Safety Assessment of *Salix alba* (Willow)-Derived Ingredients as Used in Cosmetics

Status:	Draft Report for Panel Review
Release Date:	February 17, 2026
Panel Meeting Date:	March 12 – 13, 2026

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Samuel M. Cohen, M.D., Ph.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume, M.B.A. This safety assessment was prepared by Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR.

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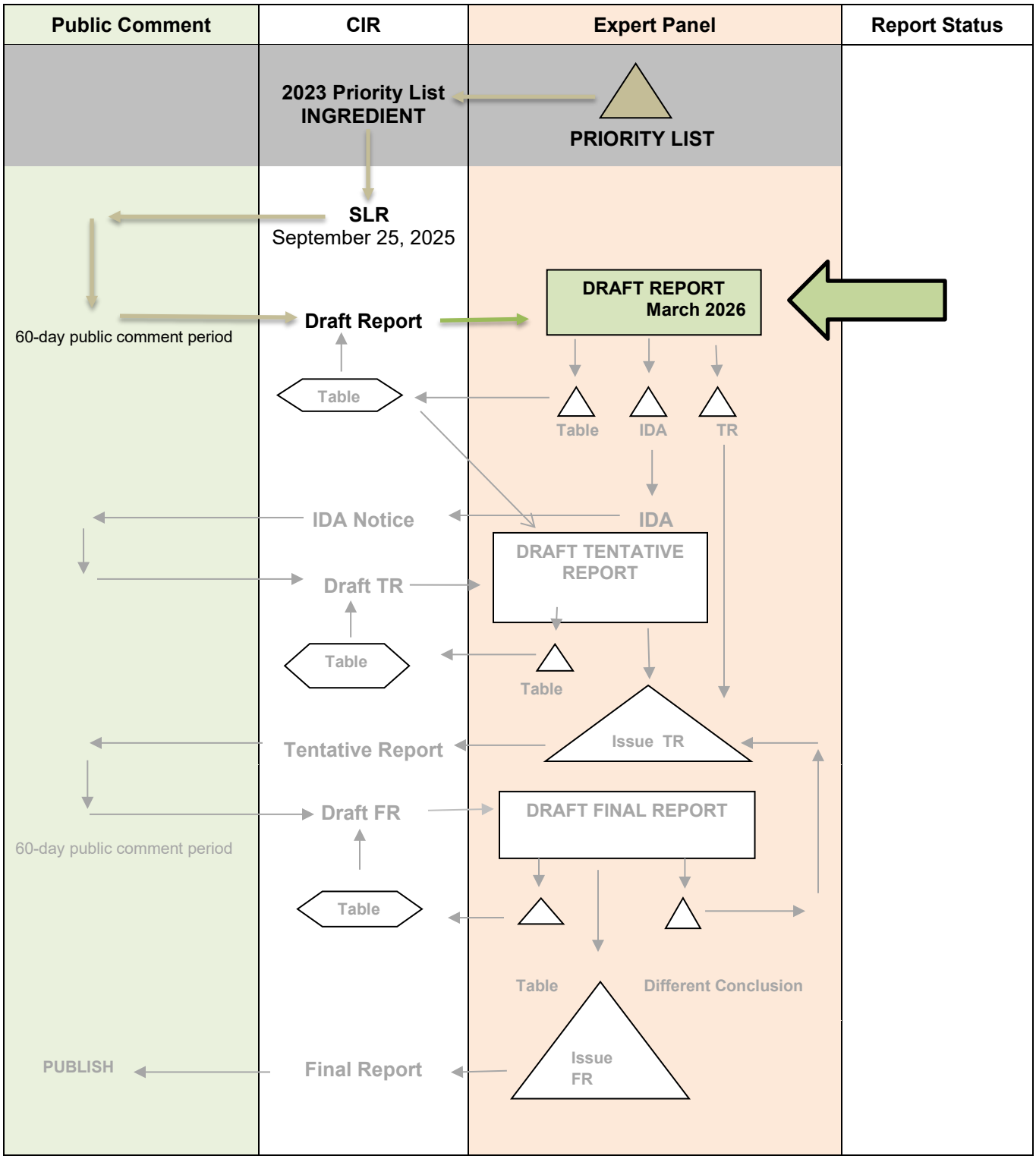
SAFETY ASSESSMENT FLOW CHART

INGREDIENT/FAMILY *Salix alba* (Willow)-Derived Ingredients
MEETING March 2026



Cosmetic Ingredient Review

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Memorandum

To: Expert Panel for Cosmetic Ingredient Safety Members and Liaisons
From: Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR
Date: February 17, 2026
Subject: Safety Assessment of *Salix alba* (Willow)-Derived Ingredients

Enclosed is the Draft Report on the Safety Assessment of *Salix alba* (Willow)-Derived Ingredients as Used in Cosmetics. (It is identified as *report_Willow_032026* in the pdf document). This is the first time the Panel is reviewing this report on the following 6 ingredients:

Salix Alba (Willow) Bark Extract	Salix Alba (Willow) Extract
Salix Alba (Willow) Bark Powder	Salix Alba (Willow) Flower Extract
Salix Alba (Willow) Bark Water	Salix Alba (Willow) Leaf Extract

Results of a 2025 concentration of use survey using FDA cosmetic product categories under MoCRA were received and incorporated (*data1_Willow_032026*). In addition, since the issuing of the Scientific Literature Review (SLR), the following data were received, and have been incorporated into the report:

- HRIPT on a serum containing 0.0025% Salix Alba (Willow) Bark Extract (*data2_Willow_032026*)
- summary data on Salix Alba (Willow) Bark Extract (*data3_Willow_032026*)
  - method of manufacturing
  - composition and impurities
  - acute oral toxicity
  - repeated dose dermal toxicity
  - dermal irritation
  - dermal sensitization
- summary data on Salix Alba (Willow) Leaf Extract (*data4_Willow_032026*)
  - composition
  - impurities
  - acute oral toxicity
  - in vitro genotoxicity
  - cytotoxicity
  - dermal irritation
  - in vitro dermal sensitization
  - in vitro phototoxicity
  - ocular irritation
- various data on Salix Alba (Willow) Bark Extract (*data5_Willow_032026*)
  - manufacturing
  - composition and specifications
  - in vitro genotoxicity
  - steroidogenesis
  - in vitro dermal irritation
  - in chemico dermal sensitization
  - in vitro dermal sensitization
  - in vitro ocular irritation
  - HRIPT
  - phototoxicity

It is important to note that some of these data were received after this Draft Report was submitted to Panel for the December 2025 meeting (with those submissions indicated above with blue highlighting). Because that meeting was delayed and the report is now being reviewed in March 2026, newly identified data and changes since the last version submitted to the Panel are included in this report and highlighted in blue for ease of review. In addition, since that time, 2025 RLD have been received and incorporated into the report.

Other information included in this packet:

- comments on the Scientific Literature Review (SLR) from Council (*PCPCcomments_Willow_032026*)
- responses to comments on SLR (*response-PCPCcomments_Willow_032026*)
- flow chart (*flow_ Willow_032026*)
- report history (*history_ Willow_032026*)
- search strategy (*search_ Willow_032026)*
- data profile (*dataprofile_Willow_032026)*

If no further data are needed to reach a conclusion of safety, the Panel should formulate a Discussion and issue a Tentative Report. However, if additional data are required, the Panel should be prepared to identify those needs and issue an Insufficient Data Announcement.

Salix alba (Willow)-Derived Ingredients History

September 2025

SLR posted

October 2025

2025 concentration of use data received

Comments on SLR from Council received

Summary HRIPT on 0.0025% Salix Alba (Willow) Bark Extract received

November 2025

Summary data received on Salix Alba (Willow) Bark Extract (method of manufacturing, composition and impurities, acute toxicity, dermal irritation, dermal sensitization)

Summary data received on Salix Alba (Willow) Leaf Extract (composition, impurities, ocular irritation, cutaneous tolerance, cutaneous sensitization, acute toxicity, mutagenicity, phototoxicity)

Studies received on Salix Alba (Willow) Bark Extract (composition, manufacturing, specifications, genotoxicity, steroidogenesis, ocular and dermal irritation, sensitization, phototoxicity)

March 2026

Panel reviews Draft Report

Salix alba (Willow)-Derived Ingredients Data Profile* – March 2026 – Writer, Priya Ferguson																														
					Toxico-kinetics		Acute Tox			Repeated Dose Tox			DART		Genotox		Carci		Dermal Irritation			Dermal Sensitization				Ocular Irritation		Clinical Studies	
	Reported Use	GRAS	Method of Mfg	Constituents/ Impurities	Dermal Penetration	ADME	Dermal	Oral	Inhalation	Dermal	Oral	Inhalation	Dermal	Oral	In Vitro	In Vivo	Dermal	Oral	In Vitro	Animal	Human	In Vitro/ In Chemico	Animal	Human	Phototoxicity	In Vitro	Animal	Retrospective/ Multicenter	Case Reports
Salix Alba (Willow) Bark Extract	X		X	X				X		X	X			X	X	X			X	X		X	X	X	X	X			X
Salix Alba (Willow) Bark Powder	X																													
Salix Alba (Willow) Bark Water	X																													
Salix Alba (Willow) Extract	X																													
Salix Alba (Willow) Flower Extract	X																													
Salix Alba (Willow) Leaf Extract	X		X	X				X							X					X		X	X		X		X		

* "X" indicates that data were available in a category for the ingredient

blue highlight indicate data that were added in these categories after the submission of December 2025 documents

Salix alba (Willow)-Derived Ingredients

Ingredient	CAS #	PubMed	FDA	CompTox	ChemPort	NIOSH	NTIS	NTP	FEMA	EU	ECHA	SIDS	SCCS	AICIS	FAO	WHO	Web
Salix Alba (Willow) Bark Extract	84082-82-6	X	X								X						X
Salix Alba (Willow) Bark Powder																	
Salix Alba (Willow) Bark Water																	
Salix Alba (Willow) Extract		X															
Salix Alba (Willow) Flower Extract																	
Salix Alba (Willow) Leaf Extract	84082-82-6	X															

X= data found

Botanical and/or Fragrance Websites

Ingredient	CAS #	Dr. Duke's	Taxonomy	GRIN	Sigma-Aldrich	AHPA	AGRICOLA	IFRA	RIFM
Salix Alba (Willow) Bark Extract	84082-82-6	X	X	X					
Salix Alba (Willow) Bark Powder			X	X					
Salix Alba (Willow) Bark Water			X	X					
Salix Alba (Willow) Extract			X	X					
Salix Alba (Willow) Flower Extract			X	X					
Salix Alba (Willow) Leaf Extract	84082-82-6	X	X	X					

Search Strategy

The following search terms were searched in PubMed:

Salix alba
Salix alba leaf
Salix alba bark
Salix alba leaf extract
Salix alba bark extract
Willow leaf extract
Willow bark extract
Salix alba bark water
Willow bark water
Salix alba extract
Salix alba flower
84082-82-6
Salix alba cosmetic
Salix alba metabolism
Salix alba penetration
Salix alba dermal
Salix alba skin
Salix alba allergy
Salix alba case report
Salix alba anaphylaxis
Salix alba ocular
Salix alba clinical
Salix alba cosmetic

The following search terms were used in all links other than PubMed:

Salix alba
Willow
84082-82-6

Search Engines

- Pubmed - http://www.ncbi.nlm.nih.gov/pubmed
  - appropriate qualifiers are used as necessary
  - search results are reviewed to identify relevant documents
- CompTox: https://comptox.epa.gov/dashboard/chemical/pubmed-abstract-sifter/DTXSID3039242; https://www.epa.gov/comptox-tools/downloadable-computational-toxicology-data#LM
- eChemPortal: https://www.echemportal.org/echemportal/
- DeepDyve: https://www.deepdyve.com/
- Connected Papers - https://www.connectedpapers.com/

Pertinent Websites

- wINCI - https://incipedia.personalcarecouncil.org/winci/ingredient-custom-search/
- FDA Cosmetics page - https://www.fda.gov/cosmetics
- eCFR (Code of Federal Regulations) - https://www.ecfr.gov/
- FDA search databases: https://www.fda.gov/industry/fda-basics-industry/search-databases
- Substances Added to Food (formerly, EAFUS): https://www.fda.gov/food/food-additives-petitions/substances-added-food-formerly-eafus
- GRAS listing: https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras
- SCOGS database: https://www.fda.gov/food/generally-recognized-safe-gras/gras-substances-scogs-database
- Inventory of Food Contact Substances Listed in 21 CFR: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=IndirectAdditives
- Drug Approvals and Database: https://www.fda.gov/drugs/development-approval-process-drugs/drug-approvals-and-databases
- FDA Orange Book: https://www.fda.gov/drugs/drug-approvals-and-databases/approved-drug-products-therapeutic-equivalence-evaluations-orange-book
- OTC Monographs - https://dps.fda.gov/omuf
- Inactive Ingredients Approved For Drugs: https://www.accessdata.fda.gov/scripts/cder/iig/
- FEMA (Flavor & Extract Manufacturers Association) GRAS: https://www.femaflavor.org/fema-gras
- NIOSH (National Institute for Occupational Safety and Health) - http://www.cdc.gov/niosh/
- NTIS (National Technical Information Service) - http://www.ntis.gov/
  - technical reports search page: https://ntrl.ntis.gov/NTRL/
- NTP (National Toxicology Program) - http://ntp.niehs.nih.gov/
- EUR-Lex - https://eur-lex.europa.eu/homepage.html
- Scientific Committees (SCCS, etc) opinions: https://health.ec.europa.eu/scientific-committees_en https://health.ec.europa.eu/scientific-committees/scientific-committee-consumer-safety-sccs_en
- ECHA (European Chemicals Agency – REACH dossiers) – https://echa.europa.eu/
- European Medicines Agency (EMA) - http://www.ema.europa.eu/ema/
- OECD SIDS (Organisation for Economic Co-operation and Development Screening Info Data Sets)- http://webnet.oecd.org/hpv/ui/Search.aspx
- EFSA (European Food Safety Authority) - https://www.efsa.europa.eu/en
- ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) - http://www.ecetoc.org
- AICIS (Australian Industrial Chemicals Introduction Scheme)- https://www.industrialchemicals.gov.au/
- International Programme on Chemical Safety http://www.inchem.org/
- Office of Dietary Supplements https://ods.od.nih.gov/
- FAO (Food and Agriculture Organization of the United Nations) - http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/en/
- WHO (World Health Organization) IRIS library - https://apps.who.int/iris/
- a general Google and Google Scholar search should be performed for additional background information, to identify references that are available, and for other general information - www.google.com https://scholar.google.com/

Botanical Websites

- Dr. Duke's - https://phytochem.nal.usda.gov/
- Taxonomy database - http://www.ncbi.nlm.nih.gov/taxonomy
- GRIN (U.S. National Plant Germplasm System) - https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysimple.aspx
- Sigma Aldrich plant profiler- http://www.sigmaaldrich.com/life-science/nutrition-research/learning-center/plant-profiler.html
- American Herbal Products Association Botanical Safety Handbook (2nd Edition; 2013) - http://abc.herbalgram.org/site/DocServer/AHPABotanicalSafety_FMexcerpt.pdf?docID=4601
- National Agricultural Library NAL Catalog (AGRICOLA) https://agricola.nal.usda.gov/
- The Seasoning and Spice Association List of Culinary Herbs and Spices
- http://www.seasoningandspice.org.uk/ssa/background_culinary-herbs-spices.aspx

Fragrance Websites

- IFRA (International Fragrance Association) – https://ifrafragrance.org/
- Research Institute for Fragrance Materials (RIFM) - https://www.rifm.org/#gsc.tab=0 http://fragrancematerialsafetyresource.elsevier.com/

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Kimberly Norman, Ph.D., DABT, ERT
Industry Liaison to the CIR Expert Panel

DATE: October 10, 2025

SUBJECT: Scientific Literature Review: Safety Assessment of *Salix alba* (Willow)-Derived Ingredients as Used in Cosmetics (release date September 25, 2025)

The Personal Care Products Council respectfully submits the following comments on the scientific literature review, Safety Assessment of *Salix alba* (Willow)-Derived Ingredients as Used in Cosmetics.

Introduction – It would be helpful to state that the ECHA dossier was on a *Salix alba* bark extract that is used in fertilizers.

Since salicin (glucose bound to salicylic acid) is an important component of the *Salix alba*-derived ingredients, it would be helpful to note that Salicylic Acid and a number of salicylates have been reviewed by CIR (final 2019) and found "safe in cosmetics in the present practices of use and concentration described in the safety assessment, when formulated to be non-irritating and non-sensitizing, which may be based on a quantitative risk assessment (QRA)".

Chemical Properties; Table 2 – In addition to the information on the fertilizer ingredient provided in the ECHA dossier, the information on the cosmetic ingredient (from reference 10) should be included in the report, e.g., white to off-white free flowing powder, pH of a 3% solution in water 4-6) (see: https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-Specification-v13.pdf)

Composition and Impurities – In addition to discussing possible heavy metal impurities, it would be helpful to also discuss salicylates, the components for which *Salix* species are used in cosmetics (and fertilizers). In this section, either provide the structure of salicin, or describe how it is related to salicylic acid.

Composition and Impurities, Salix Alba (Willow) Bark Extract – Please indicate that the cosmetic ingredient that is normalized to 53-65% salicylates is a powder of an extract.

Please provide the units for the composition information for bark.

Composition and Impurities, Salix Alba (Willow) Leaf Extract – Please provide the units for the components found in the leaf extract.

Cosmetic Use; Summary; Table 5 – The maximum use concentration of the Bark Extract is 1% in skin cleansing products (although 0.5% in other suntan preparations is the maximum leave-on use concentration). In the text, please also describe the use of Salix Alba (Willow) Leaf Extract.

Cosmetic Use – Because salicylates are important components of these ingredients, please consider including the EU limits for use of salicylates in cosmetics (found in both Annex III and Annex V, preservatives).

Annex III has the following limitations:
a) Rinse-off hair products: Maximum 3%

b) Other products except body lotion, eye shadow, mascara, eyeliner, lipstick, roll-on deodorant: Maximum 2%

c) Body lotion, eye shadow, mascara, eyeliner, lipstick, roll-on deodorant: Maximum 0.5%

Other:
a) b) c) Not to be used in preparations for children under 3 years of age. Not to be used in applications that may lead to exposure of the end-user's lungs by inhalation. Not to be used in oral products. For purposes other than inhibiting the development of microorganisms in the product. This purpose has to be apparent from the presentation of the product.

These levels are inclusive of any use of salicylic acid.

Wording – Warnings/Cautionary:
a) b) c) Not to be used for children under three years of age (Soley for products which might be used for children under three years of age.)

Annex V has the following limitations when Salicylic Acid and salts are used as preservatives:
Salicylic Acid 0.5% (acid)
Salts 0.5% (acid)

Salicylic Acid: Not to be used in products for children under 3 years of age.

Not to be used in oral products.

Not to be used in applications that may lead to exposure of the end-user's lungs by inhalation.
Salts: Not to be used in products for children under 3 years of age, except for shampoos.

Wording – Warnings/Cautionary:
Salicylic Acid: Not to be used for children under 3 years of age (Solely for products which might

be used for children under 3 years of age.)

Salicylic Acid Salts: Not to be used for children under 3 years of age (Solely for products which might be used for children under 3 years of age and which remain in prolonged contact with the skin.)

Toxicokinetic Studies – Although the species of willow is not stated in the abstract, the following study which examines the metabolism and excretion of salicin from an oral dose of willow bark extract given to 10 human volunteers, should be added to the Toxicokinetic Studies section.

Schmid B, Kòtter I, Heide L. 2001. Pharmacokinetics of salicin after oral administration of a standardized willow bark extract. Eur J Clin Pharmacol 57(5):387-391.
DOI: 10.1007/s002280100325

Abstract

Objective: To evaluate the pharmacokinetics of salicin and its major metabolites in humans after oral administration of a chemically standardised willow bark extract.

Methods: Willow bark extract corresponding to 240 mg salicin (1,360 mg, 838 micromol) was ingested by ten healthy volunteers in two equal doses at times 0 h and 3 h. Over a period of 24 h, urine and serum levels of salicylic acid and its metabolites, i.e. gentisic acid and salicyluric acid, were determined using reverse-phase high-performance liquid chromatography. Renal excretion rate, elimination half-life and total bioavailability of salicylates were calculated.

Results: Salicylic acid was the major metabolite of salicin detected in the serum (86% of total salicylates), besides salicyluric acid (10%) and gentisic acid (4%). Peak levels were reached within less than 2 h after oral administration. Renal elimination occurred predominantly in the form of salicyluric acid. Peak serum levels of salicylic acid were on average 1.2 mg/l, and the observed area under the serum concentration time curve (AUC) of salicylic acid was equivalent to that expected from an intake of 87 mg acetylsalicylic acid.

Conclusion: Willow bark extract in the current therapeutic dose leads to much lower serum salicylate levels than observed after analgesic doses of synthetic salicylates. The formation of salicylic acid alone is therefore unlikely to explain analgesic or anti-rheumatic effects of willow bark.

Genotoxicity – Please indicate that the comet assay in peripheral blood mononuclear cells was negative at 5 µg/ml.

Dermal Irritation and Sensitization – It should be made clear that the hydroalcoholic *Salix alba* bark extract tested in the *in vitro* assays is used as a fertilizer ingredient.

Phototoxicity – It should be stated that the materials tested in the phototoxicity assays were cosmetic ingredients. For reference 28, it should also be stated that Salix Alba (Willow) Bark Extract was tested in the in vitro assay because it "produced significant absorbance [i.e., >0.1 absorbance units (AU)] in the wavelengths of interest between 290 nm and 700 nm (data not shown)."

Summary – Information about composition (salicylates) should be included in the Summary.

Table 1 – As there are no structures in this table, "idealized structures" should be deleted from

the title of the table.

Table 2 – It should be noted that the information from reference 2 is for an extract used in fertilizers. Information about the cosmetic ingredient (reference 10) should be added to this table.

Table 6 – In the results column of the comet assay in peripheral blood mononuclear cells, it would be helpful to state that the results were negative at 5 µg/ml.

Table 7 – It should be made clear that the material tested in reference 29 was the powder of a willow bark extract that contained between 53-65% salicylates.

Reference 29 – It is not clear why this reference is dated 2025 when the bottom of each page includes “Version#1/12-28-16/Form#57” (which indicates the summary was prepared in 2016).

***Salix alba* (Willow)-derived ingredients – March 2026 – Priya Ferguson**	
Comment Submitter: Personal Care Products Council **Date of Submission: October 10, 2025**	
Comment	**Response/Action**
Introduction – It would be helpful to state that the ECHA dossier was on a Salix alba bark extract that is used in fertilizers.	addressed
Since salicin (glucose bound to salicylic acid) is an important component of the Salix alba - derived ingredients, it would be helpful to note that Salicylic Acid and a number of salicylates have been reviewed by CIR (final 2019) and found "safe in cosmetics in the present practices of use and concentration described in the safety assessment, when formulated to be non-irritating and non-sensitizing, which may be based on a quantitative risk assessment (QRA)"	addressed
Chemical Properties; Table 2 – In addition to the information on the fertilizer ingredient provided in the ECHA dossier, the information on the cosmetic ingredient (from reference 10) should be included in the report, e.g., white to off-white free flowing powder, pH of a 3% solution in water 4-6) (see: https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-Specification-v13.pdf)	addressed
Composition and Impurities – In addition to discussing possible heavy metal impurities, it would be helpful to also discuss salicylates, the components for which Salix species are used in cosmetics (and fertilizers). In this section, either provide the structure of salicin, or describe how it is related to salicylic acid.	The amount of salicylates in Salix Alba (Willow) Bark Extract has been provided in the report – no further details have been included as details regarding potential components are not typically provided in CIR reports.
Composition and Impurities, Salix Alba (Willow) Bark Extract – Please indicate that the cosmetic ingredient that is normalized to 53-65% salicylates is a powder of an extract.	addressed
Please provide the units for the composition information for bark	addressed
Composition and Impurities, Salix Alba (Willow) Leaf Extract – Please provide the units for the components found in the leaf extract	addressed
Cosmetic Use; Summary; Table 5 – The maximum use concentration of the Bark Extract is 1% in skin cleansing products (although 0.5% in other suntan preparations is the maximum leave-on use concentration). In the text, please also describe the use of Salix Alba (Willow) Leaf Extract.	report updated with 2025 concentration of use
Cosmetic Use – Because salicylates are important components of these ingredients, please consider including the EU limits for use of salicylates in cosmetics (found in both Annex III and Annex V, preservatives)	addressed

***Salix alba* (Willow)-derived ingredients – March 2026 – Priya Ferguson**	
Comment Submitter: Personal Care Products Council **Date of Submission: October 10, 2025**	
Comment	**Response/Action**
Annex III has the following limitations: a) Rinse-off hair products: Maximum 3% b) Other products except body lotion, eye shadow, mascara, eyeliner, lipstick, roll-on deodorant: Maximum 2% c) Body lotion, eye shadow, mascara, eyeliner, lipstick, roll-on deodorant: Maximum 0.5% Other: a) b) c) Not to be used in preparations for children under 3 years of age. Not to be used in applications that may lead to exposure of the end user's lungs by inhalation. Not to be used in oral products. For purposes other than inhibiting the development of microorganisms in the product. This purpose has to be apparent from the presentation of the product. These levels are inclusive of any use of salicylic acid. Wording – Warnings/Cautionary: a) b) c) Not to be used for children under three years of age (Soley for products which might be used for children under three years of age.) Annex V has the following limitations when Salicylic Acid and salts are used as preservatives: Salicylic Acid 0.5% (acid) Salts 0.5% (acid) Salicylic Acid: Not to be used in products for children under 3 years of age. Not to be used in oral products. Not to be used in applications that may lead to exposure of the end-user's lungs by inhalation. Salts: Not to be used in products for children under 3 years of age, except for shampoos. Wording – Warnings/Cautionary: Salicylic Acid: Not to be used for children under 3 years of age (Solely for products which might be used for children under 3 years of age.) Salicylic Acid Salts: Not to be used for children under 3 years of age (Solely for products which might be used for children under 3 years of age and which remain in prolonged contact with the skin.	

