto-reductase
ALARA	–	as low as reasonably achievable
ALAT	–	alanine aminotransferase
ALP	–	alkaline phosphatase
ALT	–	alanine aminotransferase
AST	–	aspartate aminotransferase
BCPA	–	β-caryophyllene alcohol
BCPO	–	β-caryophyllene oxide
BMDL	–	benchmark dose lower confidence limit
BrdU-FCM	–	5-bromo-2-deoxyuridine-flow cytometry method
BSO	–	DL-buthionine sulfoximine
bw	–	body weight
CAT	–	catalase
CB	–	cannabinoid receptor
CBR	–	carbonyl reductase
CMC	–	carboxymethyl cellulose
COX	–	cyclooxygenase
CYP	–	cytochrome P450
DIO	–	diet-induced obese
DLT	–	dose-limiting toxicity
DMSO	–	dimethyl sulfoxide
DTU	–	Technical University of Denmark
EC	–	European Commission
EDI	–	estimated daily intake
EFSA	–	European Food Safety Authority
EMA	–	European Medicines Agency
EO	–	essential oil
ERK	–	extracellular signal regulated kinase
ESCO	–	European Food Safety Authority (EFSA) Scientific Cooperation
EU	–	European Union
FEMA	–	Flavor and Extract Manufacturers Association of USA
FSH	–	follicle stimulating hormone
FST	–	forced swimming test
GC-FID	–	gas chromatography-flame ionization detector
GC-MS	–	gas chromatography-mass spectrometry
GDI	–	genetic damage index
GGT	–	γ-glutamyltranspeptidase
GLP	–	good laboratory practice
GPT	–	glutamic pyruvic transaminase
GRAS	–	generally recognized as safe
GSH	–	glutathione
GST	–	glutathione *S*-transferase
GTP	–	glutamyl transpeptidase
HBGV	–	health-based guidance value
HDL	–	high-density lipoprotein
h-hCG	–	human hyperglycosylated chorionic gonadotropin
hPL	–	human placental lactogen

IARC	–	International Agency for Research on Cancer
i.d.	–	intradermal
i.g.	–	intragastric
IL	–	interleukin
i.m.	–	intramuscular
IMFCSD	–	indirect mode forced convection solar dryer
iNOS	–	inducible nitric oxide synthase
i.p.	–	intraperitoneal
ISAR	–	index of severity of adverse reactions
ISSR	–	inter-simple sequence repeat
i.v.	–	intravenous
JECFA	–	Joint FAO/WHO Expert Committee on Food Additives
L.	–	Linn.
LC-MS/MS	–	liquid chromatography with tandem-mass spectrometry analysis
LD50	–	lethal dose 50%
LDH	–	lactate dehydrogenase
LDL	–	low-density lipoprotein
LH	–	luteinizing hormone
LLNA	–	local lymph node assay
LOAEL	–	lowest observed adverse effect level
LQ	–	Lordosis quotient
MCH	–	mean corpuscular hemoglobin
MCHC	–	mean corpuscular hemoglobin concentration
MDP	–	monocyte chemokine protein
MIF	–	macrophage migration inhibitory factor
MMR	–	mismatch repair
MN	–	micronucleus/micronuclei
MOE	–	margin of exposure
MPL	–	maximum permitted level
MTD	–	maximum tolerated dose
NER	–	nucleotide excision repair
NESIL	–	no expected sensitization induction level
NFC	–	natural flavour complex
NFκB	–	nuclear factor kappa B
NFSA	–	Norwegian Food Safety Authority
NIDDM	–	non-insulin-dependent diabetes mellitus
NIPH	–	Norwegian Institute of Public Health
NOAEL	–	no observed adverse effect level
NQO	–	NADPH-quinone oxireductase
NRU	–	neutral red uptake
NTP	–	U.S. National Toxicology Program
O.	–	*Ocimum* sp.
OECD	–	Organization for Economic Cooperation and Development
OS	–	*O. sanctum Linn.*
OSE	–	50% ethanolic leaf extract of *O. sanctum* Linn. or only *O. Sanctum Linn.*
OSHE	–	*O. sanctum* Linn. hydroalcoholic extract
OtEOMT	–	*Ocimum tenuiflorum* biotype 2 eugenol O-methyltransferase
PAPS	–	3'-phosphoadenosine-5'-phosphosulphate
PBBK	–	physiologically-based biokinetics
PBK	–	physiologically based kinetics
PCA	–	principal component analysis
PCNA	–	proliferating cell nuclear antigen

PFS	–	plant food supplement
PIF	–	photo-irritation factor
p.o.	–	per oral
PXR	–	pregnane X receptor
QPS	–	qualified presumption of safety
QSAR	–	quantitative structure-activity relationship
RASFF	–	European Rapid Alert System for Food and Feed
REP	–	interim relative potency
RIA	–	radioimmunoassay
RIFM	–	Research Institute for Fragrance Materials
s.c.	–	subcutaneous
SCE	–	sister chromatid exchange
SCF	–	Scientific Committee on Food
SEM	–	scanning electron microscopy
SGOT	–	aspartate aminotransferase or glutamate oxalacetate transaminase
SGPT	–	alanine aminotransferase or glutamate pyruvate transaminase
SOD	–	superoxide dismutase
SULT	–	sulfotransferase
TCDD	–	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCM	–	traditional Chinese medicine
TDI	–	tolerable daily intake
TEM	–	transmission electron microscopy
TEQ	–	toxic equivalency
Tlr	–	Toll-like receptor
TNF	–	tumour necrosis factor
TST	–	tail suspension test
TTC	–	threshold of toxicological concern
UAE	–	ultrasonic-assisted extraction
UDS	–	unscheduled DNA synthesis
UF	–	uncertainty factor
UGT	–	uridine 5'-diphospho (UDP)-glucuronosyltransferase
UPLC	–	ultra-performance liquid chromatography
UVA	–	ultraviolet A
VSD	–	virtual safe dose
WHO	–	World Health Organization
WOE	–	weight of evidence
YFAS-S	–	Yale Food Addiction Scale Score

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