***Salix alba* (Willow)-derived ingredients – March 2026 – Priya Ferguson**	
Comment Submitter: Personal Care Products Council **Date of Submission: October 10, 2025**	
Comment	**Response/Action**
Toxicokinetic Studies – Although the species of willow is not stated in the abstract, the following study which examines the metabolism and excretion of salicin from an oral dose of willow bark extract given to 10 human volunteers, should be added to the Toxicokinetic Studies section. Schmid B, Kòtter I, Heide L. 2001. Pharmacokinetics of salicin after oral administration of a standardized willow bark extract. Eur J Clin Pharmacol 57(5):387-391.	Not included in the report as the species was not stated and data on components are typically not included.
Genotoxicity – Please indicate that the comet assay in peripheral blood mononuclear cells was negative at 5 µg/ml	addressed
Dermal Irritation and Sensitization – It should be made clear that the hydroalcoholic Salix alba bark extract tested in the in vitro assays is used as a fertilizer ingredient	These types of distinctions are not typically made in CIR reports.
Phototoxicity – It should be stated that the materials tested in the phototoxicity assays were cosmetic ingredients. For reference 28, it should also be stated that Salix Alba (Willow) Bark Extract was tested in the in vitro assay because it "produced significant absorbance [i.e., >0.1 absorbance units (AU)] in the wavelengths of interest between 290 nm and 700 nm (data not shown)."	It is clear that the substances tested were cosmetic ingredients due to the use of *Dictionary* nomenclature (Salix Alba (Willow) Bark Extract).
Summary – Information about composition (salicylates) should be included in the Summary.	Not typically included in composition section.
Table 1 – As there are no structures in this table, "idealized structures" should be deleted from the title of the table	addressed
Table 2 – It should be noted that the information from reference 2 is for an extract used in fertilizers. Information about the cosmetic ingredient (reference 10) should be added to this table	The table has been updated to represent the properties of the cosmetic ingredient vs. non-cosmetic ingredient.
Table 6 – In the results column of the comet assay in peripheral blood mononuclear cells, it would be helpful to state that the results were negative at 5 µg/ml	addressed
Table 7 – It should be made clear that the material tested in reference 29 was the powder of a willow bark extract that contained between 53-65% salicylates	Not typically included in CIR reports, in addition, the composition section states that Salix Alba (Willow) Bark Extract is standardized to contain 53 – 65% salicylates.
Reference 29 – It is not clear why this reference is dated 2025 when the bottom of each page includes "Version#1/12-28-16/Form#57" (which indicates the summary was prepared in 2016)	addressed

Safety Assessment of *Salix alba* (Willow)-Derived Ingredients as Used in Cosmetics

Status: Draft Report for Panel Review
Release Date: February 17, 2026
Panel Meeting Date: March 12 – 13, 2026

The Expert Panel for Cosmetic Ingredient Safety members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; David E. Cohen, M.D.; Samuel M. Cohen, M.D., Ph.D.; Curtis D. Klaassen, Ph.D.; Allan E. Rettie, Ph.D.; David Ross, Ph.D.; Paul W. Snyder, D.V.M., Ph.D.; and Susan C. Tilton, Ph.D. The Cosmetic Ingredient Review (CIR) Executive Director is Bart Heldreth, Ph.D., and the Senior Director is Monice Fiume, M.B.A. This safety assessment was prepared by Priya Ferguson, M.S., Senior Scientific Analyst/Writer, CIR.

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ABBREVIATIONS

CIR	Cosmetic Ingredient Review
Council	Personal Care Products Council
CTFA	Cosmetic, Toiletry, and Fragrance Association
Dictionary	*International Cosmetic Ingredient Dictionary and Handbook*
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPRA	direct peptide reactivity assay
EC	European Commission
$EC_{1.5}$	effective concentration of a test chemical that induces a 1.5-fold increase in luciferase activity
ECHA	European Chemicals Agency
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FDA	Food and Drug Administration
GARD	genomic allergen rapid detection
GHS	Globally Harmonized System
HepG2	human hepatoma cell line
HL-60	human leukemia-60
Hprt	hypoxanthine-guanine phosphoribosyltransferase
IFRA	International Fragrance Association
IL-6	interleukin-6
I_{max}	mean maximal luciferase activity
IC_{50}	inhibitory concentration 50%
LD_{50}	median lethal dose
l.o.	leave-on
log K_{ow}	n-octanol/water partition coefficient
MoCRA	Modernization of Cosmetics Regulation Act
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	not applicable
ND	not detected
NOAEL	no-observed-adverse-effect-level
NR	not reported
OECD	Organisation for Economic Co-operation and Development
Panel	Expert Panel for Cosmetic Ingredient Safety
RIFM	Research Institute for Fragrance Materials
RLD	Registration and Listing Data
RNA	ribonucleic acid
r.o.	rinse-off
SIRC	Statens Seruminstitut Rabbit Cornea
TG	test guideline
US	United States
USP	United States Pharmacopeia
UVA	ultraviolet A
Vis	visible light
Xprt	xanthine-guanine phosphoribosyltransferase

INTRODUCTION

This assessment reviews the safety of the following 6 *Salix alba*-derived ingredients as used in cosmetics formulations:

Salix Alba (Willow) Bark Extract	Salix Alba (Willow) Extract
Salix Alba (Willow) Bark Powder	Salix Alba (Willow) Flower Extract
Salix Alba (Willow) Bark Water	Salix Alba (Willow) Leaf Extract

According to the web-based *International Cosmetic Ingredient Dictionary and Handbook* (*Dictionary*), 4 of these ingredients, including Salix Alba (Willow) Bark Extract, are reported to function as skin-conditioning agents in cosmetics; other functions of these ingredients may be found in Table 1.[1] It should be noted that Salix Alba (Willow) Bark Water is reported to only function as a fragrance ingredient in cosmetics, and the Expert Panel for Cosmetic Ingredient Safety (Panel) does not typically review ingredients that function only as fragrance ingredients, because, as fragrances, the evaluation of the safety of these ingredients is the purview of the Research Institute for Fragrance Materials (RIFM). However, it appears that this ingredient is not included in their review process, and therefore, the Panel is reviewing its safety.

Botanicals, such as the *Salix alba* (willow)-derived ingredients reviewed herein, may contain hundreds of constituents. In this assessment, the Panel is evaluating the potential toxicity of each of the *Salix alba*-derived ingredients as a whole, complex substance; toxicity from single components may not predict the potential toxicity of botanical ingredients.

Salicin, a glucose-bound form of salicylic acid, and a component of *Salix alba* (willow)-derived ingredients, has not been reviewed by the Panel. However, the Panel has previously reviewed salicylic acid and 17 salicylates in an amended report finalized in 2019.[2] In that report, the Panel concluded that salicylic acid and the 17 salicylates reviewed in that report are safe in cosmetics, in the present practices of use and concentration described in that safety assessment, when formulated to be non-irritating and non-sensitizing, which may be based on a quantitative risk assessment.

This safety assessment includes relevant published and unpublished data that are available for each endpoint that is evaluated. Published data are identified by conducting an extensive search of the world's literature; a search was last conducted January 2026. A listing of the search engines and websites that are used and the sources that are typically explored, as well as the endpoints that the Panel typically evaluates, is provided on the Cosmetic Ingredient Review (CIR) website (https://www.cir-safety.org/supplementaldoc/preliminary-search-engines-and-websites; https://www.cir-safety.org/supplementaldoc/cir-report-format-outline). Unpublished data are provided by the cosmetics industry, as well as by other interested parties.

The cosmetic ingredient names, according to the *Dictionary*, are written as listed above, without italics and without abbreviations. When referring to the plant from which these ingredients are derived, the standard scientific practice of using italics will be followed (i.e., *Salix alba*). Often in the published literature, the general name willow is used. If it is not known whether the substance being discussed is equivalent to the cosmetic ingredient, the test substance will be identified by the name used in the publication that is being cited (e.g., *Salix alba* (willow) bark extract). However, if it is known that the substance is a cosmetic ingredient, the *Dictionary* nomenclature (e.g., Salix Alba (Willow) Bark Extract) will be used.

Furthermore, it should also be noted that the terms "willow" and "willow bark extract" may refer to several different species of willow (e.g., *Salix purpurea*, *Salix fragilis*) in the literature. For the purposes of this report, only studies explicitly using *Salix alba* - derived plant parts were included. However, a few case reports describing allergic reactions to dietary supplements containing "white willow bark" were included, since these products may in fact contain bark derived from *Salix alba*, and these case reports may assist the Panel in evaluating the potential allergenicity of *Salix alba* - derived ingredients.

Much of the data included in this safety assessment was found on the European Chemicals Agency (ECHA) website.[3] The cited information pertains to a *Salix alba* (willow) bark extract that was reported for use in a fertilizer. Please note that the ECHA website provides summaries of information generated by industry, and it is those summary data that are reported in this safety assessment when ECHA is cited.

CHEMISTRY

Definition and Plant Identification

The definitions of the ingredients included in this review are provided in Table 1. *Salix alba* is a fast-growing deciduous tree native to Europe, Asia, and North Africa, most often occurring in riparian habitats such as riverbanks, wetlands, and other moist soils.[4] These plants are capable of reaching heights up to 30 m with trunks exceeding 1 m in diameter. The leaves are narrow and lanceolate, displaying silver-grey upper surfaces and dense silky white hairs on the underside, while the bark is dark grey and deeply fissured (younger bark may be greenish-yellow).[5] The flowers of the *Salix alba* plant are produced in catkins that emerge in early spring, appearing before or with the leaves.[6] The species is dioecious, meaning male and female catkins are produced on separate trees. Male catkins are typically larger and yellowish due to exposed anthers while female catkins are smaller and greenish.

Chemical Properties

Salix Alba (Willow) Bark Extract is a white to off-white free-flowing powder with a characteristic odor.[7] The octanol/water partition coefficient (log K_{ow}) of a *Salix alba* (willow) bark extract is < 0.3.[3] Other chemical properties of this ingredient may be found in Table 2.

Method of Manufacture

The majority of methods below are general to the processing of *Salix alba* (willow)-derived ingredients. It is unknown if these apply to cosmetic ingredient manufacturing.

Salix Alba (Willow) Bark Extract

One source reports that Salix Alba (Willow) Bark Extract is prepared by extracting the bark of *Salix alba* with ethanol, followed by purification, filtration, and drying.[8] The resulting residue is then processed with 1,3-butylene glycol and filtered to obtain the final product. No synthetic reagents, preservatives, or chemical modifications are used, and residual solvent levels comply with International Fragrance Association (IFRA)/Cosmetic, Toiletry, and Fragrance Association (CTFA; now known as the Personal Care Products Council) specifications and general cosmetic ingredient quality guidelines. According to a supplier, Salix Alba (Willow) Bark Extract is processed by the grinding of plant bark and aqueous extraction at a controlled pH and temperature for a specified duration, followed by filtration, concentration, and spray drying.[9] Throughout production, batch adjustments are made as needed, with initial and final quality control testing, including microbiological sampling.

In order to prepare a *Salix alba* (willow) bark extract, plant bark was first dried and pulverized into a fine powder.[10] The powder was then extracted in a Soxhlet apparatus using ethanol as the solvent for 7 h. The solution was filtered and evaporated to obtain the bark extract.

Salix Alba (Willow) Leaf Extract

A *Salix alba* (willow) leaf extract was prepared by first collecting, rinsing, and air-drying the leaves.[11] Following drying, the leaves were pulverized via an electric grinder. Extraction was performed via cold maceration of 500 g ethyl acetate with intermittent shaking. The resulting filtrate was concentrated using rotary vapor. To remove chlorophyll, the extract was washed with a non-polar solvent, after which it was concentrated and stored.

Composition and Impurities

Salix species are known for their capacity to concentrate toxic heavy metals.[12] Therefore, the *Salix alba* (willow) plant and plant parts (including the bark and leaves) may contain heavy metals such as cadmium, lead, and zinc.[13]

Salix Alba (Willow) Bark Extract

A cosmetic ingredient supplier states that Salix Alba (Willow) Bark Extract is standardized to contain 53 – 65% salicylates (this ingredient is a powder of an extract).[14] According to the same supplier, the ingredient should contain 2 – 5% nitrogen and should not contain more than 20 ppm heavy metals, 10 ppm lead, 2 ppm arsenic, 1 ppm mercury, or 1 ppm cadmium.[7] According to another source, Salix Alba (Willow) Bark Extract is a mixture composed of water (68.25%), butylene glycol (29.25%), and Salix Alba (Willow) Bark Extract (2.5%).[8] For this ingredient, heavy metals and pesticide residues comply with cosmetic ingredient standards (heavy metals < 20 ppm; arsenic < 2 ppm), and no synthetic additives or preservatives are present. Salix Alba (Willow) Bark Extract should not contain certain pesticides (e.g., alachlor, dieldrin) or the allergens listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (European Commission) No. 1223/2009.[15] Salix Alba (Willow) Bark Extract has also been reported to be a 1:5 extract (for every 1 part plant material used, 5 parts solvent is used) containing 9.8 – 11.5% salicylic acid or 18 – 22% salicylic acid.[16,17]

Data from the scientific literature on *Salix alba* (willow) bark report broader phytochemical compositions. In *Salix alba* (willow) bark extracts, the content of various compounds was measured in both young and mature bark.[18] For young bark, the total salicylate derivative, polyphenol (expressed as gallic acid), flavone (expressed as rutin), and tannin (expressed as pyrogallol) content in extracts of young and mature *Salix alba* (willow) bark were determined to be 15, 55, 4.5, and 16 mg/g, respectively.[18] In mature bark, the respective values were 19, 65, 3, and 16.6 mg/g.

The total phenolic content of a hot ethanolic extract of *Salix alba* (willow) bark was determined to be 162 mg/g.[10] The amounts of salicylates, flavonoids, flavan-3-ols, and phenolic acids in a methanolic *Salix alba* (willow) bark extract have been summarized in Table 3.[19] In addition, the phenolic acid, flavanol, and procyanidin content of a hydromethanolic extract of *Salix alba* (willow) bark may be found in Table 4.[20] A chemical analysis of *Salix alba* (willow) bark reported the following structural component contents: 70.18% holocellulose, 40.32% α-cellulose, and 33.81% Klason lignin.[21]

According to the World Health Organization, *Salix alba* (willow) bark used in herbal medicines must conform to the following specifications: not more than 3% of twigs with a diameter greater than 10 mm, and not more than 2% other foreign matter, not more than 10% total ash, not more than 3% acid-insoluble ash, not less than 10% water-soluble extractive, and not more than 11% loss on drying.[22] The recommended maximum limit of aldrin and dieldrin is not more than 0.05 mg/kg. In addition, the product must comprise ≥ 1.5% of total salicylate derivatives expressed as salicin by high-performance liquid chromatography.

Salix Alba (Willow) Leaf Extract

Salix Alba (Willow) Leaf Extract at 4 – 6% in water is composed of 32% sugar, 29.5% mineral ashes, 3.9% proteins, and 3.1% polyphenols.[23] Specifications indicate that heavy metal impurities (including antimony, arsenic, cadmium, chromium, cobalt, mercury, nickel, lead, and vanadium) are each present at concentrations ≤ 0.5 ppm.

The total polyphenols (expressed as gallic acid), flavones (expressed as rutin), and tannins (expressed as pyrogallol) content in the extract of young *Salix alba* (willow) leaves were determined to be 55, 8.5, and 12 mg/g, respectively.[18] In mature leaves, the respective compositions were 70, 8, and 10 mg/g. The composition of a hydromethanolic extract of *Salix alba* (willow) leaves may be found in Table 4.[20]

The major compounds present in an ethyl acetate *Salix alba* (willow) leaf extract were determined via gas chromatography-mass spectrometry analysis.[11] The detected compounds and their relative abundances (as percentages) were: heptacosan-1-ol (15.59%), tetracosanal (14.93%), n-octacosyl acetate (9.57%), cholesterol (5.11%), octacosylheptafluoro-butyrate (5.09%), octacosyl acetate (4.81%), stearyl aldehyde (4.45%), and cholest-4-en-3-one (4.08%).

USE

Cosmetic

The safety of the cosmetic ingredients addressed in this assessment is evaluated based on data received from the US Food and Drug Administration (FDA) and the cosmetics industry on the expected use of *Salix alba* (willow)-derived ingredients in cosmetics. Registration and Listing Data (RLD) obtained from the FDA report frequency of use, and responses to a survey conducted by the Personal Care Products Council (Council) indicate maximum reported concentrations of use; it is these values that define the present practices of use and concentration that are assessed by the Panel. Since 2024, as a result of the Modernization of Cosmetics Regulation Act (MoCRA) of 2022, manufacturers and processors are required to register facilities and list their products (and ingredients therein) with the FDA (i.e., RLD). An exception is made for small businesses (average gross annual sales in the US of cosmetic products for the previous 3-yr period is less than $1,000,000, adjusted for inflation), which are exempt from MoCRA reporting for most cosmetic product categories. Eye area products, injected products, internal use products, or products that alter appearance for more than 24 h, and the facilities that manufacture these products, are not included in this exemption.[24]

According to RLD obtained in 2025[25,26] and concentration of use data submitted in response to a concentration of use survey conducted by PCPC in 2025,[27] Salix Alba (Willow) Bark Extract has the highest frequency and concentration of use of the ingredients in this group. This ingredient is used in 3060 formulations at up to 1.1% (in leave-on face and neck products; Table 5). (Although all 6 ingredients have use reported in the RLD, Salix Alba (Willow) Leaf Extract is the only other ingredient with a reported concentration of use.)

These ingredients may be incidentally ingested as they are used in products used in the mouth (e.g., Salix Alba (Willow) Bark Extract is used in mouthwashes and breath fresheners; concentration not reported). In addition, these ingredients may result in mucous membrane exposure (e.g., Salix Alba (Willow) Bark Extract is used bath soaps and body washes at 0.004%). Lastly, these ingredients are reported to be used in baby products (e.g., Salix Alba (Willow) Bark Extract is used in other baby products; concentration not reported).

These ingredients may be incidentally inhaled as they are used in spray (e.g., Salix Alba (Willow) Bark Extract is used in hair spray; concentration not reported) and powder formulations (e.g., Salix Alba (Willow) Bark Extract is used in face powders; concentration not reported). In practice, as stated in the Panel's respiratory exposure resource document (https://www.cir-safety.org/cir-findings), most droplets/particles incidentally inhaled from cosmetic sprays be deposited in the nasopharyngeal and tracheobronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount. Conservative estimates of inhalation exposures to respirable particles during the use of loose powder cosmetic products are 400-fold to 1000-fold less than protective regulatory and guidance limits for inert airborne respirable particles in the workplace.

Some products containing *Salix alba* (willow)-derived ingredients may be marketed for use with airbrush delivery systems. With the advent of MoCRA and the current product categories outlined by the FDA, it is now mandatory that cosmetic products used in airbrush delivery systems be reported as such for some, but not all, product categories in the RLD. In other words, a reliable source of frequency of use data regarding the use of cosmetic ingredients in conjunction with airbrush delivery systems is now available, in some instances. Some of the reported product categories for these categories listed in the RLD do require designation if airbrush application is used, and this type of application was reported (e.g., Salix Alba (Willow) Bark Extract is reported to be used in airbrush foundation formulations and in professional airbrush indoor tanning preparations (concentrations not provided)). Additionally, the Council currently surveys the cosmetic industry for maximum reported use concentrations of ingredients in products which may be used in conjunction with an airbrush delivery system; thus, this type of data may also be available, when submitted. Please note that no concentration of use data were provided indicating airbrush application. Nevertheless, no consumer habits and practices data or particle size data are publicly available to evaluate the exposure associated with this use type, thereby preempting the ability to evaluate risk or safety. Without information regarding the consumer habits and practices data or product particle size data (or other relevant particle data, e.g., diameter) related to this use technology, the data profile is incomplete, and the Panel is not able to

determine safety for use in airbrush formulations. Accordingly, the data are insufficient to evaluate the exposure resulting from cosmetics applied via airbrush delivery systems.

None of the *Salix alba* (willow)-derived ingredients named in the report are restricted from use in any way under the rules governing cosmetic products in the European Union (EU); however, the EU limits the use of salicylates in cosmetics.[28] According to European Commission (EC) Regulation No 1223/2009, Annex III permits salicylic acid for purposes other than as a preservative at concentrations up to 3% in rinse-off hair products, up to 2% in most other products, and up to 0.5% in body lotion, eye shadow, mascara, eyeliner, lipstick, and roll-on deodorant. Under this Annex, salicylic acid should not be used in preparations for children under the age of 3, should not be used in applications that may lead to exposure of the end-user's lungs by inhalation, and should not be used in oral products. Annex V permits salicylic acid and its salts to be used as preservatives at a maximum concentration of 0.5% (as acid), also with the same restrictions regarding children under 3, oral products, and inhalation exposure; however, salicylic acid salts are permitted in shampoos for children under 3.

Non-Cosmetic

Salix alba (willow) bark has historically been used to treat various ailments such as pain, fever, inflammation, flu, and headaches; the analgesic, anti-pyretic, and anti-inflammatory properties are primarily attributed to salicin (an analogous precursor to acetylsalicylic acid) content.[10,29] Currently, *Salix alba* (willow) is used in dietary supplements (e.g., weight loss supplements), herbal treatments used for pain management, and fertilizers [12,30-32] These ingredients are also present in acne patches, wart treatments, nail fungal treatments, vitamin C serums, and various homeopathic treatments.[33,34]

According to the European Medicines Agency, willow bark supplements have several contraindications. They should be avoided by individuals with hypersensitivity to salicin, salicylates, or other non-steroidal anti-inflammatory drugs, those with active peptic ulcers, third-trimester pregnancy, glucose-6-phosphate dehydrogenase deficiency, severe liver or kidney dysfunction, coagulation disorders, and children and adolescents under 18 due to the risk of Reye's syndrome.[35] While this guidance specifically refers to willow bark derived from *Salix purpurea*, *Salix daphnoides*, and *Salix fragilis*, it is included herein, as *Salix alba* also contains salicylates and may warrant similar precautions. Similar precautions are required on labels of products containing *Salix alba* (willow) bark according to United States Pharmacopeia (USP) specifications.[5] Labels are required to state that these products should not be used in children, women who are pregnant or nursing, or by persons with known sensitivity to aspirin.

TOXICOKINETIC STUDIES

No relevant toxicokinetics studies on *Salix alba*-derived ingredients were found in the published literature, and unpublished data were not submitted. In general, toxicokinetics data are not expected to be found on botanical ingredients because each botanical ingredient is a complex mixture of constituents.

TOXICOLOGICAL STUDIES

Acute Toxicity Studies

Animal

Salix Alba (Willow) Bark Extract

No mortality or signs of toxicity were observed in mice following oral administration of Salix Alba (Willow) Bark Extract at 2000 mg/kg bw.[8] The extract was a mixture composed of water (68.25%), butylene glycol (29.25%), and Salix Alba (Willow) Bark Extract (2.5%) and was administered to 2 groups of mice (5/group; strain and sex not stated).[8]

In an acute toxicity assay performed according to Organisation for Economic Co-operation and Development (OECD) test guideline (TG) 423, a hydroalcoholic *Salix alba* (willow) bark extract (2000 mg/kg bw; 100% purity; water as vehicle) was administered by gavage to female Wistar rats (3/group).[3] The median lethal dose (LD_{50}) was determined to be > 2000 mg/kg bw.

Salix Alba (Willow) Leaf Extract

An acute oral toxicity assay was performed according to OECD TG 401 using Salix Alba (Willow) Leaf Extract (4 – 6% in water).[23] The test substance was considered to be non-toxic. No other details were provided.

Short-Term Toxicity Studies

Animal

Dermal

Salix Alba (Willow) Bark Extract

Salix Alba (Willow) Bark Extract (mixture composed of water (68.25%), butylene glycol (29.25%), and Salix Alba (Willow) Bark Extract (2.5%)) was applied to the clipped skin of 5 guinea pigs (strain and sex not stated), 5x/wk, for 2 wk.[8] The test material was diluted to 3% in water, resulting in a final Salix Alba (Willow) Bark Extract concentration of 0.075%. No erythema or edema were observed during the observation period (prior to daily dosing and 24 h after final dosing).

Oral

Salix Alba (Willow) Bark Extract

A combined repeated-dose toxicity study with a reproductive/developmental toxicity test was performed in Wistar rats (12/sex/group) according to OECD TG 422.[3] Results regarding the reproductive toxicity parameters evaluated in this study can be found in the Developmental and Reproductive Toxicity section of this report. Animals were treated with 100, 300, or 1000 mg/kg bw/d of a hydroalcoholic *Salix alba (*willow) bark extract (water as vehicle; 100% purity), once a day, via gavage (controls given vehicle only). Males were treated for 28 d and females were treated for approximately 41 d. No test substance-related adverse effects were observed in this assay, and the no-observed-adverse-effect-level (NOAEL) for systemic toxicity was determined to be 1000 mg/kg bw/d.

DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Animal

Salix Alba (Willow) Bark Extract

As stated previously, a combined repeated-dose toxicity study with a reproductive/developmental toxicity test was performed in Wistar rats (12/sex/group) according to OECD TG 422.[3] Results regarding the systemic toxicity evaluated in this study can be found in the Short-Term Toxicity Studies section of this report. Males were treated for 28 d (14 d pre-mating and 14 d during/post-mating) and females were treated for 14 d pre-mating, for up to 14 d during the mating period and through gestation, and for up to 13 d post-partum. Treatments occurred daily via gavage using a hydroalcoholic *Salix alba* (willow) bark extract (100, 300, and 1000 mg/kg bw/d; water as vehicle; controls were treated with the vehicle only). There were no differences between control- and test item-treated groups with regard to reproductive ability, mating, or gestation indices. The parental NOAEL was determined to be 1000 mg/kg bw/d. In the F1 generation, statistically significantly lower thyroid gland weights were observed in the mid- and high-dosed groups compared to control pups. However, thyroid gland weights were well within the historical range, and no effects on thyroid hormone concentration levels were observed. No developmental or endocrine changes were observed in pups. The NOAEL for reproductive effects and pup development/survival was determined to be 1000 mg/kg bw/d.

GENOTOXICITY STUDIES

Details regarding the genotoxicity studies summarized herein are found in Table 6. Salix Alba (Willow) Bark Extract (20 – 100%; up to 5000 µg/plate; in water) and hydroalcoholic *Salix alba* (willow) bark extracts (up to 5000 µg/plate; in water), yielded negative results in bacterial reverse mutation assays (performed with and without metabolic activation).[3,36-38,38] Negative results were also obtained for a hydroalcoholic *Salix alba* (willow) bark extract in an in vitro mammalian cell gene mutation test (up to 5000 µg/ml; in water), and in an in vitro mammalian cell micronucleus assay (at up to 2000 µg/ml; in saline).[29] All assays were performed with and without metabolic activation. A statistically significant elevation in deoxyribonucleic acid (DNA) damage was observed in peripheral blood mononuclear cells at concentrations of 50 µg/ml and higher in a comet assay using *Salix alba* (willow) bark extract (use of metabolic activation not stated; negative results at 5 µg/ml). However, no genotoxicity was observed in a comet assay performed in human hepatoma cell line (HepG2) cells using *Salix alba* (willow) bark extract (up to 100 µg/ml; use of metabolic activation and vehicle not stated). No genotoxicity was observed in a cytokinesis-block micronucleus assay performed in human peripheral blood leukocytes and HepG2 cells using *Salix alba* (willow) bark extract (up to 100 µg/ml; use of metabolic activation and vehicle not stated). Salix Alba (Willow) Leaf Extract (4 – 6% in water) was determined to be non-mutagenic in an Ames assay performed according to OECD TG 471 (no other details provided).[23] Similarly, no genotoxicity was observed in an in vivo comet assay or an in vivo micronucleus assay. In both in vivo assays, *Salix alba* (willow) bark extract was given to mice via gavage at up to 2000 mg/kg bw in dimethyl sulfoxide (DMSO).

CARCINOGENICITY STUDIES

Carcinogenicity studies on *Salix alba*-derived ingredients were found in the published literature, and unpublished data were not submitted.

ANTI-CARCINOGENICITY STUDIES

In Vitro

Salix Alba (Willow) Bark Extract

The ability of an ethanolic *Salix alba* (willow) bark extract to induce cytotoxicity in human leukemia-60 (HL-60) cells was evaluated.[10] Cells were incubated with 1, 2, 4, 6, 8, or 10 µg of the test substance for up to 24 h. The percent viability of cells after 24 h treatment with the negative control, 1, 2, 4, 6, 8, and 10 µg of extract was 98, 93, 87, 38, 50, and 16%, respectively.

OTHER RELEVANT STUDIES

Effect on Interleukin-6 (IL-6)

Salix Alba (Willow) Bark Extract

The effect of Salix Alba (Willow) Bark Extract on IL-6 levels was evaluated using cultured human dermal fibroblasts in an IL-6 enzyme-linked immunosorbent assay (ELISA).[39] Cells were treated with the test substance at concentrations of 0.01, 0.1, and 1% and incubated for 24 h. Appropriate positive and negative controls were used. IL-6 concentrations following exposure to 0.01, 0.1, and 1% of the test substance were 2860, 2600, and 1087 pg/ml, respectively. IL-6 concentrations following positive and negative control exposure were 2599 and 1423 pg/ml, respectively.

Effect on Pigmentation

Salix Alba (Willow) Bark Extract

The effect of 2% Salix Alba (Willow) Bark Extract (in a base lotion) was evaluated in artificially pigmented forearm skin sites (n = 10 subjects).[40] Skin was pigmented using a dye containing dihydroxyacetone. Five skin sites were identified: subject – site 1 was an untreated comparative site, site 2 received dye only, site 3 received base lotion only, site 4 received 1% salicylic acid in base lotion, and site 5 received 2% Salix Alba (Willow) Bark Extract in base lotion, with approximately 0.2 g applied to each site Baseline pigmentation readings were taken about 24 h after dye application, prior to test substance application. After application, site evaluations occurred every 24 h until pigmentation returned to baseline. After 3 d, the untreated dye control and base lotion control resulted in a 4% reduction in pigmentation (compared to baseline), while 1% salicylic acid and 2% Salix Alba (Willow) Bark Extract resulted in decreases of 6 and 9%, respectively.

Cytotoxicity

Salix Alba (Willow) Leaf Extract

A neutral red release assay was performed on Statens Seruminstitut Rabbit Cornea (SIRC) cells using Salix Alba (Willow) Leaf Extract (4 – 6% in water).[23] The test substance was non-cytotoxic. No other details were provided.

Steroidogenesis Assay

Salix Alba (Willow) Bark Extract

An H295R steroidogenesis assay (OECD TG 456) was performed to evaluate the potential for Salix Alba (Willow) Bark Extract in DMSO to induce or inhibit the production of 17β-estradiol and testosterone in human adrenocarcinoma H295R cells.[41] Cells were exposed to the test substance at multiple concentrations (0.01, 0.1, 1, 10, 100, 1000, and 10,000 µM), alongside positive, negative, and solvent controls. Hormone levels in the culture media were quantified by ELISA after 48 h of exposure. The test substance was non-cytotoxic and did not induce or inhibit 17β-estradiol or testosterone production at any test concentration, indicating no evidence of endocrine disruption via this steroidogenesis pathway.

DERMAL IRRITATION AND SENSITIZATION STUDIES

Details regarding the following dermal irritation, sensitization, and phototoxicity studies summarized below are found in Table 7. In vitro dermal irritation assays using reconstructed human epidermis showed Salix Alba (Willow) Bark Extract (tested at concentrations of 20% and 100%), as well as a hydroalcoholic *Salix alba* bark extract (tested at 100%), did not reduce tissue viability below the threshold for irritation, with mean viabilities ranging from approximately 85 to 111%.[3,42-44] No erythema or edema were observed when Salix Alba (Willow) Bark Extract was applied to the skin of guinea pigs (final test concentration, 0.075% aqueous; level of occlusion not stated).[8] Salix Alba (Willow) Leaf Extract (4 – 6% in water) was non-irritating in a dermal irritation assay performed according to OECD TG 404 (no details provided; likely performed in rabbits).[23] In several direct peptide reactivity assays (DRPAs), Salix Alba (Willow) Bark Extract (20 – 100% in acetonitrile) was predicted to be non-sensitizing.[42-47] KeratinoSens™ assays using 20 – 100% Salix Alba (Willow) Bark Extract (in DMSO; tested at up to 2000 µM) and Salix Alba (Willow) Leaf Extract (4 - 6% in water) yielded negative results; however, positive results were obtained in vitro sensitization assays (KeratinoSens™ and genomic allergen rapid detection (GARD) assays) using an aqueous *Salix alba* (willow) bark extract (in DMSO; tested at up to 400 µg/ml) and hydroalcoholic *Salix alba* (willow) bark extract (in DMSO; tested at up to 500 µg/ml).[3,23,48-50] Salix Alba (Willow) Bark Extract (final test concentration, 0.075%) was non-sensitizing in a guinea pig sensitization assay performed under occlusive conditions.[8] No sensitization was observed in a guinea pig maximization assay using Salix Alba (Willow) Leaf Extract (4 – 6% in water; no other details provided).[23] No irritation or sensitization was observed in HRIPTs performed in 50 subjects using 5% Salix Alba (Willow) Bark Extract and a serum containing 0.0025% Salix Alba (Willow) Bark Extract (tested neat).[51,52]

Phototoxicity

In Vitro

A 3T3 neutral red reuptake phototoxicity assay showed that 1% Salix Alba (Willow) Bark Extract in ethanol (phototoxicity was evaluated in this ingredient as it produced significant absorbance in the wavelengths of interest (290 and 700 nm)) was non-phototoxic under with 5 J/cm^2 ultraviolet A (UVA)/visible light (Vis).[53] EpiDerm™ phototoxicity assays using 0.5 – 10% Salix Alba (Willow) Bark Extract (including a 20% extract tested at final concentrations of 0.025 – 0.5%) yielded negative results (tissues irradiated with 6 J/cm^2 UVA).[54,55] Salix Alba (Willow) Leaf Extract (4 – 6% in water) was

evaluated in a neutral red reuptake phototoxicity assay performed according to OECD TG 432 and was considered to be non-phototoxic.[23]

OCULAR IRRITATION STUDIES

Details regarding the ocular irritation studies summarized below are found in Table 8. Salix Alba (Willow) Bark Extract (20 – 100%) was predicted to be non-irritating in EpiOcular™ ocular irritation assays.[42-44] Conversely, a hydroalcoholic *Salix alba* (willow) bark extract (tested at 100%) was predicted to be irritating in an EpiOcular™ assay (mean cell viability was 14% (below 60% is classified as irritating)).[3] In addition, an vitro ocular irritation assay using isolated chicken eyes was performed using an undiluted hydroalcoholic *Salix alba* (willow) bark extract.[3] The test substance was not classified as a severe irritant (Globally Harmonized System (GHS) category 1) and was also not classified as a non-irritant; therefore, it was concluded that further information is required for classification. Salix Alba (Willow) Leaf Extract tested at 4 – 6% in water was a non-irritant when evaluated in an in vivo acute ocular irritation assay (likely performed in rabbits as stated in OECD TG 405).[23]

CLINICAL STUDIES

Case Reports

Salix Alba (Willow) Bark Extract

A 61-yr-old female presented with a sudden onset of shortness of breath and non-productive cough 30 min after taking a white willow bark supplement.[30] Evaluation revealed oxygen desaturation, severe hypoxemia, metabolic acidosis, bilateral interstitial infiltrates which led to a diagnosis of acute hypoxic respiratory failure secondary to severe acute respiratory distress syndrome from reaction to white willow bark. The patient improved following treatment with steroids, antihistamines, and oxygen.

A 25-yr-old woman presented to the emergency department with anaphylaxis requiring epinephrine, antihistamines, steroids, and volume resuscitation.[56] Medication history revealed that the patient ingested 2 capsules of weight loss supplement containing white willow bark. The patient reported a history of allergy to acetylsalicylic acid. No other causes for anaphylaxis were identified.

SUMMARY

The safety of 6 *Salix alba* (willow)-derived ingredients is reviewed in this safety assessment. According to the *Dictionary*, 4 of these ingredients are reported to function in skin-conditioning agents in cosmetics.

According to 2025 RLD and concentration of use data, Salix Alba (Willow) Bark Extract has the highest frequency and concentration of use. This ingredient is reported to be used in 3060 formulations at up 1.1% in leave-on face and neck products (not spray).

No signs of toxicity were observed in an acute toxicity assay in which mice were administered Salix Alba (Willow) Bark Extract via gavage at 2000 mg/kg bw/d. An acute oral toxicity assay was performed using a hydroalcoholic *Salix alba* (willow) bark extract given to rats via gavage. The LD_{50} in this assay was determined to be > 2000 mg/kg bw. Salix Alba (Willow) Leaf Extract (4 – 6% in water) was considered to be non-toxic in an acute oral toxicity assay performed according to OECD TG 401. No erythema or edema were observed in a 2-wk dermal assay in which Salix Alba (Willow) Bark Extract (final concentration, 0.075%) was applied to the clipped skin of guinea pigs. No test substance-related systemic toxicity was observed in a combined repeated-dose and reproductive/developmental toxicity assay in which rats were given up to 1000 mg/kg bw/d of a *Salix alba* (willow) bark extract. The test substance was administered via gavage for 28 d in males (pre- and post-mating) and for 41 d in females (pre-mating, gestation, and post-partum). When reproductive and developmental toxicity parameters were evaluated in this assay, the NOAEL for reproductive effects and pup development/survival was determined to be 1000 mg/kg bw/d.

Salix Alba (Willow) Bark Extract (20 – 100%; up to 5000 µg/plate) and hydroalcoholic *Salix alba* (willow) bark extracts (up to 5000 µg/plate) were negative in bacterial reverse mutation assays, with and without metabolic activation. Hydroalcoholic Salix alba (willow) bark extract was also negative in in vitro mammalian cell gene mutation (up to 5000 µg/ml) and micronucleus assays (up to 2000 µg/ml). In a comet assay using peripheral blood mononuclear cells, *Salix alba* (willow) bark extract increased DNA damage at ≥ 50 µg/ml but not at 5 µg/ml; however, no genotoxicity was observed in comet or cytokinesis-block micronucleus assays in HepG2 cells or human leukocytes at up to 100 µg/ml. Salix Alba (Willow) Leaf Extract (4–6%) was non-mutagenic in an Ames assay, and *Salix alba* (willow) bark extract was negative in in vivo comet and micronucleus assays in mice at doses up to 2000 mg/kg bw.

An ethanolic *Salix alba* (willow) bark extract resulted in a decrease in cell viability of HL-60 cells in a concentration-dependent manner. Cells were incubated with 1 – 10 µg of the test substance.

The effect of Salix Alba (Willow) Bark Extract on IL-6 levels was evaluated in an IL-6 ELISA. IL-6 concentrations following exposure to 0.01, 0.1, and 1% of the test substance were 2860, 2600, and 1087 pg/ml, respectively. IL-6 concentrations following positive and negative control exposure were 2599 and 1423 pg/ml, respectively.

Application of 2% Salix Alba (Willow) Bark Extract in base lotion to artificially pigmented skin resulted in a 9% reduction in pigmentation compared to baseline after 3 d. This effect exceeded that of the 1% salicylic acid positive control (6% reduction), and the base lotion and untreated dye controls (4% reduction).

A neutral red release assay was performed on SIRC cells using Salix Alba (Willow) Leaf Extract (4 – 6% in water). The test substance was non-cytotoxic.

An H295R steroidogenesis assay was performed to evaluate the potential for Salix Alba (Willow) Bark Extract (up to 10,000 µM) to induce or inhibit the production of 17β-estradiol and testosterone in human adrenocarcinoma H295R cells. The test substance did not inhibit or induce 17β-estradiol and testosterone production.

Salix Alba (Willow) Bark Extract was non-irritating in reconstructed human epidermis assays at 20% and 100%, and a hydroalcoholic Salix alba bark extract (tested at 100%) also did not significantly reduce tissue viability (mean viabilities approximately 85 – 111%). Salix Alba (Willow) Bark Extract (0.075%; assay performed in guinea pigs) and Salix Alba (Willow) Leaf Extract (4 – 6%; assay likely performed in rabbits) were non-irritating in dermal irritation assays. Salix Alba (Willow) Bark Extract (20 – 100%) and Salix Alba (Willow) Leaf Extract (4 – 6%) were negative in DPRA and KeratinoSens™ assays; however, positive in vitro sensitization responses were reported for aqueous and hydroalcoholic Salix alba (willow) bark extracts at up to 400 – 500 µg/ml. Salix Alba (Willow) Bark Extract (0.075%) and Salix Alba (Willow) Leaf Extract (4 – 6%) were non-sensitizing in guinea pig assays, and no irritation or sensitization was observed in HRIPTs using 5% Salix Alba (Willow) Bark Extract or a formulation containing 0.0025% Salix Alba (Willow) Bark Extract. A 3T3 neutral red reuptake phototoxicity assay showed that 1% Salix Alba (Willow) Bark Extract in ethanol was non-phototoxic. EpiDerm™ phototoxicity assays using 0.5 – 10% Salix Alba (Willow) Bark Extract (including a 20% extract tested at final concentrations of 0.025 – 0.5%) yielded negative results. Salix Alba (Willow) Leaf Extract (4 – 6% in water) evaluated in a neutral red reuptake phototoxicity assay was considered to be non-phototoxic.

Salix Alba (Willow) Bark Extract (20 – 100%) was predicted to be non-irritating in EpiOcular™ ocular irritation assays. A hydroalcoholic *Salix alba* bark extract (tested at 100%) showed irritation potential in one in vitro assay and inconclusive results in an isolated chicken eye test, indicating that further evaluation may be needed. Salix Alba (Willow) Leaf Extract was predicted to be non-irritating in an in vivo ocular irritation assay (likely performed in albino rabbits).

A 61-yr-old woman developed acute hypoxic respiratory failure secondary to severe acute respiratory distress syndrome after ingesting a white willow bark supplement. Her condition improved with treatment that included steroids, antihistamines, and oxygen. Additionally, a published case report described a 25-yr-old patient with a known allergy to acetylsalicylic acid who presented to the emergency department with anaphylaxis following ingestion of a supplement containing white willow bark.

DISCUSSION

To be developed.

CONCLUSION

To be determined.

TABLES

Table 1. Definitions and reported functions[1]

Ingredient/CAS No.	Definition	Function(s)
Salix Alba (Willow) Bark Extract (84082-82-6)	Salix Alba (Willow) Bark Extract is the extract of the bark of *Salix alba*.	hair conditioning agents skin conditioning agents - occlusive
Salix Alba (Willow) Bark Powder	Salix Alba (Willow) Bark Powder is the powder obtained from the dried, ground bark of *Salix alba.*	absorbents exfoliants skin protectants
Salix Alba (Willow) Bark Water	Salix Alba (Willow) Bark Water is the aqueous solution of the steam distillate obtained from the bark of *Salix alba*.	fragrance ingredients
Salix Alba (Willow) Extract	Salix Alba (Willow) Extract is the extract of the whole plant, *Salix alba*.	antifungal agent skin-conditioning agents – emollient skin-conditioning agents – humectant
Salix Alba (Willow) Flower Extract	Salix Alba (Willow) Flower Extract is the extract of the flowers of *Salix alba.*	skin-conditioning agents – misc
Salix Alba (Willow) Leaf Extract (84082-82-6)	Salix Alba (Willow) Leaf Extract is the extract of the leaves of *Salix alba*.	skin conditioning agents - misc

Table 2. Chemical properties

Property	Value	References
Salix Alba (Willow) Bark Extract		
Physical Form	free-flowing powder	7
Color	white to off-white	7
Odor	characteristic	7
pH (3% solution in water)	4.0 – 6.0	7
a *Salix alba* (willow) bark extract		
Physical Form	solid particulate/powder	3
Color	brown	3
Odor	characteristic	3
Density (g/ml @ 20ºC)	1.48	3
Vapor Pressure (Pa @ ≤ 61ºC)	< 0.001	3
Melting Point (ºC)	≥ 160	3
Water Solubility (mg/l @ 20ºC & pH = 7)	50	3
log K_{ow} (@ 25ºC)	< 0.3 (measured); - 2.2 (extrapolated)	3
Mass Median Aerodynamic Diameter (µm)	21	3

Table 3. Composition of a methanolic Salix alba (willow) bark extract[19]

Constituent	Amount (mg/g dry weight)
Salicylates	
salicin	0.91
salicortin	0.89
total salicylate content	1.80
Flavonoids	
ampelopsin	0.07
quercetin-hexoside	0.04
other quercetin-derivatives	0.02
total flavonoids	0.13
Flavan-3-ols	
catechin	1.26
procyanidin B3	0.24
total flavan-3-ols	1.49
Phenolic Acid Derivatives	
chlorogenic acid	0.40
coumaric acid derivative	0.02
neochlorogenic acid	0.33
total phenolic acid derivatives	0.75
Other Phenolic Compounds	
syringin	0.30
triadrin	0.67
total other phenolic compounds	0.97

Table 4. Composition of hydromethanolic extracts of Salix alba (willow) bark and leaves[20]

Constituent	Bark (mg/100 g dry weight)	Leaves (mg/100 g dry weight)
Phenolic Acids		
caffeoylhexose I	57.71	2.98
caffeoylhexose II	24.71	ND
caffeoxyl hexose-deoxyhexoside I	140	ND
caffeoyl hexose-deoxyhexoside II	ND	875.32
1-*O*-caffeoylquinic acid	ND	117.43
3-*O*-caffeoylquinic acid	41.96	386.31
caffeoylhexose III	240.49	ND
caffeoylquinic acid dimer I	ND	60.63
caffeoylquinic acid dimer II	ND	10.11
5-*O*-caffeoylquinic acid	ND	37.85
Flavanols and Procyanidins		
(epi)catechin-(epi)gallocatechin I	130.67	ND
(epi)catechin-(epi)gallocatechin II	10.09	ND
(epi)catechin-(epi)gallocatechin III	59.79	ND
A-type procyanidin dimer digallate	48.66	ND
A-type procyanidin dimer I	224.33	ND
A-type procyanidin dimer II	22.33	241.31
A-type procyanidin dimer III	9.22	ND
B-type procyanidin trimer I	22.90	241.43
A-type procyanidin trimer I	25.87	ND
A-type procyanidin dimer IV	14.27	ND
(+)-catechin	63.79	ND
(-)-epicatechin	176.49	ND
epigallocatechin I	ND	319.52
A-type procyanidin trimer digallate	99.25	ND
A-type procyanidin trimer II	15.07	ND
B-type procyanidin trimer II	40.79	ND
B-type procyanidin trimer III	ND	124.37
epigallocatechin II	ND	324.69
B-type procyanidin tetramer I	25.54	ND
B-type procyanidin trimer IV	ND	246.46
B-type procyanidin tetramer II	56.07	ND
A-type procyanidin dimer V	198.24	ND
B-type procyanidin pentamer	ND	118.11
B-type procyanidin dimer I	ND	395.23
(epi)catechin methyl-hexoside I	68.55	ND
(epi)catechin methyl-hexoside II	56.78	ND
A-type procyanidin tetramer	33.45	ND
A-type procyanidin dimer VI	94.91	ND
A-type procyanidin trimer III	103.61	ND
(epi)catechin methyl-hexoside III	96.21	ND
(epi)catechin methyl-hexoside IV	28.52	ND
(epi)catechin-ethyl trimer	70.45	ND
Flavanols		
quercetin 3-*O*-rutinoside	ND	92.91
quercetin methyl-pentoside	ND	9.11
quercetin acylated-deoxylhexoside I	ND	166.51
quercetin acylated-deoxyhexoside II	ND	148.93
isorhamnetin 3-O-rutinoside	ND	459.80
quercetin 3-O-galactoside	ND	59.27
quercetin 3-O-glucoside	ND	98.43
quercetin-acylated-hexoside I	ND	180.03
quercetin-acylated-hexoside II	ND	413.14
quercetin 3-O-hexoside	10.47	ND
isorhamnetin-acylated-hexoside I	ND	4.65
isorhamnetin-3-galactoside	ND	145.89
kaempferol pentoside	6.58	ND
isorhamnetin acylated-hexoside II	ND	32.62
isorhamnetin acylated-hexoside III	ND	234.36
kaempferol 3-O-galactoside	12.54	ND
isorhamnetin acylated-hexoside IV	ND	28.56

ND = not detected

Table 5. Frequency and concentration of use according to likely duration and exposure and by product category[25-27]

	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)
	Salix Alba (Willow) Bark Extract		Salix Alba (Willow) Bark Powder		Salix Alba (Willow) Bark Water	
Totals*	**3060**	**0.00002 – 1.1**	**43**	**NR**	**31**	**NR**
summarized by likely duration and exposure**						
Duration of Use						
Leave-On	*2505*	*0.00002 – 1.1*	*NR*	*NR*	*28*	*NR*
Rinse-Off	*1375*	*0.001 – 1*	*43*	*NR*	*9*	*NR*
Diluted for (Bath) Use	*1*	*NR*	*NR*	*NR*	*NR*	*NR*
Permanent Tattoo Ink	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Unknown	*65*	*NR*	*NR*	*NR*	*NR*	*NR*
Exposure Type						
Baby Products	36	NR	NR	NR	NR	NR
Children's Makeup	NR	NR	NR	NR	NR	NR
Eye Area	41	NR	NR	NR	2	NR
Incidental Ingestion	28	NR	NR	NR	NR	NR
Mucous Membrane	191	0.004 – 0.005	1	NR	1	NR
Incidental Inhalation-Spray	29; 1153[a]; 1514[b]	0.025[a]; 0.00002 – 0.00003[b]	1[a]	NR	10[a]; 19[b]	NR
Incidental Inhalation-Airbrush	2	NR	NR	NR	NR	NR
Incidental Inhalation-Powder	8; 1514[b]; 16[c]	0.00002 – 0.00003[b]; 0.001 – 1.1[c]	NR	NR	19[b]	NR
Dermal Contact	3322	0.001 – 1.1	43	NR	37	NR
Deodorant (underarm)	11	NR	NR	NR	NR	NR
Hair - Non-Coloring	525	0.00002 – 0.01	NR	NR	NR	NR
Hair-Coloring	6	NR	NR	NR	NR	NR
Nail	7	NR	NR	NR	NR	NR
Tattoo Preparations	NR	NR	NR	NR	NR	NR
Other Preparations (Unknown Exposure Type)	65	NR	NR	NR	NR	NR
as reported by product category						
Baby Products						
Baby Shampoos	3	NR				
Baby Lotions/Oils/Powders/Creams	16	NR				
Other Baby Products	11 (l.o.); 6 (r.o.)	NR				
Bath Preparations (diluted for use)						
Bath Oils, Tablets, and Salts	3	NR				
Bubble Baths	4	NR				
Other Bath Preparations	3	NR				
Eye Makeup Preparations (not children's)						
Eye Shadow	1	NR				
Eye Lotion	15	NR			1	NR
Eye Makeup Remover	5	NR				
Eyelash and Eyebrow Adhesives/Glues/Sealants	1	NR				
Eyelash and Eyebrow Preparations (primers, conditioners, serums, fortifiers)	13	NR				
Eyelash Cleansers	1	NR				
Other Eye Makeup Preparations	5	NR			1	NR
Fragrance Preparations						
Perfumes	1	NR				
Other Fragrance Preparation	10	NR				

Table 5. Frequency and concentration of use according to likely duration and exposure and by product category[25-27]

	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)
Hair Preparations (non-coloring)						
Hair Conditioners	27 (l.o.); 78 (r.o.)	0.002 (l.o.); 0.01 (r.o.)				
Hair Sprays (aerosol fixatives)	14	NR				
Hair Straighteners	1	NR				
Rinses (non-coloring)	18	NR				
Shampoos (non-coloring)	3 (l.o.); 125 (r.o.)	0.01 (r.o.)				
Tonics, Dressings, Other Hair Grooming Aids	102	0.00003				
Wave Sets	1	NR				
Other Hair Preparations	100 (l.o.); 53 (r.o.)	0.00002 (l.o.)				
Hair Coloring Preparations						
Hair Tints	1	NR				
Hair Rinses (coloring)	1 (l.o.); 1 (r.o.)	NR				
Other Hair Coloring Preparation	2 (l.o.); 1 (r.o.)	NR				
Makeup Preparations (not eye or children's)						
Blushers and Rouges (all types)	22	NR				
Face Powders	8	NR				
Foundations	29 (traditional application); 1 (airbrush application)	0.001 (traditional application)				
Leg and Body Paints						
Lipsticks and Lip Glosses	25	NR				
Makeup Bases	7 (traditional application)	0.0063 (traditional application)				
Makeup Fixatives	4	NR				
Other Makeup Preparations	9 (traditional application)	0.0053 (traditional application)				
Manicuring Preparations						
Cuticle Softeners	1	NR				
Nail Creams and Lotions	3	NR				
Other Manicuring Preparations	3	NR				
Oral Hygiene Products						
Dentifrices	1	NR				
Mouthwashes and Breath Fresheners	1	NR				
Other Oral Products	1	NR				
Personal Cleanliness						
Bath Soaps and Body Washes	96	0.004	1	NR		
Deodorants (underarm)	11	NR				
Feminine Deodorants	1 (l.o.); 2 (r.o.)	NR				
Disposable Wipes	5	NR				
Other Personal Cleanliness Products	12 (l.o.); 37 (r.o.)	0.005			1 (r.o.)	NR
Shaving Preparations						
Aftershave Lotions	31	NR				
Beard Softeners	14	NR				
Pre-shave Lotions (all types)	1	NR				
Shaving Cream (aerosol, brushless, lather)	9	NR				
Other Shaving Preparations	13	NR				
Skin Care Preparations						
Cleansing	447	0.001 – 0.03			3	NR

Table 5. Frequency and concentration of use according to likely duration and exposure and by product category[25-27]

	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)
Depilatories	5					
Face and Neck (excluding shaving preps)	928 (l.o.); 256 (r.o.)	0.001–1.1 (l.o.; not spray); 0.005 – 1 (r.o.; not spray)			18 (l.o.); 2 (r.o.)	NR
Body and Hand (excluding shaving preps)	157 (l.o.); 32	0.53 (l.o.; not spray)				
Foot Powders and Sprays	2	NR				
Moisturizing	500	0.002 – 0.053 (not spray)			6	NR
Night	34	0.4 (not spray)				
Paste Masks (mud packs)	77	NR	41	NR	2	NR
Skin Fresheners	138	NR			1	NR
Other Skin Care Preparations	208 (l.o.); 102 (r.o.)	0.025 (r.o)	1 (r.o.)	NR	1 (l.o.); 1 (r.o.)	NR
Suntan Preparations						
Suntan Gels, Creams, and Liquids	23	NR				
Indoor Tanning Preparations	1; 1 (professional airbrush application); 4 (professional spray application)	NR				
Other Suntan Preparations	2	NR				
Other Preparations (i.e., those that do not fit another category)	65	NR				
	Salix Alba (Willow) Extract		**Salix Alba (Willow) Flower Extract**		**Salix Alba (Willow) Leaf Extract**	
Totals*	**8**	**NR**	**34**	**NR**	**76**	**0.00002**
summarized by likely duration and exposure**						
Duration of Use						
Leave-On	*11*	*NR*	*22*	*NR*	*67*	*0.00002*
Rinse-Off	*1*	*NR*	*11*	*NR*	*17*	*NR*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Permanent Tattoo Ink	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Unknown	*NR*	*NR*	*1*	*NR*	*1*	*NR*
Exposure Type						
Baby Products	NR	NR	NR	NR	NR	NR
Children's Makeup	NR	NR	NR	NR	NR	NR
Eye Area	NR	NR	NR	NR	NR	NR
Incidental Ingestion	NR	NR	5	NR	NR	NR
Mucous Membrane	NR	NR	5	NR	3	NR
Incidental Inhalation-Spray	4[a]; 7[b]	NR	9[a]; 8[b]	NR	14[a]; 35[b]	0.00002[b]
Incidental Inhalation-Airbrush	NR	NR	NR	NR	NR	NR
Incidental Inhalation-Powder	7[b]	NR	8[b]	NR	35[b]	0.00002[b]
Dermal Contact	11	NR	28	NR	80	NR
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	1	NR	NR	NR	4	0.00002[b]
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Tattoo Preparations	NR	NR	NR	NR	NR	NR
Other Preparations (Unknown Exposure Type)	NR	NR	1	NR	1	NR
as reported by product category						
Baby Products						
Baby Shampoos						

Table 5. Frequency and concentration of use according to likely duration and exposure and by product category[25-27]

	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)
Baby Lotions/Oils/Powders/Creams						
Other Baby Products						
Bath Preparations (diluted for use)						
Bath Oils, Tablets, and Salts						
Bubble Baths						
Other Bath Preparations						
Eye Makeup Preparations (not children's)						
Eye Shadow						
Eye Lotion						
Eye Makeup Remover						
Eyelash and Eyebrow Adhesives/Glues/Sealants						
Eyelash and Eyebrow Preparations (primers, conditioners, serums, fortifiers)						
Eyelash Cleansers						
Other Eye Makeup Preparations						
Fragrance Preparations						
Perfumes						
Other Fragrance Preparation						
Hair Preparations (non-coloring)						
Hair Conditioners					1 (r.o.)	NR
Hair Sprays (aerosol fixatives)						
Hair Straighteners	1	NR				
Rinses (non-coloring)						
Shampoos (non-coloring)						
Tonics, Dressings, Other Hair Grooming Aids						
Wave Sets						
Other Hair Preparations					3 (l.o.)	0.00002
Hair Coloring Preparations						
Hair Tints						
Hair Rinses (coloring)						
Other Hair Coloring Preparation						
Makeup Preparations (not eye or children's)						
Blushers and Rouges (all types)						
Face Powders						
Foundations					20 (traditional application)	NR
Lipsticks and Lip Glosses			5	NR		
Makeup Bases						
Makeup Fixatives			1	NR	1	NR
Other Makeup Preparations					1 (traditional application)	NR
Manicuring Preparations						
Cuticle Softeners						
Nail Creams and Lotions						
Other Manicuring Preparations						
Oral Hygiene Products						
Dentifrices						
Mouthwashes and Breath Fresheners						
Other Oral Products						

Table 5. Frequency and concentration of use according to likely duration and exposure and by product category[25-27]

	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)	# of Uses RLD (2025)	Max Conc of Use % (2025)
Personal Cleanliness						
Bath Soaps and Body Washes					3	NR
Deodorants (underarm)						
Feminine Deodorants						
Disposable Wipes						
Other Personal Cleanliness Products						
Shaving Preparations						
Aftershave Lotions			1	NR		
Beard Softeners						
Pre-shave Lotions (all types)						
Shaving Cream (aerosol, brushless, lather)						
Other Shaving Preparations						
Skin Care Preparations						
Cleansing			2	NR	9	NR
Depilatories						
Face and Neck (excluding shaving preps)	6 (l.o.)	NR	3 (l.o.); 1 (r.o.)	NR	28 (l.o.); 4 (r.o.)	NR
Body and Hand (excluding shaving preps)	1 (l.o.)	NR	1 (r.o.)	NR	4 (l.o.)	NR
Foot Powders and Sprays						
Moisturizing	4	NR	7	NR	4	NR
Night					6	NR
Paste Masks (mud packs)			7	NR		
Skin Fresheners						
Other Skin Care Preparations			5 (l.o.)	NR		
Suntan Preparations						
Suntan Gels, Creams, and Liquids						
Indoor Tanning Preparations						
Other Suntan Preparations						
Other Preparations (i.e., those that do not fit another category)			1	NR	1	NR

NR – not reported

l.o. – leave-on; r.o. – rinse-off

*The sum of the counts given for duration of use and by exposure type, and the sum of the frequency reported by product category, may not equal the sum of total uses because each ingredient may be used in cosmetic formulations that are reported under more than one product category.

**Likely duration and exposure are derived from survey data based on product category (see Use Categorization https://www.cir-safety.org/cir-findings)

[a] It is possible these products are sprays, but it is not specified whether the reported uses are sprays.

[b] Not specified whether a spray or a powder, but it is possible the use can be as a spray or a powder, therefore the information is captured in both categories

[c] It is possible these products are powders, but it is not specified whether the reported uses are powders.

Table 6. Genotoxicity studies

Test Article	Vehicle	Concentration/Dose	Test System	Protocol	Results	Reference
IN VITRO						
20% Salix Alba (Willow) Bark Extract	water	1.5, 5, 15, 50, 150, 500, 1500, and 5000 µg/plate	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and *Escherichia coli* WP2	OECD TG 471; bacterial reverse mutation assay; performed with and without metabolic activation; appropriate positive and negative controls used	non-mutagenic; controls gave expected results	37
Salix Alba (Willow) Bark Extract	water	1.5, 5, 15, 50, 150, 500, 1500, and 5000 µg/plate	*S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2	OECD TG 471; bacterial reverse mutation assay; performed with and without metabolic activation; appropriate positive and negative controls used	non-mutagenic; controls gave expected results	38
Salix Alba (Willow) Bark Extract	water	1.5, 5, 15, 50, 150, 500, 1500, and 5000 µg/plate	*S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2	OECD TG 471; bacterial reverse mutation assay; performed with and without metabolic activation; appropriate positive and negative controls used	non-mutagenic; controls gave expected results	36
hydroalcoholic *Salix alba* (willow) bark extract	water	0.5, 1.58, 5, 15.81, 50, 158.1, 500, 1581, and 5000 µg/plate	*S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2	OECD TG 471; bacterial reverse mutation assay; performed with and without metabolic activation; appropriate positive and negative controls used	non-mutagenic; controls gave expected results	3
hydroalcoholic *Salix alba* (willow) bark extract	water	62.5, 125, 250, 500, 1000, 2000, and 5000 µg/ml	Chinese hamster ovary cells	OECD TG 476; in vitro mammalian cell gene mutation test using Hprt and Xprt genes; performed with and without metabolic activation; appropriate positive and negative controls used	non-mutagenic; controls gave expected results	3
hydroalcoholic *Salix alba* (willow) bark extract	saline	without metabolic activation: 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml with metabolic activation: 125, 250, 500, 1000, 1500 and 2000 µg/ml	mouse lymphoma L5178Y cells	OECD TG 487; in vitro mammalian cell micronucleus assay; performed with and without metabolic activation; appropriate positive and negative controls used	non-genotoxic; controls gave expected results	3
Salix alba (willow) bark extract	NR	5, 50, or 100 µg/ml	peripheral blood mononuclear cells	comet assay; 4-h incubation; use of metabolic activation not stated; appropriate positive and negative controls used	a statistically significant elevation in DNA damage was found between the negative control and groups treated with 50 or 100 µg/ml; results were negative at 5 µg/ml	29
Salix alba (willow) bark extract	NR	5, 50, or 100 µg/ml	HepG2 cells	comet assay; 4-h incubation; use of metabolic activation not stated; appropriate positive and negative controls used	non-genotoxic; controls gave expected results	29
Salix alba (willow) bark extract	NR	5, 50, or 100 µg/ml	human peripheral blood leukocytes	cytokinesis-block micronucleus assay; 28-h exposure; use of metabolic activation not stated; micronuclei analysis performed	non-genotoxic; controls gave expected results	29
Salix alba (willow) bark extract	NR	5, 50, or 100 µg/ml	HepG2 cells	cytokinesis-block micronucleus assay; 24-h exposure; use of metabolic activation not stated; micronuclei analysis performed	non-genotoxic; controls gave expected results	29
Salix Alba Willow Leaf Extract (4 – 6%)	water	NR	not stated	OECD TG 471 (Ames assay); no other details provided	non-mutagenic	23
IN VIVO						
Salix alba (willow) bark extract	DMSO	500, 1000, or 2000 mg/kg bw	male Swiss albino mice (6/group)	in vivo assay; gavage administration 1/x for 7 d; appropriate positive and negative controls used; animals killed 4 h after last treatment and cells (bone marrow cells) evaluated; micronucleus assay performed on cells	non-genotoxic; controls gave expected results	57

Table 6. Genotoxicity studies

Test Article	Vehicle	Concentration/Dose	Test System	Protocol	Results	Reference
Salix alba (willow) bark extract	DMSO	500, 1000, or 2000 mg/kg bw	male Swiss albino mice (6/group)	in vivo assay; gavage administration 1/x for 7 d; appropriate positive and negative controls used; animals killed 4 h after last treatment and cells (peripheral blood, heart and testes cells); comet assay performed on cells	non-genotoxic; controls gave expected results	[57]

DMSO = dimethyl sulfoxide; DNA = deoxyribonucleic acid; HepG2 = human hepatoma cell line; HPRT = hypoxanthine-guanine phosphoribosyltransferase; NR = not reported; OECD = Organisation for Economic Co-operation and Development; TG = test guideline; Xprt = xanthine-guanine phosphoribosyltransferase

Table 7. Dermal irritation, sensitization, and phototoxicity studies

Test Article	Vehicle	Concentration/ Dose	Test Population/ System	Protocol	Results	Reference
IRRITATION						
IN VITRO						
Salix Alba (Willow) Bark Extract	NR	20%; 25 mg	reconstructed human epidermis	EpiDerm™ assay; 60 min incubation; tissue viability evaluated via MTT assay; appropriate positive and negative controls used	non-irritating; mean tissue viability of approximately 85% (viability ≤ 50 indicates positive results); controls gave expected results	[42]
Salix Alba (Willow) Bark Extract	none	100%; 25 mg	reconstructed human epidermis	EpiDerm™ assay; 60 min incubation; tissue viability evaluated via MTT assay; appropriate positive and negative controls used	non-irritating; mean tissue viability of approximately 95% (viability ≤ 50 indicates positive results); controls gave expected results	[44]
Salix Alba (Willow) Bark Extract	none	100%; 25 mg	reconstructed human epidermis	EpiDerm™ assay; 60 min incubation; tissue viability evaluated via MTT assay; appropriate positive and negative controls used	non-irritating; mean tissue viability of 100% (viability ≤ 50 indicates positive results); controls gave expected results	[43]
a hydroalcoholic *Salix alba* (willow) bark extract (100% purity)	none	100%; 10 mg	reconstructed human epidermis (n = 3)	OECD TG 439; in vitro irritation reconstructed human epidermis assay; appropriate positive and negative controls used	non-irritating; mean tissue viability was 111.2% (viability ≤ 50 indicate positive results); controls gave expected results	[3]
ANIMAL						
Salix Alba (Willow) Bark Extract (mixture composed of water (68.25%), butylene glycol (29.25%), Salix Alba (Willow) Bark Extract (2.5%))	water	3% (final test concentration of Salix Alba (Willow) Bark Extract, 0.075%)	guinea pigs (n = 3; strain and sex not stated)	test substance applied topically to animals (level of occlusion not stated); animals evaluated for erythema and edema 24, 48, and 72 h after dosing	non-irritating; no erythema or edema observed	[8]
Salix Alba (Willow) Leaf Extract	water	4 – 6%	not stated; however, likely 1 – 3 albino rabbits (as suggested by OECD TG 404 guidelines)	OECD TG 404; dermal irritation assay; no other details provided	non-irritating	[23]
SENSITIZATION						
IN CHEMICO						
20% Salix Alba (Willow) Bark Extract	acetonitrile	5 mM for cysteine peptide assay; 25 mM for lysine peptide assay	cysteine and lysine peptides	OECD TG 442C: direct peptide reactivity assay; appropriate positive and negative controls used	not predicted to be sensitizing The mean percent depletion of cysteine and lysine was 3.13% causing minimal reactivity. The controls gave expected results	[47]
Salix Alba (Willow) Bark Extract	acetonitrile	5 mM for cysteine peptide assay; 25 mM for lysine peptide assay	cysteine and lysine peptides	OECD TG 442C: direct peptide reactivity assay; appropriate positive and negative controls used	not predicted to be sensitizing The mean percent depletion of cysteine and lysine was 3.22% causing minimal reactivity. The controls gave expected results	[46]

Table 7. Dermal irritation, sensitization, and phototoxicity studies

Test Article	Vehicle	Concentration/ Dose	Test Population/ System	Protocol	Results	Reference
Salix Alba (Willow) Bark Extract	acetonitrile	5 mM for cysteine peptide assay; 25 mM for lysine peptide assay	cysteine and lysine peptides	OECD TG 442C: direct peptide reactivity assay; appropriate positive and negative controls used	not predicted to be sensitizing The mean percent depletion of cysteine and lysine was 3.08% causing minimal reactivity. The controls gave expected results	45
				IN VITRO		
20% Salix Alba (Willow) Bark Extract	DMSO	0.98, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µM	immortalized human skin keratinocytes	OECD TG 442D; KeratinoSens™ assay; appropriate positive and negative controls used	not predicted to be sensitizing The I_{max} was determined to be 0.40 and the IC_{50} was > 10,000 µM. Cell viability was lower than 70% at all relevant concentrations, indicating a negative prediction for sensitization. Positive and negative controls gave expected results.	48
Salix Alba (Willow) Bark Extract	DMSO	0.98, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µM	immortalized human skin keratinocytes	OECD TG 442D; KeratinoSens™ assay; appropriate positive and negative controls used	not predicted to be sensitizing The I_{max} was determined to be 0.34 and the IC_{50} was > 10,000 µM. Cell viability was lower than 70% at all relevant concentrations, indicating a negative prediction for sensitization. Positive and negative controls gave expected results.	50
Salix Alba (Willow) Bark Extract	DMSO	0.98, 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1000, and 2000 µM	immortalized human skin keratinocytes	OECD TG 442D; KeratinoSens™ assay; appropriate positive and negative controls used	not predicted to be sensitizing The I_{max} was determined to be 0.33 and the IC_{50} was > 10,000 µM. Cell viability was lower than 70% at all relevant concentrations, indicating a negative prediction for sensitization. Positive and negative controls gave expected results.	49
an aqueous *Salix alba* (willow) bark extract (100% purity)	DMSO	0.2, 0.39, 0.78. 1.56, 3.1, 6.3, 13, 25, 50, 100, 200, and 400 µg/ml	immortalized human skin keratinocytes	OECD TG 442D; KeratinoSens™ assay; appropriate positive and negative controls used	predicted to be sensitizing The I_{max} was determined to be 5.51 and the $EC_{1.5}$ concentration was 152.33 µg/ml. The cell viability at the $EC_{1.5}$ concentration was higher than 70%, indicating a positive prediction for sensitization. Positive and negative controls gave expected results	3
an aqueous *Salix alba* (willow) bark extract (100% purity)	DMSO	NR	immortalized human skin keratinocytes	OECD TG 442D; KeratinoSens™ assay; appropriate positive and negative controls used	predicted to be sensitizing The I_{max} values and $EC_{1.5}$ values were determined to be 3.99/7.03 and 118.1/179.55 µg/ml, respectively. At the $EC_{1.5}$, in both repetitions, cell viability was higher than 70%. Positive and negative controls gave expected results.	3
a hydroalcoholic *Salix alba* (willow) bark extract (100% purity)	DMSO	1, 5 10, 50, 100, 200, 300,400, and 500 µg/ml	dendritic cell line	GARD assay (predicts sensitization by evaluating changes in genomic biomarker expression; positive reactions observed if test substance triggers sensitization-related pathways); 24-h incubation, cell viability measured and RNA extracted and evaluated (RNA expression profiles analyzed); appropriate positive and negative controls used	predicted to be sensitizing The mean decision value was 1.76. Values > 0 indicate positive results. Positive and negative controls gave expected results.	3
Salix Alba (Willow) Leaf Extract	water	4 – 6%	immortalized human skin keratinocytes	KeratinoSens™ assay; no details provided	predicted to be non-sensitizing	23

Table 7. Dermal irritation, sensitization, and phototoxicity studies

Test Article	Vehicle	Concentration/ Dose	Test Population/ System	Protocol	Results	Reference
ANIMAL						
Salix Alba (Willow) Bark Extract (mixture composed of water (68.25%), butylene glycol (29.25%), Salix Alba (Willow) Bark Extract (2.5%))	Freund-type adjuvant and water	3% (final test concentration of Salix Alba (Willow) Bark Extract, 0.075%)	guinea pigs (10/group; strain and sex not stated)	guinea pig sensitization assay; intradermal injections given for first induction period, followed by a second induction using occlusive patches, and occlusive patch challenge; negative control group used; animals observed for signs of erythema and edema at 24 and 48 h after challenge patch removal; no other details provided	non-sensitizing; no erythema or edema observed	8
Salix Alba (Willow) Leaf Extract	water	4 – 6%	guinea pigs (number, sex, and strain not stated)	guinea pig maximization assay performed according to Magnusson and Kligman methods; no other details provided	non-sensitizing	23
HUMAN						
Salix Alba (Willow) Bark Extract	water	5%; 0.2 ml	50 subjects	HRIPT; 9 induction applications followed by a 10 – 14 d non-treatment period and challenge patch; reactions scored 24 and 48 h after application; open patch conditions	non-irritating and non-sensitizing	52
serum containing 0.0025% Salix Alba (Willow) Bark Extract	none	100%	50 subjects	HRIPT; no details provided	non-irritating and non-sensitizing	51
PHOTOTOXICITY						
IN VITRO						
Salix Alba (Willow) Bark Extract	ethanol	1%	Balb/c 3T3 (clone A31) mouse fibroblast cells	OECD TG 432; 3T3 neutral red reuptake phototoxicity assay; cells incubated for 60 min then irradiated or left non-irradiated with UVA/Vis, 5 J/cm^2 for 50 min; appropriate positive and negative controls used	non-phototoxic	53
20% Salix Alba (Willow) Bark Extract	NR	0.5, 1.5, 5, and 10% (final test concentrations of 0.025, 0.075, 0.25, and 0.5%)	reconstructed human epidermis	EpiDerm^{TM} model assay; test substance applied to tissues and incubated overnight; appropriate tissues were irradiated with UVA (6 J/cm^2) for 60 min; some tissues non-irradiated for use as control; appropriate positive and negative controls used	non-phototoxic	54
Salix Alba (Willow) Bark Extract	NR	0.5, 1.5, 5, and 10%	reconstructed human epidermis	EpiDerm^{TM} model assay; test substance applied to tissues and incubated overnight; appropriate tissues were irradiated with UVA (6 J/cm^2) for 60 min; some tissues non-irradiated for use as control; appropriate positive and negative controls used	non-phototoxic	55
Salix Alba (Willow) Leaf Extract	water	4 – 6%	Balb/c 3T3 (clone A31) mouse fibroblast cells	OECD TG 432; 3T3 neutral red reuptake phototoxicity assay; no other details provided	non-phototoxic	23

DMSO = dimethyl sulfoxide; $EC_{1.5}$ = effective concentration of a test chemical that induces a 1.5-fold increase in luciferase activity; GARD = genomic allergen rapid detection; HRIPT = human repeat insult patch test; I_{max} = mean maximal luciferase activity; IC_{50} = inhibitory concentration 50%; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR = not reported; OECD = Organisation for Economic Co-operation and Development; RNA = ribonucleic acid; TG = test guideline; UVA = ultraviolet A; Vis = visible light

Table 8. Ocular irritation studies

Test Article	Vehicle	Concentration/Dose	Test Population	Protocol	Results	Reference
IN VITRO						
Salix Alba (Willow) Bark Extract	none	20%; 50 mg	reconstructed human cornea-like epithelial tissue (2 replicates/group)	EpiOcular™ assay; 90 min incubation; appropriate positive and negative controls used	predicted to be non-irritating mean cell viability was approximately 90%; below 60% is classified as irritating controls gave expected results	42
Salix Alba (Willow) Bark Extract	none	100%; 50 mg	reconstructed human cornea-like epithelial tissue (2 replicates/group)	EpiOcular™ assay; 90 min incubation; appropriate positive and negative controls used	predicted to be non-irritating mean cell viability was approximately 90%; below 60% is classified as irritating controls gave expected results	43
Salix Alba (Willow) Bark Extract	none	100%; 50 mg	reconstructed human cornea-like epithelial tissue (2 replicates/group)	EpiOcular™ assay; 90 min incubation; appropriate positive and negative controls used	predicted to be non-irritating mean cell viability was approximately 95%; below 60% is classified as irritating controls gave expected results	44
hydroalcoholic *Salix alba* (willow) bark extract	none	100%; 50 mg	reconstructed human cornea-like epithelial tissue (2 replicates/group)	OECD TG 492; EpiOcular™ assay; 6-h incubation; appropriate positive and negative controls used	predicted to be an irritant mean cell viability was 14%; below 60% is classified as irritating controls gave expected results	3
hydroalcoholic *Salix alba* (willow) bark extract	none	100%; 30 mg	isolated chicken eyes (n = 3 per test substance treatment; 1 for negative control)	OECD TG 438; in vitro ocular irritation assay; parameters evaluated: mean corneal opacity, corneal swelling, fluorescein retention; 75-min incubation; appropriate positive and negative controls used	the test substance was not classified as a severe irritant (GHS category 1) and was also not classified as a non-irritant; therefore, it was concluded that further information is required for classification mean corneal opacity score: 0.5/4 corneal swelling: 0.5% (values <5% indicate minimal or no irritation) fluorescein retention score: 1.67 (falls within range indicative of severe irritation) positive and negative controls gave expected results	3
ANIMAL						
Salix Alba (Willow) Leaf Extract	water	4 – 6%	likely albino rabbits (no details provided)	OECD TG 405; no details provided; test substance applied to the conjunctival sac of one eye, second eye used as control	non-irritating	23

DPRA = direct peptide reactivity assay; GHS = Globally Harmonized System

REFERENCES

1. Nikitakis J, Venema J. 2026. *International Cosmetic Ingredient Dictionary and Handbook*. https://incipedia.personalcarecouncil.org/winci/. Date Accessed: January 9, 2026.

2. Johnson WJ, Zhu J, Bergfeld WF, et al. 2019. Amended Safety Assessment of Salicylic Acid and Salicylates as Used in Cosmetics. https://cir-reports.cir-safety.org/. Date Accessed: November 6, 2025.

3. European Chemicals Agency. 2025. Willow, *Salix alba* ext. https://chem.echa.europa.eu/100.074.548/dossier-view/4719c574-3486-4832-9e05-5d379034b80e/1ba6be16-8afe-477c-a349-03b60bcdb8d6_1ba6be16-8afe-477c-a349-03b60bcdb8d6?searchText=willow. Date Accessed: September 4, 2025.

4. Durrant TH, de Rigo D, Caudullo G. *Salix Alba* in Europe: Distribution, Habitat, Usage, and Threats. In: *European Atlas of Forest Tree Species.* Publication Office of the European Union, Luxembourg; 2016.

5. United States Pharmacopeial Convention. 2025. USP-NF Online. https://www.uspnf.com/?utm_source=chatgpt.com. Date Accessed: September 25, 2025.

6. Tawfeek N, Mahmoud MF, Hamdan DI, et al. Phytochemistry, pharmacology and medicinal uses of plants of the genus *Salix*: an updated review. *Front Pharmacol*. 2021;12.

7. Active Concepts. 2025. Product specification: ABS White Willow Bark Extract Powder. https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-Specification-v13.pdf. Date Accessed: October 21, 2025.

8. Anonymous. 2025. Summary information Salix Alba (Willow) Bark Extract. [Unpublished data submitted by Personal Care Products Council on November 17, 2025].

9. Active Concepts LLC. 2016. Manufacturing Flow Chart - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

10. Sulaiman GM, Hussien NN, Marzoog TR, Awad HA. Phenolic content, antioxidant, antimicrobial and cytotoxic activities of ethanolic extract of *Salix alba*. *AJBB*. 2013;9(1):41–46.

11. Qureshi MA, Khatoon F, Rizvi MA, Zafaryab M. Ethyl acetate *Salix alba* leaves extract-loaded chitosan-based hydrogel film for wound dressing applications. *J Biomater Sci Polym Ed*. 2015;26(18):1452–1464.

12. Matyjaszczyk E, Schumann R. Risk assessment of white willow (*Salix alba*) in food. *EFSA J*. 2018;16(Suppl 1):e16081.

13. Tőzsér D, Magura T, Simon E. Heavy metal uptake by plant parts of willow species: a meta-analysis. *Journal of Hazardous Materials*. 2017;336:101–109.

14. Active Concepts. 2025. ABS White Willow Bark Extract Powder Technical Data Sheet. https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-NewTechnicalDataSheet-v4.pdf. Date Accessed: September 21, 2025.

15. Active Concepts LLC. 2020. Product Specification - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

16. Active Concepts LLC. 2023. Compositional Breakdown - ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

17. Active Concepts LLC. 2023. Compositional Breakdown - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

18. Neagu Codreanu A, Baicea C, Popescu A, Tomescu J, Bordei N, Raluca S. Bioactive phenolic compounds from white willow (*Salix alba*) bark, leaves and branches. *UPB Scientific Bulletin, Series B: Chemistry and Materials Science*. 2021;83:41–50.

19. Köhler A, Förster N, Zander M, Ulrichs C. Inter- and intraspecific diversity of *Salix* bark phenolic profiles – a resource for the pharmaceutical industry. *Fitoterapia*. 2023;170:105660.

20. Piątczak E, Dybowska M, Płuciennik E, Kośla K, Kolniak-Ostek J, Kalinowska-Lis U. Identification and accumulation of phenolic compounds in the leaves and bark of *Salix alba* (L.) and their biological potential. *Biomolecules*. 2020;10(10):1391.

21. Dönmez I, Salman H. Chemical compositon of willow (*Salix alba* L.) wood and bark. *Turkish Journal of Forestry | Türkiye Ormancılık Dergisi*. 2021;22:38–42.

22. World Health Organization. 2005. WHO Monographs on Selected Medicinal Plants. https://iris.who.int/bitstream/handle/10665/42052/9789241547055_eng.pdf?sequence=4 . Date Accessed: September 21, 2025.

23. Anonymous. 2025. Summary information Salix Alba (Willow) Leaf Extract. [Unpublished data submitted by Personal Care Products Council on November 24, 2025].

24. United States Food and Drug Administration. Federal Food, Drug, and Cosmetic Act Section 612 Title 21.

25. Hicks J., Eisenmann C., Nikitakis J., Kim D., Flores W. 2025. Personal Care Products Council (PCPC) RLD Mapping Project Report.Washington, DC. [Analysis results provided as a courtesy to CIR].

26. U.S. Food and Drug Administration Office of Colors and Cosmetics (OCAC). 2025. Data from: Registration and Listing of Cosmetic Product Facilities and Products.College Park, MD. [Obtained under the Freedom of Information Act].

27. Personal Care Products Council. 2025. Concentration of use data by FDA product category. [Unpublished data submitted by Personal Care Products Council on October 20, 2025].

28. EUR-Lex. 2025. Access to European Union Law. https://eur-lex.europa.eu/homepage.html. Date Accessed: April 21, 2025.

29. Maistro EL, Terrazzas PM, Perazzo FF, Gaivão IODM, Sawaya ACHF, Rosa PCP. *Salix alba* (white willow) medicinal plant presents genotoxic effects in human cultured leukocytes. *J Toxicol Environ Health A*. 2019;82(23-24):1223–1234.

30. Srivali N, Cheungpasitporn W, Chongnarungsin D, Edmonds LC. White willow bark induced acute respiratory distress syndrome. *N Am J Med Sci*. 2013;5(5):330.

31. Oltean H, Robbins C, van Tulder MW, Berman BM, Bombardier C, Gagnier JJ. Herbal medicine for low-back pain. *Cochrane Database Syst Rev*. 2014;2014(12):CD004504.

32. Lin C, Tsai SHL, Wang C, et al. Willow bark (*Salix* spp.) used for pain relief in arthritis: a meta-analysis of randomized controlled trials. *Life (Basel)*. 2023;13(10):2058.

33. US Food and Drug Administration. 2025. FDA Label Search. https://labels.fda.gov/ingredientname.cfm . Date Accessed: September 21, 2025.

34. National Library of Medicine. 2025. DailyMed. https://dailymed.nlm.nih.gov/dailymed/index.cfm. Date Accessed: September 25, 2025.

35. European Medicines Agency. 2017. European Union herbal monograph on *Salix* [various species including *S. purpurea* L., *S. daphnoides* Vill., *S. fragilis* L.] cortex. https://www.ema.europa.eu/en/documents/herbal-monograph/final-european-union-herbal-monograph-salix-various-species-including-s-purpurea-l-s-daphnoides-vill-s-fragilis-l-cortex_en.pdf . Date Accessed: September 21, 2025.

36. Active Concepts LLC. 2018. Bacterial Reverse Mutation Test - ABS White Willow Bark Extract Powder. [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

37. Active Concepts LLC. 2017. Bacterial Reverse Mutation Test - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

38. Active Concepts LLC. 2023. Bacterial Reverse Mutation Test - ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

39. Active Concepts. 2014. ABS White Willow Bark Extract Powder IL-6 ELISA Analysis. https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-IL-6ELISAAnalysis-v3.pdf.

40. Active Concepts. 2021. ABS White Willow Bark Extract Powder Cellular Renewal Study. . Date Accessed: November 6, 2025.

41. Active Concepts LLC. 2024. OECD TG 456: H295R Steroidogenesis Assay - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2026].

42. Active Concepts LLC. 2017. Dermal and Ocular Irritation Tests - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

43. Active Concepts LLC. 2013. Dermal and Ocular Irritation Tests - ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

44. Active Concepts LLC. 2013. Dermal and Ocular Irritation Tests - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

45. Active Concepts LLC. 2021. OECD TG 442C: In Chemico Skin Sensitization - ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

46. Active Concepts LLC. 2016. OECD TG 442C: In Chemico Skin Sensitization - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

47. Active Concepts LLC. 2023. OECD 442C: In Chemico Skin Sensitization - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

48. Active Concepts LLC. 2023. OECD 442D: In Vitro Skin Sensitization - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

49. Active Concepts LLC. 2021. OECD TG 442D: In Vitro Skin Sensitization - ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

50. Active Concepts LLC. 2016. OECD TG 442D: In Vitro Skin Sensitization - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

51. Anonymous. 2021. Summary information: HRIPT data for a serum containing 0.0025% Salix Alba (Willow) Bark Extract. [Unpublished data submitted by Personal Care Products Council on October 24, 2025].

52. AMA Laboratories. 2002. 50 Human Subject Repeat Insult Open Patch Test Skin Irritation/Sensitization Evaluation (open patch) - ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

53. Hilberer A, Hoffman L, Madrid M, Labib R, Costin G. Assessment of phototoxicity potential of botanicals as cosmetic ingredients using the in vitro 3T3 neutral red uptake phototoxicity test. *Regulatory Toxicology and Pharmacology*. 2025;163:105940.

54. Active Concepts LLC. 2023. Phototoxicity Assay Analysis - ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract). [Unpublished data submitted by Personal Care Products Council on November 26, 2025].

55. Active Concepts. 2016. Phototoxicity analysis report: ABS White Willow Bark Extract Powder. https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-PhototoxicityAssayAnalysisReport-v1.pdf https://activeconceptsllc.com/wp-content/uploads/2022/10/10229-ABSWhiteWillowBarkExtractPowder-PhototoxicityAssayAnalysisReport-v1.pdf. Date Accessed: September 4, 2025.

56. Boullata JI, McDonnell PJ, Oliva CD. Anaphylactic reaction to a dietary supplement containing willow bark. *Ann Pharmacother*. 2003;37(6):832–835.

57. Maistro EL, Terrazzas PM, Sawaya ACHF, Rosa PCP, Perazzo FF, de Mascarenhas Gaivão IO. In vivo toxicogenic potential of *Salix alba* (Salicaceae) bark extract. *J Toxicol Environ Health A*. 2022;85(3):121–130.

Concentration of Use by FDA Product Category[1] - *Salix alba* (Willow)-Derived Ingredients*

Salix Alba (Willow) Bark Extract
Salix Alba (Willow) Leaf Extract
Salix Alba (Willow) Bark Powder
Salix Alba (Willow) Bark Water
Salix Alba (Willow) Extract
Salix Alba (Willow) Flower Extract

Ingredient	Product Category	Maximum Concentration of Use
Salix Alba (Willow) Bark Extract	Hair conditioners Leave-on Rinse-off	 0.002% 0.01%
Salix Alba (Willow) Bark Extract	Shampoos (noncoloring) Rinse-off	 0.01%
Salix Alba (Willow) Bark Extract	Tonics, dressings, and other hair grooming aids	0.00003%
Salix Alba (Willow) Bark Extract	Other hair preparations (noncoloring) Leave-on	 0.00002%
Salix Alba (Willow) Bark Extract	Foundations Traditional	 0.001%
Salix Alba (Willow) Bark Extract	Makeup bases Traditional	 0.0063%
Salix Alba (Willow) Bark Extract	Other makeup preparations Traditional	 0.0053%
Salix Alba (Willow) Bark Extract	Bath soaps and body washes	0.004%
Salix Alba (Willow) Bark Extract	Other personal cleanliness products Rinse-off	 0.005%
Salix Alba (Willow) Bark Extract	Skin cleansing (cold creams, cleansing lotions, liquids and pads	0.001-0.03%
Salix Alba (Willow) Bark Extract	Face and neck products (not spray) Leave-on Rinse-off	 0.001-1.1% 0.005-1%
Salix Alba (Willow) Bark Extract	Body and hand products (not spray) Leave-on	 0.53%
Salix Alba (Willow) Bark Extract	Moisturizing products (not spray)	0.002-0.053%
Salix Alba (Willow) Bark Extract	Night products (not spray)	0.4%
Salix Alba (Willow) Bark Extract	Other skin care preparations Rinse-off	 0.025%
Salix Alba (Willow) Leaf Extract	Other hair preparations (noncoloring) Leave-on	 0.00002%

*Ingredients included in the title of the table but not found in the table were included in the concentration of use survey, but no uses were reported.

Information collected in 2025
Table prepared: October 20, 2025

[1] The FDA cosmetic product categories under MoCRA were used for this survey.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: October 24, 2025

SUBJECT: Salix Alba (Willow) Bark Extract

Anonymous. 2021. Summary information: HRIPT data for the serum containing 0.0025% Salix Alba (Willow) Bark Extract.

Summary Information

HRIPT data for the serum containing 0.0025% Salix Alba (Willow) Bark Extract

Test procedure: Human repeated insult patch test

Date of final report: March 24, 2021

Test article: Lotion containing 0.0025% Salix Alba (Willow) Bark Extract

Test subject： 50 subjects (17 males and 33 females)

Results: All 50 subjects did not exhibit any dermal reactions throughout the course of the entire study and had scores of “0”.

The following Dermal Scoring System was used:

Dermal Score	Description
0	No visible skin reaction
±	Barely perceptible erythema
1+	Mild erythema
2+	Well defined erythema
3+	Severe erythema and edema
4+	Erythema and edema with vesiculation

Conclusion: The test material did not demonstrate a potential for eliciting dermal irritation or inducing sensitization.

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Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 17, 2025

SUBJECT: Salix Alba (Willow) Bark Extract

Anonymous. 2025. Summary information Salix Alba (Willow) Bark Extract.

November 2025

Summary Information Salix Alba (Willow) Bark Extract

1. Method of Manufacturing

Add ethanol solution to the bark of *Salix alba* L.(Salicaceae), extract, purify, filter, and dry.

Add 1,3-butylene glycol solution to the residue, and filter to obtain the final product.

No synthetic reagents, preservatives, or chemical modifications are used. Residual solvent levels meet the specifications described in IFRA/CTFA and general cosmetic ingredient quality guidelines.

2. Composition and Impurities

As provided to the cosmetic industry, it is a mixed material, which is composed of Water : Butylene Glycol : Salix Alba (Willow) Bark Extract (68.25 : 29.25 : 2.50).

Heavy metals and pesticide residues comply with cosmetic ingredient standards (Heavy metals <20 ppm, Arsenic <2 ppm). (JSQI)

No synthetic additives or preservatives are present.

3. Acute and Repeated-Dose Toxicity

2000 mg/kg dose of the sample was orally administered through gavage to 2 groups (5×2 mice).

The animals were dosed once and were observed for mortality and signs of toxicity for 14 days following dosing.

No major signs of toxicity were observed after dosing in any of the animals of the 2000 mg/kg group.

Animal body weight increased gradually in all groups throughout the 14-day observation period.

ALD : Not less than 2000 mg/kg.

The diluted sample for 3.0w/w% water solution (of the water, butylene glycol, Salix Alba (Willow) Bark Extract described above) was applied topically on the clipped skin of 5 guinea pigs once a day for 2 weeks (five times a week).

The animals were examined for signs of erythema and edema before dosing every day and at 24 hours after final dosing.

No erythema and no edema were observed on the clipped skin of guinea pigs treated for 2 weeks.

4. Dermal Irritation and Sensitization

The diluted sample for 3.0w/w% water solution (of the water, butylene glycol, Salix Alba (Willow) Bark Extract described above) was applied topically on the clipped skin of 3 guinea pigs.

The animals were examined for signs of erythema and edema at 24, 48 and 72 hours after dosing.

No erythema and no edema were observed on the skin of guinea pigs at 24, 48 and 72 hours after dosing.

An emulsion of FCA with water (E-FCA), the diluted sample for 3.0w/w% water solution, and an emulsion of FCA with the diluted sample for 3.0w/w% water solution were injected intradermally into clipped dorsal skin area.

Then occlusive patch with the diluted sample for 3.0w/w% water solution (of the water, butylene glycol, Salix Alba (Willow) Bark Extract described above) was applied on the same sites. (Induction)

Occlusive patches with the diluted sample for 3.0w/w% water solution were applied on the clipped flank skin of guinea pigs in the test sample group (10 guinea pigs) and negative control group (10 guinea pigs). (Challenge)

The animals were examined for signs of erythema and edema at 24 and 48 hours after removal of the challenge patch.

No erythema and no edema were observed on the skin of guinea pigs in the test sample group and negative control group at 24 and 48 hours after challenge.

5. Summary and Conclusion

The available manufacturing, compositional, and toxicological data indicate that Salix Alba (Willow) Bark Extract as used in cosmetic formulations is safe under current use conditions.

Personal Care Products Council
Committed to Safety, Quality & Innovation

Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 24, 2025

SUBJECT: Salix Alba (Willow) Leaf Extract

Anonymous. 2025. Summary information Salix Alba (Willow) Leaf Extract.

November 2025

Summary Information Salix Alba (Willow) Leaf Extract

The composition of a Salix Alba (Willow) Leaf Extract at 4-6% in water is the following:

- Sugars 32.0%
- Mineral ashes 29.5 %
- Proteins 3.9%
- Polyphenols 3.1%

Impurities:

Heavy metals	N°CAS	Threshold (ppm)
Antimony	7440-36-0	≤0.5
Arsenic	7440-38-2	≤0.5
Cadmium	7440-43-9	≤0.5
Chromium	7440-47-3	≤0.5
Cobalt	7440-48-4	≤0.5
Mercury	7439-97-6	≤0.5
Nickel	7440-02-0	≤0.5
Lead	7439-92-1	≤0.5
Vanadium	7440-62-2	≤0.5

Toxicological studies completed in 2000 the ingredient Salix alba (Willow) Leaf Extract at 4-6% in water (all tests listed below were conducted on the ingredient undiluted):

- Assessment of ocular tolerance in rabbits (OECD 405): non irritant
- Assessment of cutaneous tolerance in rabbits (OECD 404): non irritant
- Assessment of cutaneous sensitizing power in Guinea pigs (following Magnusson and Kligman test): not sensitizing
- Acute toxicity (OECD 401): not toxic
- Mutagenicity test (OECD 471): Non-mutagenic

Toxicological studies completed in 2018 on the ingredient Salix alba (Willow) Leaf Extract at 4-6% in water (all tests listed below were conducted on the ingredient undiluted):

- Evaluation of a test item irritant potential by cytotoxicity study according to neutral red release method on SJRC cell line : not cytotoxic
- In vitro sensitization test: KeratinoSens : not sensitizing
- Phototoxicity (OECD 432) : not phototoxic

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Memorandum

TO: Bart Heldreth, Ph.D.
Executive Director - Cosmetic Ingredient Review

FROM: Carol Eisenmann, Ph.D.
Personal Care Products Council

DATE: November 26, 2025

SUBJECT: Salix Alba (Willow) Bark Extract

Active Concepts LLC. 2024. Composition Breakdown. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2016. Manufacturing Flow Chart. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2020. Product Specification. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2018. Bacterial Reverse Mutation Test. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2024. OECD 456: H295R Steroidogenesis Assay. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2013. Dermal and Ocular Irritation Tests. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2016. OECD TG 442C: In Chemico Skin Sensitization. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2016. OECD TG 442D: In Vitro Skin Sensitization. ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

AMA Laboratories, Inc. 2002. 50 Human Subject Repeat Insult Open Patch Test Skin Irritation/Sensitization Evaluation (open patch). ABS White Willow Bark Extract Powder (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. Compositional Breakdown. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2014. Manufacturing Flow Chart. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2018. Product Specification. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. Bacterial Reverse Mutation Test. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2013. Dermal and Ocular Irritation Tests. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2021. OECD TG 442C: In Chemico Skin Sensitization. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2021. OECD TG 442D: In Vitro Skin Sensitization. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2021. Phototoxicity Assay Analysis. ABS White Willow Bark Extract (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. Compositional Breakdown. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2010. Manufacturing Flow Chart. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2017. Product Specification. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2017. Bacterial Reverse Mutation Test. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2017. Dermal and Ocular Irritation Tests. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. OECD 442C: In Chemico Skin Sensitization. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. OECD 442D: In Vitro Skin Sensitization. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

Active Concepts LLC. 2023. Phototoxicity Assay Analysis. ACB White Willow Bark Extract 20% (Salix Alba (Willow) Bark Extract).

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ABS White Willow Bark Extract Powder
Code: 10229

Compositional Breakdown:

Ingredient	%
Salix Alba (Willow) Bark Extract	100.00

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Manufacturing Flow Chart

ACTIVE CONCEPTS LLC
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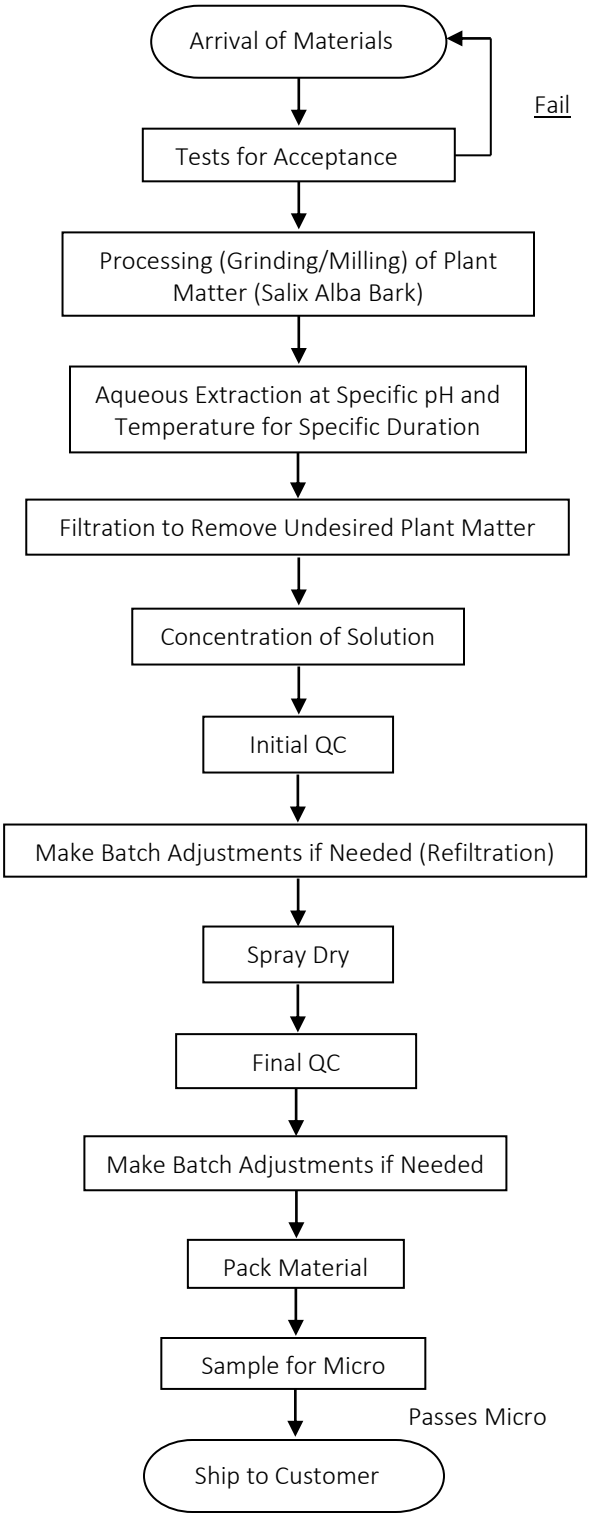
ABS White Willow Bark Extract Powder (10229)



Product Specification

ACTIVE CONCEPTS LLC
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Product Name: ABS White Willow Bark Extract Powder
Code Number: 10229
CAS #'s: 84082-82-6
EINECS #'s: 282-029-0
INCI Name: Salix Alba (Willow) Bark Extract
Status: Approved

Specification	Parameter
Appearance	White to Off-White, Free Flowing Powder*
Color (Gardner - 1% Solution in Water)	3 Maximum
Odor	Characteristic
pH (3% solution in H_2O)	4.0 – 6.0
NVM (1g, 105°C, 1hr)	94.0% Minimum
Nitrogen	2.0 – 5.0%
Salicylic Acid	53.0 – 65.0%
Heavy Metals	< 20 ppm
Lead	< 10 ppm
Arsenic	< 2 ppm
Mercury	< 1 ppm
Cadmium	< 1 ppm
Microbial Content Yeast & Mold Gram Negative Bacteria	< 100 CFU/g; No pathogens < 100 CFU/g 0 CFU/g

*HYGROSCOPIC

Bacterial Reverse Mutation Test

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Test Article: ABS White Willow Bark Extract Powder
Code Number: 10229
CAS #: 84082-82-6

Sponsor:
Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: *Erica Segura*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part3

Test Request Number: 4086

SUMMARY

A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **ABS White Willow Bark Extract Powder** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A in the presence and absence of Aroclor-induced rat liver S9. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37^0C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2*uvr*A. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

Bacterial Reverse Mutation Test

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I. Introduction

A. **Purpose**
A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

II. Materials

A. **Storage Conditions:** Room temperature (23-25C).
B. **Vehicle:** Sterile DI Water.
C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
D. **Solubility/Stability:** 100% Soluble and Stable.
E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System
Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa)*, the deletion of *uvr*B gene and the deletion in the *uvr*A gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvr*A) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	All possible transitions and transversions, small deletions.

B. Metabolic Activation
Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control
Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of reveratants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.

E. Positive Control
A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

F. Titer of the Strain Cultures:
Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to $2x10^9$/ml.

IV. Method

A. Standard Plate Incorporation Assay:
Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for *Escherichia coli* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50µl aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Evaluation

For the test solution to be evaluated as a test failure or “potential mutagen” there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* tester strain used.

VI. Results and Discussion

A. Solubility:
Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

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B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

C. Standard Plate Incorporation Assay
In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2*uvr*A in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A.The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

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Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	***TA98***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	18	22	20
	1500	24	33	29
	500	38	48	43
	150	30	57	44
	50	41	36	39
	15	39	56	48
	5.0	37	39	38
	1.5	29	46	38
Test Solution w/o S9	5000	9	19	14
	1500	38	29	34
	500	48	28	38
	150	46	44	45
	50	29	36	33
	15	42	36	39
	5.0	67	47	57
	1.5	47	33	40
DI Water w/S9		48	34	41
DI Water w/o S9		44	39	42
2-aminoanthracen w/ S9		380	347	364
2-nitrofluorene w/o S9		178	137	158
Historical Count Positive w/S9		**43-1893**		
Historical Count Positive w/o S9		**39-1871**		
Historical Count Negative w/S9		**4-69**		
Historical Count Negative w/o S9		**3-59**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	28	27	28
	1500	105	107	106
	500	100	95	98
	150	104	105	105
	50	86	93	90
	15	81	84	83
	5.0	91	99	95
	1.5	105	124	115
Test Solution w/o S9	5000	11	10	11
	1500	48	69	59
	500	91	97	94
	150	94	102	98
	50	86	107	97
	15	100	84	92
	5.0	100	84	92
	1.5	102	89	96
DI Water w/S9		94	124	109
DI Water w/o S9		85	108	97
2-aminoanthracen w/ S9		812	813	813
Sodium azide w/o S9		688	634	661
Historical Count Positive w/S9		**224-3206**		
Historical Count Positive w/o S9		**226-1837**		
Historical Count Negative w/S9		**55-268**		
Historical Count Negative w/o S9		**47-250**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	*TA1537*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	6	0	3
	1500	8	14	11
	500	10	4	7
	150	6	6	6
	50	11	5	8
	15	4	4	4
	5.0	8	6	7
	1.5	4	3	4
Test Solution w/o S9	5000	4	1	3
	1500	4	1	3
	500	1	10	6
	150	11	6	9
	50	1	6	4
	15	8	6	7
	5.0	5	5	5
	1.5	5	4	5
DI Water w/S9		4	6	5
DI Water w/o S9		5	5	5
2-aminoanthracen w/ S9		58	51	55
2-aminoacridine w/o S9		530	471	501
Historical Count Positive w/S9		**13-1934**		
Historical Count Positive w/o S9		**17-4814**		
Historical Count Negative w/S9		**0-41**		
Historical Count Negative w/o S9		**0-29**		

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*Mean = Average of duplicate plates

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	Concentration µg per Plate	*TA1535*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	3	3	3
	1500	19	18	19
	500	19	18	19
	150	11	14	13
	50	11	19	15
	15	9	10	10
	5.0	14	19	17
	1.5	10	20	15
Test Solution w/o S9	5000	8	0	4
	1500	0	4	2
	500	10	6	8
	150	11	20	16
	50	9	5	7
	15	5	14	10
	5.0	4	14	9
	1.5	5	6	6
DI Water w/S9		13	15	14
DI Water w/o S9		6	11	9
2-aminoanthracen w/ S9		86	95	91
Sodium azide w/o S9		541	601	571
Historical Count Positive w/S9		**22-1216**		
Historical Count Positive w/o S9		**47-1409**		
Historical Count Negative w/S9		**1-50**		
Historical Count Negative w/o S9		**1-45**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	27	30	29
	1500	41	29	35
	500	20	33	27
	150	48	33	41
	50	33	20	27
	15	32	32	32
	5.0	37	41	39
	1.5	37	23	30
Test Solution w/o S9	5000	19	32	26
	1500	18	36	27
	500	22	20	21
	150	22	27	25
	50	30	24	27
	15	29	19	24
	5.0	33	25	29
	1.5	28	33	31
DI Water w/S9		17	25	21
DI Water w/o S9		30	24	27
2-aminoanthracen w/ S9		130	11	121
Methylmethanesulfonate w/o S9		258	271	265
Historical Count Positive w/S9		**44-1118**		
Historical Count Positive w/o S9		**42-1796**		
Historical Count Negative w/S9		**8-80**		
Historical Count Negative w/o S9		**8-84**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

OECD 456: H295R Steroidogenesis Assay

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Tradename: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 11496

Lot #: 9396424

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
OECD 456: H295R Steroidogenesis Assay

Introduction

Endocrine disrupting agents are substances that interfere with the endocrine system and are associated with several negative health effects. Located throughout the body, endocrine glands release hormones into circulation and these signaling molecules are vital to maintaining homeostasis. Endocrine glands and hormones are extremely sensitive and small disruptions to the structure or function of any aspect of the endocrine system significantly alter physiological processes. Endocrine disruptors work through one or a combination of the following mechanisms: 1) mimicking, or partly mimicking, naturally occurring hormones, potentially leading to over production, 2) binding to a receptor and blocking a naturally occurring hormone from binding, and/or 3) interfering with or preventing the synthesis or utilization of natural hormones and/or receptors. The OECD 456: H295R Steroidogenesis Assay was developed as an *in vitro* test to screen for test article effects on steroidogenesis, specifically the production of 17β-estradiol (E2) and testosterone (T).

A H295R Steroidogenesis Assay was conducted to determine the potential of **ABS White Willow Bark Extract Powder** to induce or inhibit the production of 17β-estradiol and testosterone.

Assay Principle

The *in vitro* H295R Steroidogenesis Assay utilizes a human adreno-carcinoma cell line (NCI-H295R cells) and constitutes a level 2 "*in vitro* assay, providing mechanistic data" to screen for test article effects on steroidogenesis, but does not aim to provide specific mechanistic information concerning the interaction of the test substance with the endocrine system. Moreover, the H295R assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamic pituitary-gonadal axis. The objective of the H295R Steroidogenesis Assay is to detect substances that affect production of 17β-estradiol (E2) and testosterone (T). The H295R assay is intended to identify xenobiotics that have as their target site(s) the endogenous components that comprise the intracellular biochemical pathway beginning with the sequence of reactions from cholesterol to the production of E2 and/or T. Human adreno-carcinoma H295R cells are utilized because the cell line expresses genes that encode all key steroidogenesis enzymes, enables the detection of both increases and decreases in the production of both E2 and T, and permits the direct assessment of the potential impact of a test article on cell viability/cytotoxicity. The purpose of the assay is the detection of substances that affect E2 and T production by distinguishing test articles that typically inhibit or induce key enzymes of the steroidogenesis pathway.

OECD 456: H295R Steroidogenesis Assay

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The human adreno-carcinoma H295R cells are exposed to different concentrations of the test substance. After the incubation period, the medium is removed from each well and cellular viability is immediately analyzed. Next, concentrations of E2 and T in the medium are measured.

Materials

A. Kit:	Estradiol ELISA Kit (Cayman Chemical; 501890)*; DetectX® Testosterone ELISA Kit (Arbor Assays; K032-H)*; CyQUANT™ MTT Cell Viability Assay (ThermoFisher; V13154)*
B. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
C. Equipment:	Forma Humidified Incubator; ESCO Biosafety Laminar Flow Hood; Synergy HT Microplate Reader; Pipettes; Light Microscope; Weigh Boats; Chemical Spatula; Analytical Balance
D. Cell Line:	Human Adreno-Carcinoma Cell (NCI-H295R [H295R]) (ATCC; CRL-2128)*
E. Media/Buffers:	DMEM: F-12 Medium (ATCC; 30-2006)*; ITS+ Premix Universal Culture Supplement (Corning®; 354352)*; Nu-Serum™ Growth Medium Supplement (Corning®; 355100)*; Dimethyl sulfoxide (DMSO); Deionized water
F. Reagents:	Forskolin (ThermoFisher; J63292.MF)*; Prochloraz (ThermoFisher; P21371G)* Phosphate Buffered Saline (PBS); Trypsin; Trypsin-EDTA; Methanol; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT Assay; ThermoFisher; V13154); SDS-HCl Solution (sodium dodecyl sulfate hydrochloride) (MTT Assay; ThermoFisher; V13154)
G. Culture Plate:	24 Well Tissue Culture Treated Microplates; Tissue Culture Treated Flasks
H. Software:	Excel Analysis ToolPak (Microsoft)
I. Other:	Sterile disposable pipette tips

**Or suitable alternatives, subject to change without notice based off vendor availability*

Methods

One source vial of H295R cells were initiated from cryopreservation and expanded three passages to establish a lot for screening and cells were expanded five times before used for testing or frozen for storage. Cells cultured from the expanded stock did not exceed 10 passages. Cells were passaged and maintained in Complete Media (DMEM: F-12 Medium + 1.0% ITS+ Premix Universal Culture Supplement + 2.5% Nu-Serum™ Growth Medium Supplement). The maintenance, expansion, and cryopreservation standard operating procedures were adopted directly from the guideline specifications. A quality control test plate to establish acceptable variation between replicate wells, replicate experiments, linearity and sensitivity of hormone measurement assays, and variability between replicate hormone measures of the same sample were performed and met criteria according to the guideline specifications.

Cells utilized for testing were seeded at a target density of 200,000 to 300,000 cells per mL of Complete Media resulting in approximately 50-60% confluency in the wells at 24 hours to achieve the optimal cell density for hormone production. After an acclimation period of 24 hours, cells were exposed for 48 hours to seven concentrations of **ABS White Willow Bark Extract Powder**, diluted with DMSO, in triplicate. Cells were also exposed to Complete Media and positive, negative, and solvent controls. Prepared in DMSO, Forskolin (10 µM) and Prochloraz (1.0 µM) were utilized as positive and negative controls as they are known inducers and inhibitors, respectively, of E2 and T synthesis. DMSO was utilized as the solvent control. At the end of the 48-hour exposure period, the media is removed from each well and stored in aliquots at -80°C until hormone analysis. Cell viability in each well is analyzed immediately after removal of media. Conclusions regarding the ability of a test article to affect steroidogenesis are based on at least two independent experimental runs.

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To determine cell viability, fresh Complete Media with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to all wells, except for the positive control wells which received 70% Methanol in Complete Media provided Methanol is a known cytotoxicity inducer. After a 4-hour incubation period at 37°C, the SDS-HCl solution was added to all wells and the plate was incubated again at 37°C for 4 hours after which the absorbance was read at 570 nm. Cell viability data is displayed as Mean Absorbance Units (MAU) and reflects averages of the triplicates for independent experimental runs. The results were analyzed utilizing a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Conditions demonstrating viability lower than 80% were not included in the final data analysis. The percentage of viable cells was calculated with the following equation:

$$Viable\ Cells\ (\%) = \frac{MAU_{Sample} - MAU_{Methanol}}{MAU_{DMSO} - MAU_{Methanol}} \times 100$$

After cellular viability was determined, 17β-estradiol (E2) and testosterone (T) concentration levels in the media were determined with an Estradiol ELISA Kit (Limit of Quantification 6.0 pg/mL) and DetectX® Testosterone ELISA Kit (Limit of Quantification 30.6 pg/mL), respectively. Frozen media aliquots were thawed at room temperature and both ELISAs were performed according to the manufacturers' protocols. Briefly, thawed media aliquots were loaded onto the plate along with prepared standards. Next, conjugates and antibodies were added to appropriate wells and allowed to incubate at room temperature. After the incubation, wells were washed, and antibody detection reagents were added to each well followed by stop solutions to stop the antibody detection reactions. Lastly, absorbance values were measured.

The criteria for a negative, positive, or equivocal run are provided in Diagram 1. A minimum of two independent runs of the assay should be conducted. Unless prior information such as information on solubility limits or cytotoxicity provides a basis for selecting test concentrations, it is recommended that the test concentrations for the initial run be spaced at $\log_{10}$ intervals with 10^{-3} M being the maximum concentration. If the test article is soluble, and not cytotoxic at any of the tested concentrations, and the first run was negative for all concentrations, then it is to be confirmed in one more run using the same conditions as the first run was conducted (Table 1). If the results of the first run are equivocal or positive, the test should be repeated as indicated in Table 1 and footnote 'a' to Diagram 1, by refining the selected test concentrations. Test concentrations in runs two and three (if applicable) should be adjusted on the basis of the results of the initial run bracketing concentrations that elicited an effect using ½-log concentration spacing (e.g. if the original run of 0.001, 0.01, 0.1, 1, 10, 100, 1000 µM resulted in inductions at 1 and 10 µM, the concentrations tested in the second run should be 0.1, 0.3, 1, 3, 10, 30, 100 µM), unless lower concentrations need to be employed to achieve a Lowest-Observed-Effect-Concentration (LOEC). In the latter case, at least five concentrations below the lowest concentration tested in the first run should be used in the second run using a ½-log scale. If the assay is equivocal or positive, the LOEC is reported. If the assay is negative, the highest concentration tested is reported as the No-Observed-Effect-Concentration (NOEC).

If the second run does not confirm the first run, a third experiment is to be conducted using the original testing conditions. Equivocal results in the first run are considered negative if the observed effect could not be confirmed in any of the two subsequent runs. Equivocal results are considered as weak positive responses (effect) when the response can be confirmed in at least two more runs within a ± 1 concentration increment (Diagram 1; Table 1). Non monotonic concentration response patterns are rare but possible. If reproducible effects are seen at lower concentrations which then dissipate at greater concentrations and/or return at the top concentrations, this data should not be disregarded and should be included in the study report.

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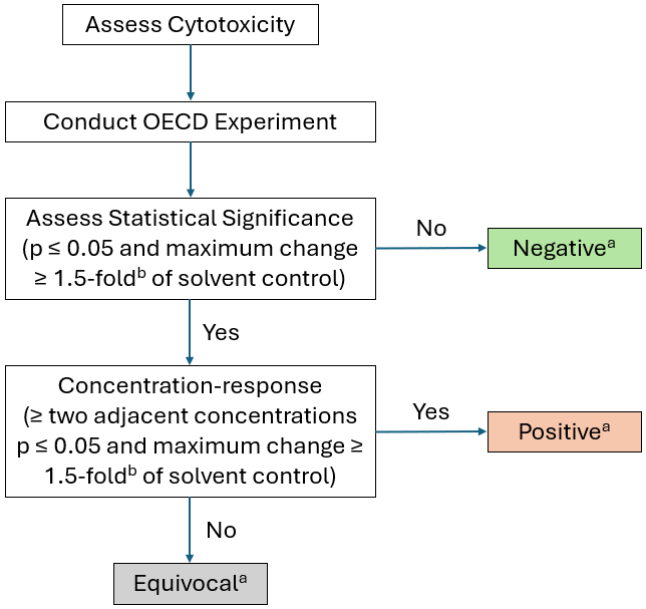

Diagram 1. Overview of the Data Interpretation Procedure within an Individual Run. Diagram from OECD 456 Test Guidelines.

[a] Conduct second or third confirmatory runs as specified in Table 1.

[b] 1.5-fold applies to both induction and inhibition of hormones.

Table 1. Decision Matrix for Possible Outcome Scenarios. Table adapted from OECD 456 Test Guidelines.

Run 1		Run 2		Run 3		Final Call
Scenario	*Decision*	*Scenario*	*Decision*	*Scenario*	*Decision*	
Negative	Confirm[a]	Negative	Stop			Negative
Negative	Confirm[a]	Positive/Equivocal[c]	Refine[b]	Negative	Stop	Negative
Negative	Confirm[a]	Positive	Refine[b]	Positive	Stop	Positive
Negative	Confirm[a]	Positive	Refine[b]	Equivocal[c]	Stop	Weak Positive
Negative	Confirm[a]	Equivocal[c]	Refine[b]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Negative	Stop	Negative
Equivocal[c]	Refine[b]	Negative	Confirm[a]	Positive/Equivocal[c]	Stop	Weak Positive
Equivocal[c]	Refine[b]	Equivocal[c]	Confirm[a]	Equivocal[c]/Positive	Stop	Weak Positive
Equivocal[c]	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Positive	Stop			Positive
Positive	Refine[b]	Negative	Confirm[a]	Positive	Stop	Positive

[a] Confirm previous run using the same experimental design/concentration-spacing.

[b] Re-run assay at ½ log concentration spacing (bracketing the concentration that tested significantly different in the preceding experiment). A smaller concentration-spacing in runs 2 and 3 is recommended given the chance of having only one concentration showing a significant response at a 10-fold increment. Applying a ½ log or even smaller spacing increment should result in at least two significant responses. If the confirmatory run at lower concentrations at the ½ log scale is positive, then the test article is positive. However, on rare occasions an "all-or-nothing" response may occur that only shows an effect at one concentration, which would be captured by this decision process.

[c] Fold-change at one concentration is statistically different from the solvent control.

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To evaluate the relative increase/decrease in test article altered hormone production, the results are expressed as the average fold change relative to the solvent control of each test plate. Relative changes were calculated as follows:

$$Relative\ Change = \frac{Hormone\ Concentration_{Sample}}{Hormone\ Concentration_{DMSO}}$$

A test article is judged to be positive if the fold induction or inhibition is statistically different ($p \leq 0.05$) and above or below the 1.5-fold induction or inhibition threshold from the solvent control at two adjacent concentrations in at least two independent runs (Table 1). The 1.5-fold threshold applies for both increase and decrease of hormone concentrations, i.e. above 150% of the solvent control and below 66.7% of the solvent control (where cytotoxicity ≥ 80%). A test article is judged to be negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in Table 1, the experimental results are not interpretable. Prior to conducting statistical analyses, the assumptions of normality and variance homogeneity were evaluated and passed. Statistical significance was accepted at $p \leq 0.05$.

Results

The data obtained from this study met criteria for valid assays and the positive, negative, and solvent controls performed as anticipated. **ABS White Willow Bark Extract Powder** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

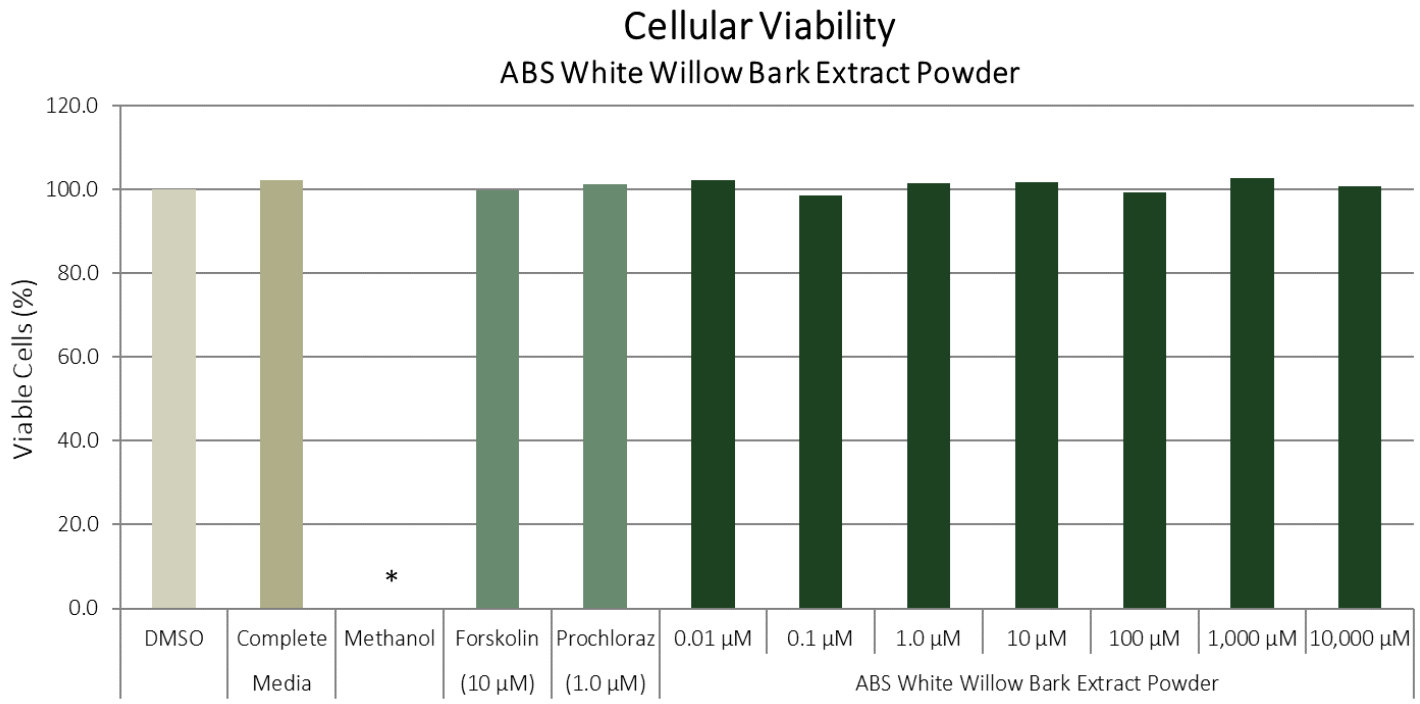

Figure 1. Cellular viability. * indicates significance ($p \leq 0.05$) compared to DMSO.

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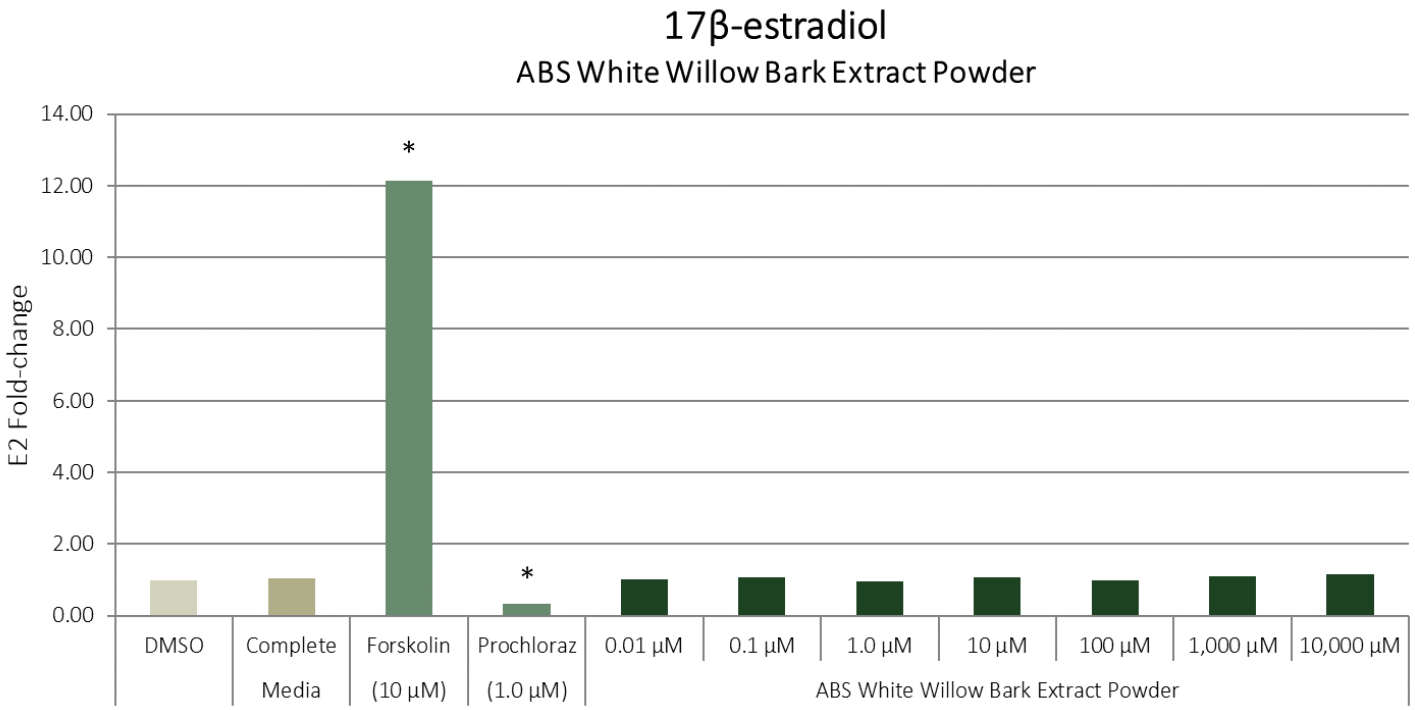

Figure 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 2. 17β-estradiol (E2) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		1.03
Forskolin		12.15*
Prochloraz		0.33*
ABS White Willow Bark Extract Powder	0.01 µM	1.01
	0.1 µM	1.07
	1.0 µM	0.96
	10 µM	1.08
	100 µM	0.98
	1,000 µM	1.09
	10,000 µM	1.17

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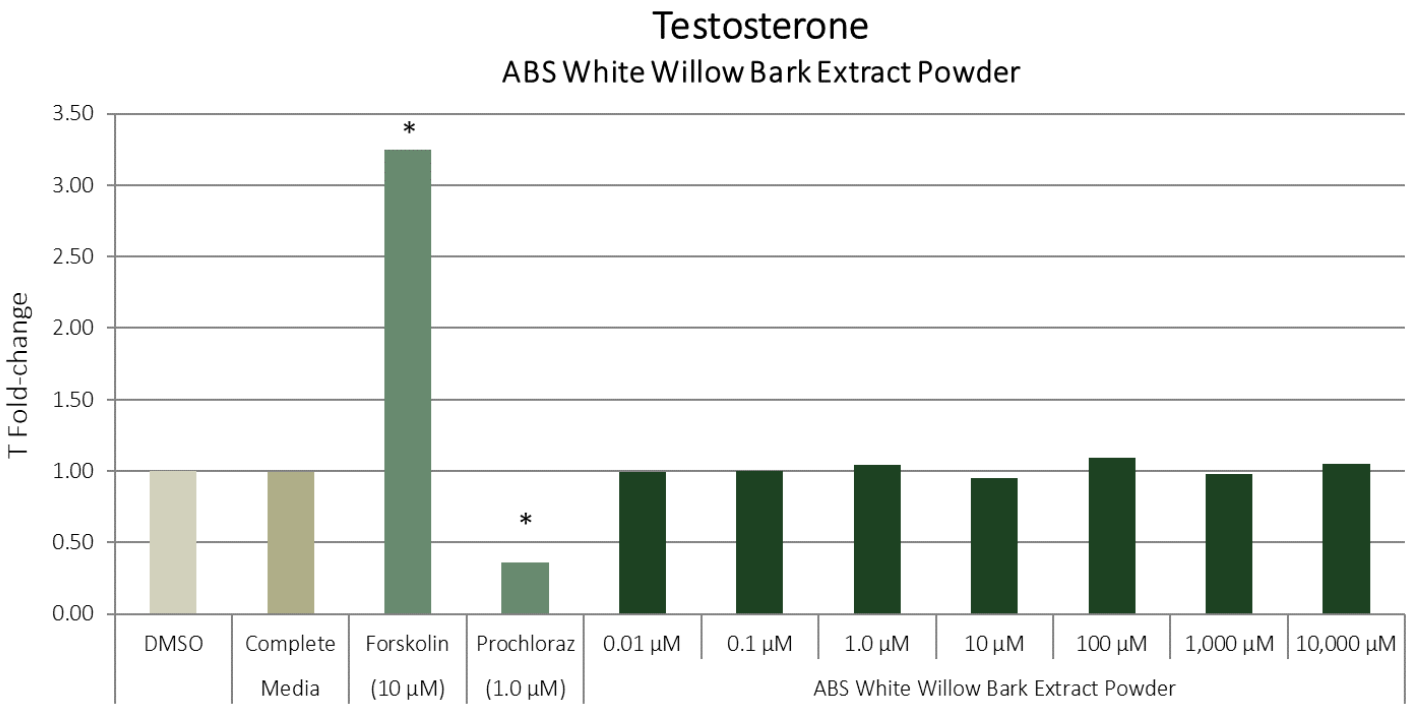

Figure 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Table 3. Testosterone (T) fold-change relative to DMSO (Solvent Control). * indicates significance ($p \leq 0.05$) compared to DMSO.

Test Article		Fold-change
DMSO		1.00
Complete Media		0.99
Forskolin		3.25*
Prochloraz		0.36*
ABS White Willow Bark Extract Powder	0.01 µM	0.99
	0.1 µM	1.00
	1.0 µM	1.04
	10 µM	0.95
	100 µM	1.09
	1,000 µM	0.98
	10,000 µM	1.05

Discussion

The objective of the H295R Steroidogenesis Assay is to screen for test article effects on cellular viability and steroidogenesis by detecting substances that affect 17β-estradiol and testosterone production. Collectively, the results demonstrate **ABS White Willow Bark Extract Powder** was non-cytotoxic and did not induce or inhibit the production of 17β-estradiol or testosterone at all concentrations tested.

Dermal and Ocular Irritation Tests

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Trade Name: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 431

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ABS White Willow Bark Extract Powder** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

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Dermal and Ocular Irritation Tests

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II. Materials

A. Incubation Conditions: 37°C at 5% CO_2 and 95% relative humidity

B. Equipment: Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes

C. Media/Buffers: DMEM based medium; DPBS; sterile deionized H_2O

D. Preparation: Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components

E. Tissue Culture Plates: Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates

F. Reagents: MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate

G. Other: Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

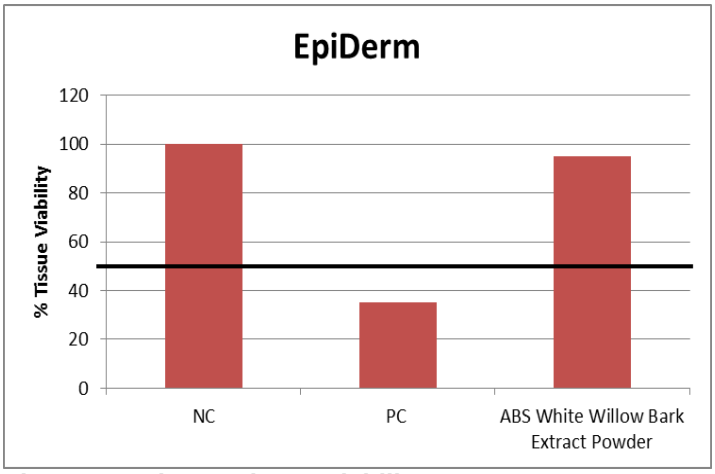

Figure 1: EpiDerm tissue viability

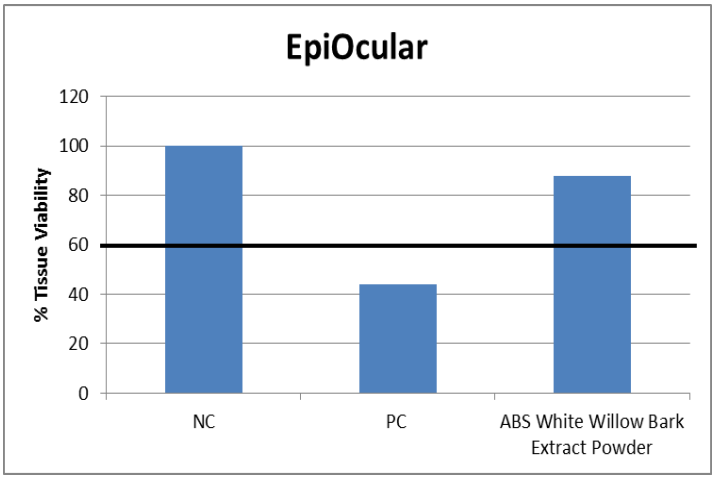

Figure 2: EpiOcular tissue viability

OECD TG 442C: *In Chemico* Skin Sensitization

Active Concepts

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Tradename: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 2210

Lot #: 45275P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD TG 442C: *In Chemico* Skin Sensitization Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact[1]. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis[2]. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)[3].

This assay was conducted to determine skin sensitization hazard of **ABS White Willow Bark Extract Powder** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

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Materials

A.	**Equipment:**	HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
B.	**HPLC/Guard Columns:**	Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5µm; Phenomenex Security Guard C18 4mm x 2mm
C.	**Chemicals:**	Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-COOH); Cinnamic aldehyde
D.	**Reagents/Buffers:**	Sodium phosphate buffer (100mM); Ammonium acetate buffer (100mM)
E.	**Other:**	Sterile disposable pipette tips

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM* **ABS White Willow Bark Extract Powder** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **ABS White Willow Bark Extract Powder**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
• 750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200µL Acetonitrile • 50µL Test Chemical Solution (or Acetonitrile for Reference Controls)	• 750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250µL Test Chemical Solution (or Acetonitrile for Reference Controls)

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Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
  - For the Cysteine peptide using the phosphate buffer, pH 7.5
  - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

Data and Reporting

Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
   a. Standard calibration curve should have an $r^2 > 0.99$.
   b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
   c. Mean peptide concentration of reference controls A should be 0.50±0.05mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.

2. The following criteria must be met for a test chemical's results to be considered valid:
   a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
   b. Mean peptide concentration of the three reference control C should be 0.50±0.05mM.

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Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.26	Minimal Reactivity	Non-sensitizer
3.30	Minimal Reactivity	Non-sensitizer
3.29	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.15	Minimal Reactivity	Non-sensitizer
3.12	Minimal Reactivity	Non-sensitizer
3.22	Minimal Reactivity	Non-sensitizer

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

$$Percent\ Peptide\ Depletion = \left[1 - \left(\frac{Peptide\ Peak\ Area\ in\ Replicate\ Injection}{Mean\ Peptide\ Peak\ Area\ in\ Reference\ Controls\ C}\right)\right] \times 100$$

Based on HPLC-UV analysis of **ABS White Willow Bark Extract Powder (10229)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.22% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD TG 442D: *In Vitro* Skin Sensitization

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Tradename: ABS White Willow Bark Extract Powder

Code: 10229

CAS #: 84082-82-6

Test Request Form #: 2074

Lot #: 47292P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD TG 442D: In Vitro Skin Sensitization *ARE-Nrf2 Luciferase Test Method*

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals[1]. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **ABS White Willow Bark Extract Powder** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013

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Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
B. Equipment:	Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Fetal Bovine Serum (FBS); Phosphate Buffered Saline (PBS); Geneticin
E. Culture Plate:	Flat bottom 96-well tissue culture treated plates
F. Reagents:	Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. Other:	Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM containing 10% FBS and 500µg/mL G418 geneticin. Twelve test concentrations of **ABS White Willow Bark Extract Powder** were prepared in DMSO with a concentration range from 0.98 - 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64 µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO_2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO_2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC_{50} and IC_{30} values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and $EC_{1.5}$ and maximum response (I_{max}) values were obtained.

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Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC1.5 value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The Imax is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC1.5 determining concentration)
3. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	$EC_{1.5}$ (µM)	IC_{50}	I_{max}
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.82
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.15
ABS White Willow Bark Extract Powder	Non-Sensitizer	No Induction	> 1000 µM	0.34

Table 1: Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values Fg.1)

Active Concepts

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OECD TG 442D: *In Vitro* Skin Sensitization

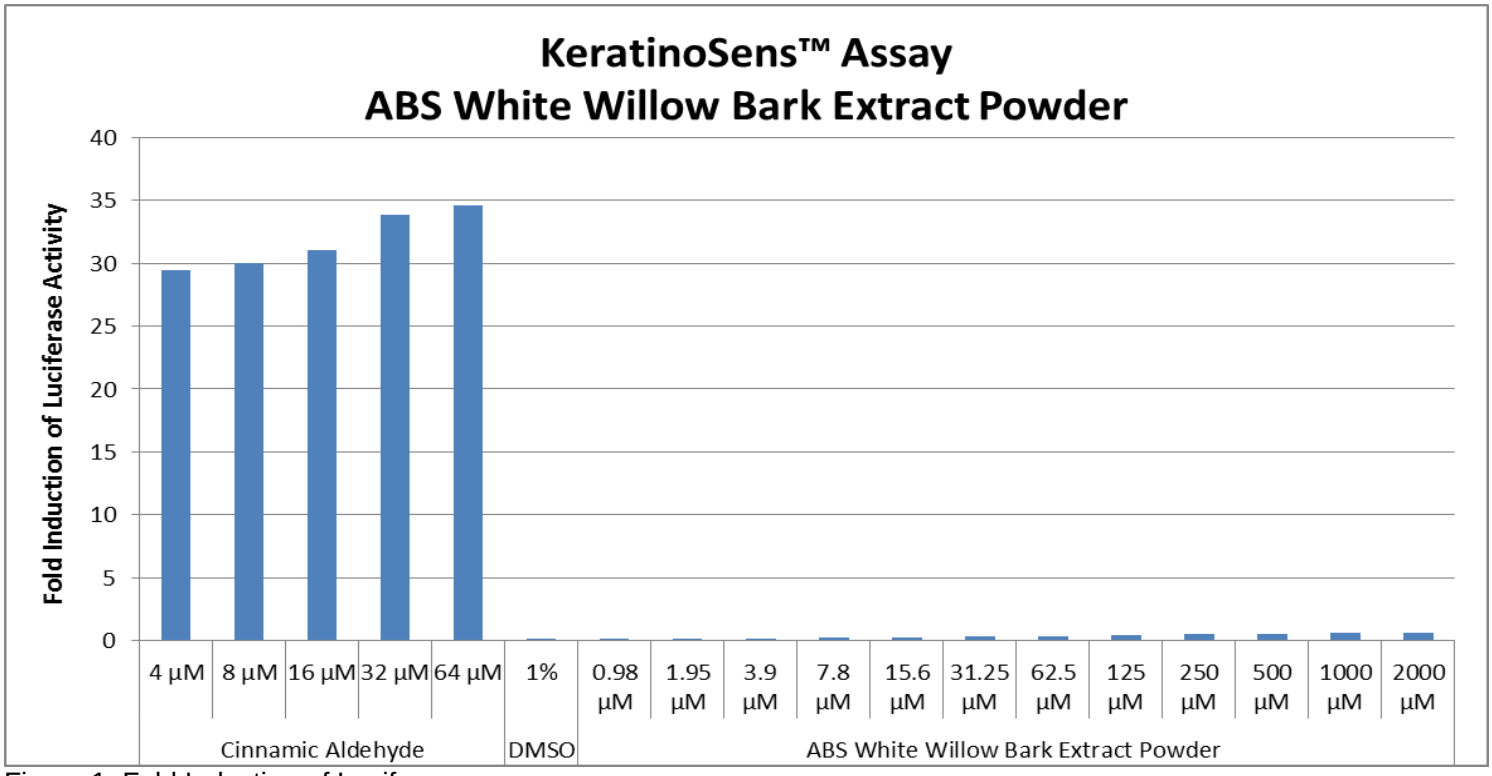

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **ABS White Willow Bark Extract Powder (10229)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **ABS White Willow Bark Extract Powder** can be safely used in cosmetics and personal care products at typical use levels.

AMA LABORATORIES, INC.
216 Congers Road, Bldg. 1
New City, NY 10956 USA
(845) 634-4300
FAX: (845) 638-4872

50 HUMAN SUBJECT REPEAT INSULT OPEN PATCH TEST SKIN IRRITATION/SENSITIZATION EVALUATION (OPEN PATCH)

Date: September 6, 2002

AMA Ref. No.: MS02.RIPT.C3859OP.50.ACTC

Sponsor: Active Concepts
43 Davis Street
South Plainfield, New Jersey 07080

1.0 Objective: Consumer products or raw materials designed for consistent reapplication to areas of the skin may, under proper conditions, prove to be contact sensitizers or irritants in certain individuals. It is the intention of a Repeat Insult Patch Test (RIPT) to provide a basis for evaluation of this irritation/ sensitization potential if such exists.

2.0 Reference: The method is modified to test 50 panelists and not the 200 cited in the reference Appraisal of the Safety of Chemicals in Food, Drugs and Cosmetics, published by The Association of Food and Drug Officials of The United States. The method also employs nine inductive patchings and not the ten cited in the reference under 'open patch' conditions.

3.0 Test Material:

3.1 Test Material Description:

On July 30, 2002 eight test samples labeled as listed below were received from Active Concepts and assigned AMA Lab Nos. as follows:

Sample Description	AMA Lab No.
[redacted]	[redacted]
[redacted]	[redacted]
ABS White Willow Bark Extract Powder, Code 10229, Lot SN020507-3	C-3861
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]
[redacted]	[redacted]

3.2 Handling:

Upon arrival at AMA Laboratories, Inc., the test material is assigned a unique laboratory code number and entered into a daily log identifying the lot number, sample description, sponsor, date and test requested.
Samples are retained for a period of three months beyond submission of final reports unless otherwise specified by the sponsor or, if sample is known to be in support of governmental applications, representative retained samples are kept two years beyond final report submission.

Sample disposition is conducted in compliance with appropriate federal, state and local ordinances.

3.3 Test Material Evaluation Prerequisite:

Prior to induction of a human test panel, animal toxicology, microbiology and other in-vivo or in-vitro performance spectra may be required to assess the feasibility of commencement as dictated by an Institutional Review Board (IRB) described in Section 4.0.

Sponsor purports that prior to sample submission to AMA the following tests were conducted with no adverse results and that the test data are on file on their premises and have not been made available to AMA personnel:

- CTFA Preservative Efficacy Test or equivalent
- 90 Day Accelerated Stability and Container Compatibility Study

4.0 Institutional Review Board:

Reference: CFR Title 21 Part 56, Subparts A, B, C, and D.
The IRB of AMA Laboratories, Inc., consists of five or more individuals, chosen from within the company for technical expertise and from the local community for lay interaction. The list of IRB members is kept on file at AMA Laboratories, Inc., and is available for inspection during the hours of operation.

5.0 Panel Selection:

5.1 Standards for Inclusion in a Study:

- Individuals who are not currently under a doctor's care.
- Individuals free of any dermatological or systemic disorder which would interfere with the results, at the discretion of the Investigator.
- Individuals free of any acute or chronic disease that might interfere with or increase the risk of study participation.
- Individuals who will complete a preliminary medical history form mandated by AMA Laboratories, Inc. and are in general good health.
- Individuals who will read, understand and sign an informed consent document relating to the specific type of study they are subscribing. Consent forms are kept on file and are available for examination on the premises of AMA Laboratories, Inc. only.
- Individuals able to cooperate with the Investigator and research staff, be willing to have test materials applied according to the protocol, and complete the full course of the study.

5.2 Standards for Exclusion from a Study:

- Individuals under 18 years of age.
- Individuals who are under doctor's care.
- Individuals who are currently taking any medication (topical or systemic) that may mask or interfere with the test results.
- Subjects with a history of any acute or chronic disease that might interfere with or increase the risk of study participation.
- Individuals diagnosed with chronic skin allergies.
- Female volunteers who indicate that they are pregnant or nursing.

5.3 Recruitment:

Panel selection is accomplished by advertisements in local periodicals, community bulletin boards, phone solicitation, electronic media or any combination thereof.

5.4 Informed Consent and Medical History Forms:

An informed consent was obtained from each volunteer prior to initiating the study describing reasons for the study, possible adverse effects, associated risks and potential benefits of the treatment and their limits of liability. Panelists signed and dated the informed consent document to indicate their authorization to proceed and acknowledge their understanding of the contents. Each subject was assigned a permanent identification number and completed an extensive medical history form. These forms along with the signed consent forms, are available for inspection on the premises of AMA Laboratories, Inc. only. Reference 21 CFR Ch. 1 Part 50, Subpart B.

6.0 Population Demographics:

Number of subjects enrolled		54
Number of subjects completing study		50
Age Range		18-72
Sex	Male	10
	Female	44
Race	Caucasian	40
	Hispanic	13
	Asian	1

7.0 Equipment:

- Acculine Surgical Marking Pen (Accu-Line Products, Inc.).
- 1 ml volumetric syringe without a needle and spatula.

8.0 Procedure:

- Subjects are requested to bathe or wash as usual before arrival at the facility.
- As per client request, [redacted] were diluted to 10% in distilled water; the test materials C-3861, [redacted] and [redacted]

were diluted to 5% in distilled water; and test material C-[redacted] was diluted to 2% in distilled water. Dilutions were freshly prepared on each application day.

- 0.2 ml or 0.2 g of the test material is dispensed directly onto a designated area of the panelist's back and allowed to air dry.

- This procedure is repeated until a series of nine consecutive 'open patch' applications have been made for every Monday, Wednesday and Friday for three consecutive weeks.

- In the event of an adverse reaction, the area of erythema and edema is measured. The edema is estimated by the evaluation of the skin with respect to the contour of the unaffected normal skin. Reactions are scored just before applications two through nine and the next test date following application nine. In most instances this is approximately 24 hours after patch removal. Clients are notified immediately in the case of adverse reaction and determination is made as to treatment program if necessary.

- Subjects are then given a 10 - 14 day rest period after which a challenge or retest dose is applied once to a previously unexposed test site. The retest dose is equivalent to any one of the original nine exposures. Reactions are scored 24 and 48 hours after application.

- Comparison is made between the nine inductive responses and the retest dose.

9.0 Results: Please refer to attached Tables.

10.0 Observations: No adverse reactions of any kind were noted during the course of this study.

11.0 Archiving: All original samples, raw data sheets, technicians notebooks, correspondence files, and copies of final reports and remaining specimens are maintained on premises of AMA Laboratories in limited access storage files marked "Archive". A duplicate disk copy of final reports is separately archived in a bank safe deposit vault.

12.0 Conclusion:

The test materials listed below when tested under 'open patch' conditions as described herein at the dilutions in distilled water listed, may be considered as **NON-PRIMARY IRRITANTS** and **NON-PRIMARY SENSITIZERS** to the skin according to the reference:

Sample Description	AMA Lab No.
[redacted]	[redacted]
ABS White Willow Bark Extract Powder, Code 10229, Lot SN020507-3 (5%)	C-3861
[redacted]	[redacted]

Shyla Cantor, Ph.D.
Study Director

P. Nou Torres, B.S.
Technician

Lynn Kimiecik, B.S.
Technician

Diana Steixner
Technician

David R. Winne, B.S.
Quality Assurance Supervisor

9/6/02
Date

TABLE 3
SUMMARY OF RESULTS
(OPEN PATCH)

AMA Lab No.: C-3861
Client No.: ABS White Willow Bark Extract Powder, Code 10229, Lot SN020507-3
Dilution: 5% in distilled water

No.	Subject ID	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	Chall. 48 HR	Score
1	86 3979	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
2	26 0599	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
3	30 1215	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
4	72 1591	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
5	24 3334	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
6	69 1708	C	F	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
7	70 3708	C	F	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
8	41 8535	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
9	50 9640	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
10	46 5842	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
11	62 1627	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
12	50 9087	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
13	36 4212	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
14	46 4213	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
15	26 7211	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
16	44 5435	C	F	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
17	42 3961	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
18	28 5046	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
19	36 5248	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
20	40 6489	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
21	60 5821	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
22	68 7038	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
23	62 6182	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
24	32 6955	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
25	60 8615	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
26	89 1786	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
27	34 7962	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
28	40 1260	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
29	50 7621	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
30	66 8570	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
31	66 8507	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
32	62 3596	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0

TABLE 3 (CONT'D)
SUMMARY OF RESULTS
(OPEN PATCH)

AMA Lab No.: C-3861
Client No.: ABS White Willow Bark Extract Powder, Code 10229, Lot SN020507-3
Dilution: 5% in distilled water

No.	Subject ID	RACE	SEX	Response 1	2	3	4	5	6	7	8	9	Chall. 24 HR	Chall. 48 HR	Score
33	22 4828	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
34	36 9096	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
35	46 7496	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
36	50 4079	C	M	0	0	0	0	0	0	0	0	0	0	0	0.0
37	38 9386	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
38	52 6559	A	F	0	0	0	0	0	0	0	0	0	0	0	0.0
39	40 0614	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
40	52 8248	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
41	36 8248	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
42	72 3637	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
43	52 3573	C	M	0	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	Dc	N/A
44	64 3640	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
45	40 6875	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
46	42 5472	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
47	44 5375	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
48	30 5720	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
49	66 9894	C	F	0	0	0	0	0	0	0	0	0	0	0	0.0
50	68 8987	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
51	79 3346	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
52	73 5431	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0
53	40 7089	H	F	0	0	0	0	0	0	0	0	0	0	0	0.0
54	49 2301	H	M	0	0	0	0	0	0	0	0	0	0	0	0.0

Evaluation Period:

This study was conducted from July 31, 2002 through September 5, 2002.

Scoring Scale And Definition Of Symbols Shown In Tables:

0 - No evidence of any effect

? - (Barely perceptible) minimal faint (light pink) uniform or spotty erythema

1 - (Mild) pink uniform erythema covering most of contact site

2 - (Moderate) pink\red erythema visibly uniform in entire contact area

3 - (Marked) bright red erythema with accompanying edema, petechiae or papules

4 - (Severe) deep red erythema with vesiculation or weeping with or without edema

D - Patch eliminated due to severe reaction

Dc - Discontinued due to absence of subject on application date

M - Patch applied to an adjacent site after strong test reaction

NA - Score is not calculated for subjects discontinued before challenge

S - Skin stained from pigment in product

T - Tan

NOTE: All technical employees of AMA LABORATORIES, INC. are required to take and pass a visual discrimination examination conducted by a Board Certified Ophthalmologist using the Farnsworth-Munsell 100 Hue Test as published; which determines a person's ability to discern color against a black background. This test was additionally modified to include a flesh tone background more nearly approaching actual use conditions, wherein erythematous skin is graded according to intensity.

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ABS White Willow Bark Extract
Code: 10230

ABS White Willow Bark Extract is a 1 : 5 extract. For every 1 part plant material used, 5 parts solvent is used.

Compositional Breakdown:

Ingredient	%
Salix Alba (Willow) Bark Extract	100.00

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

10230-ABS White Willow Bark Extract- Manufacturing Flow Chart

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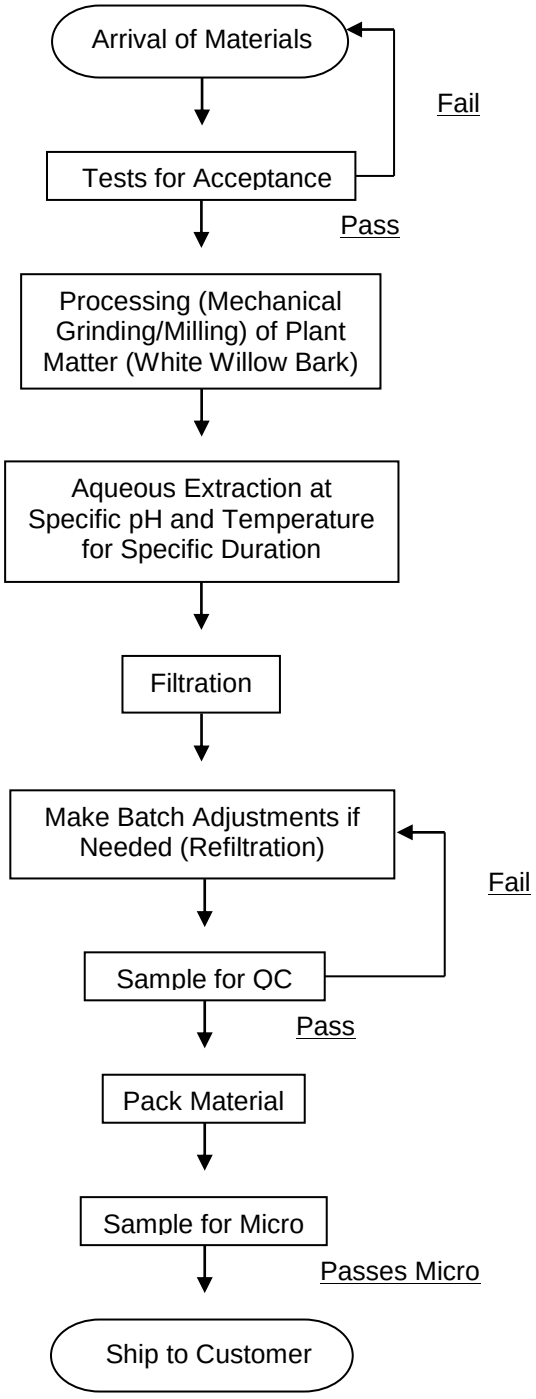

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Product Specification

Product Name: ABS White Willow Bark Extract
Code Number: 10230
CAS #'s: 84082-82-6
EINECS #'s: 282-029-0
INCI Name: Salix Alba (Willow) Bark Extract
Status: Approved

Specification	Parameter
Appearance	Colorless to Light Amber Liquid
Color	4 Gardner Maximum
Odor	Characteristic
pH (direct)	4.0 – 6.5
NVM (1g-105°C-1hr)	25.0% Minimum
Ash (600°C)	0.50% Maximum
Salicylic Acid (UV Absorption)	9.8 - 11.5%
Heavy Metals	< 20 ppm
Lead	< 10 ppm
Arsenic	< 2 ppm
Cadmium	< 1 ppm
Microbial Content Yeast & Mold Gram Negative Bacteria	< 100 CFU/g; No pathogens < 100 CFU/g 0 CFU/g

May Sediment upon Standing; Mix Well Prior to Use

**Note: Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored

Bacterial Reverse Mutation Test

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Test Article: ABS White Willow Bark Extract
Code Number: 10230
CAS #: 84082-82-6

Sponsor:
Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: *Maureen Danaher*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part 3

Test Request Number: 9718

SUMMARY

A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **ABS White Willow Bark Extract** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A in the presence and absence of Aroclor-induced rat liver S9. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37^0C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2*uvr*A. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

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Bacterial Reverse Mutation Test

I. Introduction

A. **Purpose**

A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

II. Materials

A. **Storage Conditions:** Room temperature (23-25C).
B. **Vehicle:** Sterile DI Water.
C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
D. **Solubility/Stability:** 100% Soluble and Stable.
E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System

Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa)*, the deletion of *uvr*B gene and the deletion in the *uvr*A gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvr*A) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	All possible transitions and transversions, small deletions.

B. Metabolic Activation

Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100,TA1537, TA1535 and *Escherichia coli* WP2uvrA were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control
Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of reveratants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.

E. Positive Control
A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

F. Titer of the Strain Cultures:
Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to $2x10^9$/ml.

IV. Method

A. Standard Plate Incorporation Assay:
Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for *Escherichia coli* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50µl aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Criteria for a Valid Test

For the test solution to be evaluated as a test failure or “potential mutagen” there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* and *Escherichia coli* tester strains used.

All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to $0.3x10^9$ cells/ml.

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The mean of each positive control must exhibit at least 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxicdose levels is required to evaluate assay data. A dose level is considered toxic if one of both of the following criteria are met: (1). A >50% reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2). At least a moderate reduction in the background lawn.

VI. Results and Discussion

A. Solubility:
Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

D. Standard Plate Incorporation Assay

In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2*uvr*A in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

All *Salmonella* tester strain cultures demonstrated the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 demonstrated the presence of the Pkm101 plasmid R-factor. All WP2 *uvr*A cultures demonstrated the deletion in the *uvr*A gene. All cultures demonstrated the characteristic mean number of spontaneous revertants in the vehicle controls as follows: TA98, 10-50; TA100, 80-240; TA1535, 5-45; TA1537, 3-21, WP2*uvr*A, 10-60.

VII. Conclusion

All criteria for a valid study were mete as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A.The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

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Bacterial Reverse Mutation Test

Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	12	14	13
	1500	14	17	16
	500	10	12	11
	150	25	25	25
	50	13	16	15
	15	12	24	18
	5.0	31	30	31
	1.5	42	37	40
Test Solution w/o S9	5000	13	16	15
	1500	25	23	24
	500	18	24	21
	150	22	29	26
	50	25	29	27
	15	13	14	14
	5.0	39	37	38
	1.5	21	21	21
DI Water w/S9		20	19	20
DI Water w/o S9		68	62	65
2-aminoanthracen w/ S9		142	139	141
2-nitrofluorene w/o S9		125	131	128
Historical Count Positive w/S9		**43-1893**		
Historical Count Positive w/o S9		**39-1871**		
Historical Count Negative w/S9		**4-69**		
Historical Count Negative w/o S9		**3-59**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	115	123	119
	1500	143	149	146
	500	165	142	154
	150	143	159	151
	50	162	151	157
	15	163	149	156
	5.0	132	133	133
	1.5	116	112	114
Test Solution w/o S9	5000	133	136	135
	1500	137	129	133
	500	145	188	167
	150	149	141	145
	50	111	127	119
	15	116	145	131
	5.0	122	116	119
	1.5	110	125	118
DI Water w/S9		98	116	107
DI Water w/o S9		77	84	81
2-aminoanthracen w/ S9		616	626	621
Sodium azide w/o S9		551	607	579
Historical Count Positive w/S9		**224-3206**		
Historical Count Positive w/o S9		**226-1837**		
Historical Count Negative w/S9		**55-268**		
Historical Count Negative w/o S9		**47-250**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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Bacterial Reverse Mutation Test

	Concentration µg per Plate	*TA1537*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	15	14	15
	1500	8	8	8
	500	14	18	16
	150	26	11	19
	50	15	14	15
	15	13	10	12
	5.0	8	7	8
	1.5	13	17	15
Test Solution w/o S9	5000	22	23	23
	1500	10	16	13
	500	15	19	17
	150	14	16	15
	50	17	15	16
	15	22	23	23
	5.0	29	26	28
	1.5	15	9	12
DI Water w/S9		18	14	16
DI Water w/o S9		28	22	25
2-aminoanthracen w/ S9		355	370	363
2-aminoacridine w/o S9		128	127	128
Historical Count Positive w/S9		**13-1934**		
Historical Count Positive w/o S9		**17-4814**		
Historical Count Negative w/S9		**0-41**		
Historical Count Negative w/o S9		**0-29**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	9	17	13
	1500	14	10	12
	500	14	18	16
	150	9	13	11
	50	12	12	12
	15	19	11	15
	5.0	20	22	21
	1.5	7	5	6
Test Solution w/o S9	5000	20	15	17
	1500	13	16	15
	500	16	12	14
	150	17	10	14
	50	22	12	17
	15	16	19	18
	5.0	16	8	12
	1.5	11	16	14
DI Water w/S9		38	39	39
DI Water w/o S9		11	14	13
2-aminoanthracen w/ S9		145	166	156
Sodium azide w/o S9		744	732	738
Historical Count Positive w/S9		**22-1216**		
Historical Count Positive w/o S9		**47-1409**		
Historical Count Negative w/S9		**1-50**		
Historical Count Negative w/o S9		**1-45**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	8	14	11
	1500	16	13	15
	500	26	17	22
	150	16	15	16
	50	16	13	15
	15	8	9	9
	5.0	22	19	21
	1.5	6	12	9
Test Solution w/o S9	5000	30	27	29
	1500	20	25	23
	500	26	24	25
	150	12	15	14
	50	28	29	29
	15	14	22	18
	5.0	26	21	24
	1.5	16	9	13
DI Water w/S9		25	30	28
DI Water w/o S9		25	27	26
2-aminoanthracen w/ S9		158	161	160
Methylmethanesulfonate w/o S9		240	264	252
Historical Count Positive w/S9		**44-1118**		
Historical Count Positive w/o S9		**42-1796**		
Historical Count Negative w/S9		**8-80**		
Historical Count Negative w/o S9		**8-84**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

Dermal and Ocular Irritation Tests

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Sample: ABS White Willow Bark Extract

Code: 10230

CAS #: 84082-82-6

Test Request Form/Submission #: 341

Lot #: 28075P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Meghan Darley*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ABS White Willow Bark Extract** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be a **non-irritant**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

Dermal and Ocular Irritation Tests

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II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

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Dermal and Ocular Irritation Tests

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B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

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Dermal and Ocular Irritation Tests

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B. Tissue Viability Assay

The results are summarized in Figures 1 and 2. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

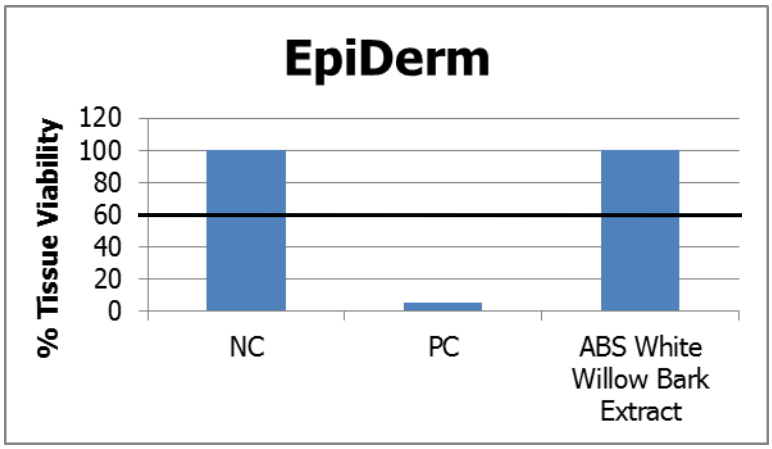

Figure 1: EpiDerm tissue viability

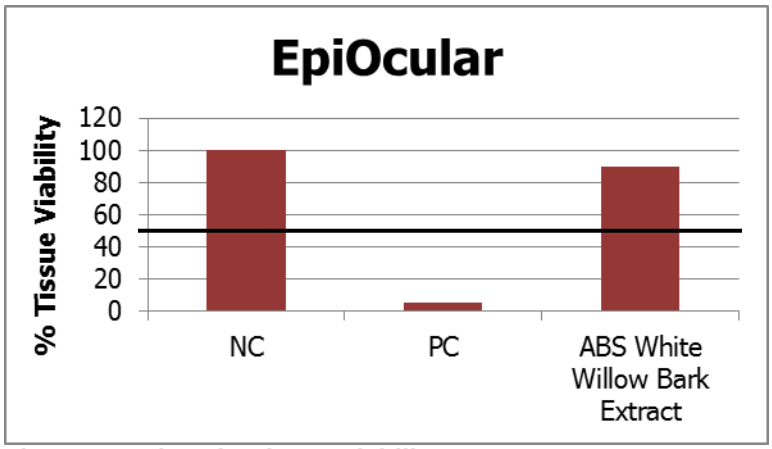

Figure 2: EpiOcular tissue viability

OECD TG 442C: *In Chemico* Skin Sensitization

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Tradename: ABS White Willow Bark Extract

Code: 10230

CAS #: 84082-82-6

Test Request Form #: 8715

Lot #: 8068400

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Daniel Shill*

Test Performed:
OECD TG 442C: *In Chemico* Skin Sensitization
Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact[1]. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis[2]. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)[3].

This assay was conducted to determine skin sensitization hazard of **ABS White Willow Bark Extract** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

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OECD TG 442C: *In Chemico* Skin Sensitization

Materials

A. Equipment:	HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
B. HPLC/Guard Columns:	Agilent Zorbax SB-C18 2.1mm x 100mm x 3.5µm; Phenomenex Security Guard C18 4mm x 2mm
C. Chemicals:	Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-COOH); Cinnamic aldehyde
D. Reagents/Buffers:	Sodium phosphate buffer (100mM); Ammonium acetate buffer (100mM)
E. Other:	Sterile disposable pipette tips

Methods

Solution Preparation:

- 0.667mM Cysteine Peptide in 100mM Phosphate Buffer (pH 7.5)
- 0.667mM Lysine Peptide in 100mM Ammonium Acetate Buffer (pH 10.2)
- 100mM Cinnamic Aldehyde in Acetonitrile
- 100mM* **ABS White Willow Bark Extract** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **ABS White Willow Bark Extract**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

Reference Controls:

- Reference Control A: For calibration curve accuracy
- Reference Control B: For peptide stability over analysis time of experiment
- Reference Control C: For verification that the solvent does not impact percent peptide depletion

Sample, Reference Control, and Co-Elution Control Preparation:

- Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
- Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5mM Peptide, 5mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5mM Peptide, 25mM Test Chemical
• 750µL Cysteine Peptide Solution (or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls) • 200µL Acetonitrile • 50µL Test Chemical Solution (or Acetonitrile for Reference Controls)	• 750µL Lysine Peptide Solution (or 100mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls) • 250µL Test Chemical Solution (or Acetonitrile for Reference Controls)

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Calibration Curve:

- Standards are prepared in a solution of 20% Acetonitrile:Buffer
  - For the Cysteine peptide using the phosphate buffer, pH 7.5
  - For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

HPLC Analysis:

- HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
- Absorbance is measured at 220nm
- Flow Conditions:

Time	Flow	%A	%B
0 minutes	0.35 mL/min	90	10
10 minutes	0.35 mL/min	75	25
11 minutes	0.35 mL/min	10	90
13 minutes	0.35 mL/min	10	90
13.5 minutes	0.35 mL/min	90	10
20 minutes	End Run		

Data and Reporting

Acceptance Criteria:

1. The following criteria must be met for a run to be considered valid:
   a. Standard calibration curve should have an $r^2 > 0.99$.
   b. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be <14.9 for the percent cysteine depletion and <11.6 for the percent lysine depletion.
   c. Mean peptide concentration of reference controls A should be 0.50±0.05mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be <15.0%.

2. The following criteria must be met for a test chemical's results to be considered valid:
   a. Maximum standard deviation should be <14.9 for percent cysteine depletion and <11.6 for percent lysine depletion.
   b. Mean peptide concentration of the three reference control C should be 0.50±0.05mM.

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Prediction Model:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Therefore the measured values of % depletion in the three separated runs for each peptide depletion assay include:

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.14	Minimal Reactivity	Non-sensitizer
3.16	Minimal Reactivity	Non-sensitizer
3.13	Minimal Reactivity	Non-sensitizer

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	**Reactivity Class**	**Prediction**
3.02	Minimal Reactivity	Non-sensitizer
2.99	Minimal Reactivity	Non-sensitizer
3.04	Minimal Reactivity	Non-sensitizer

Results and Discussion

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

Percent peptide depletion is determined by the following equation:

$$Percent\ Peptide\ Depletion = \left[1 - \left(\frac{Peptide\ Peak\ Area\ in\ Replicate\ Injection}{Mean\ Peptide\ Peak\ Area\ in\ Reference\ Controls\ C}\right)\right] \times 100$$

Based on HPLC-UV analysis of **ABS White Willow Bark Extract (10230)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.08% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD TG 442D: *In Vitro* Skin Sensitization

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Tradename: ABS White Willow Bark Extract

Code: 10230

CAS #: 84082-82-6

Test Request Form #: 8740

Lot #: 8068400

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
OECD TG 442D: In Vitro Skin Sensitization *ARE-Nrf2 Luciferase Test Method*

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals[1]. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **ABS White Willow Bark Extract** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013

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OECD TG 442D: *In Vitro* Skin Sensitization

Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
B. Equipment:	Humidified incubator; Biosafety laminar flow hood; Microplate Reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Phosphate Buffered Saline (PBS)
E. Culture Plate:	Flat bottom 96-well tissue culture treated plates
F. Reagents:	Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. Other:	Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM. Twelve test concentrations of **ABS White Willow Bark Extract** were prepared in DMSO with a concentration range from 0.98 - 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64 µM. The negative control was a 1% test concentration of DMSO.

24 hour post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO_2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO_2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC_{50} and IC_{30} values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and $EC_{1.5}$ and maximum response (I_{max}) values were obtained.

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Data and Reporting

Acceptance Criteria:

1. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
2. The EC1.5 value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
3. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

A KeratinoSens™ prediction is considered positive if the following conditions are met:

1. The Imax is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
2. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the EC1.5 determining concentration)
3. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
4. There is an apparent overall dose-response for luciferase induction

Results

Compound	Classification	$EC_{1.5}$ (µM)	IC_{50}	I_{max}
Cinnamic aldehyde	Sensitizer	19	289.19 µM	32.60
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.18
ABS White Willow Bark Extract	Non-Sensitizer	No Induction	> 1000 µM	0.33

Table 1: Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values Fg.1)

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OECD TG 442D: *In Vitro* Skin Sensitization

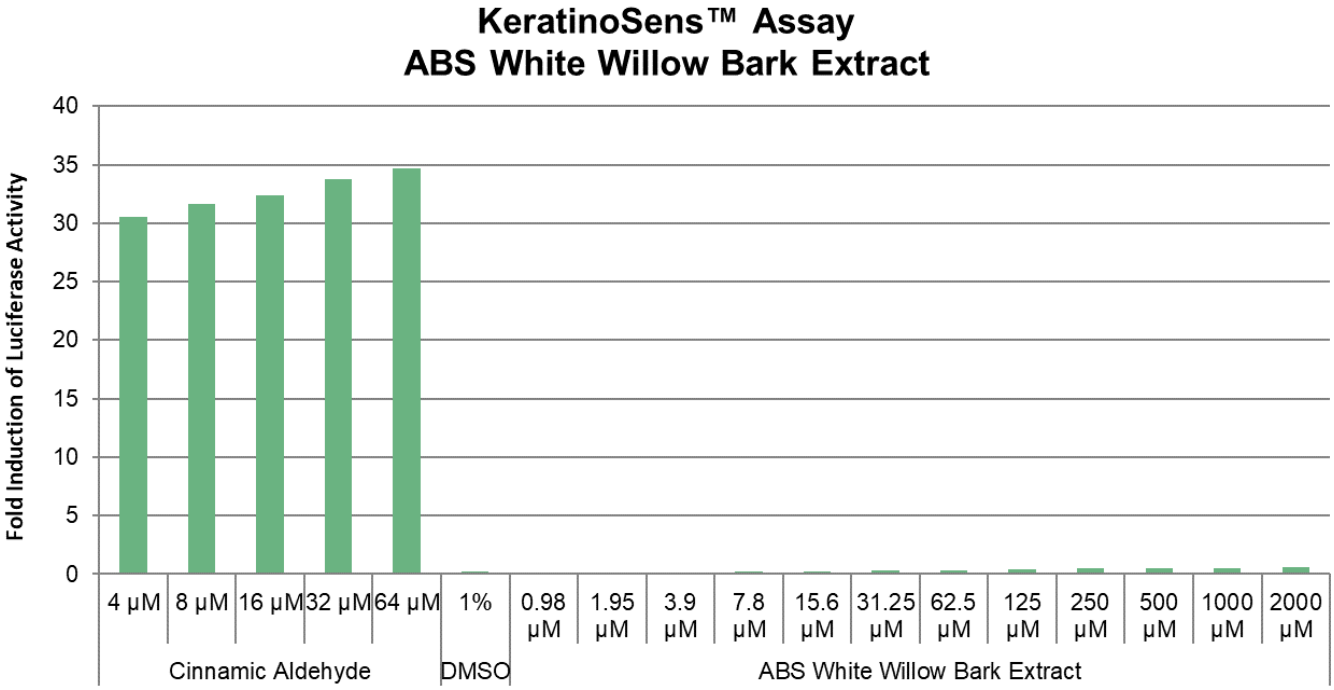

Figure 1: Fold Induction of Luciferase

Discussion

As shown in the results, **ABS White Willow Bark Extract (10230)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **ABS White Willow Bark Extract** can be safely used in cosmetics and personal care products at typical use levels.

Phototoxicity Assay Analysis

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Tradename: ABS White Willow Bark Extract

Code: 10230

CAS #: 84082-82-6

Test Request Form #: 8741

Lot #: 8068400

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In Vitro EpiDerm™ Model (EPI-200-SIT) Phototoxicity

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **ABS White Willow Bark Extract** would induce phototoxic irritation in the EpiDerm™ model assay.

The product was tested according to the manufacturer's protocol. The test article solution was found to be a non-photoirritant at concentrations of 0.5%, 1.5%, 5.0% and 10.0%. Reconstructed human epidermis was incubated in growth media for one hour to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substance was applied to the tissue inserts in four varying concentrations and incubated overnight at 37°C, 5% CO_2, and 95% relative humidity (RH). The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, the cell viability test was conducted. Cell viability was measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that was measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues.

Under the conditions of this assay, the test article was considered to be non-phototoxic at concentrations of 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

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Phototoxicity Assay Analysis

I. Introduction

A. Purpose

In vitro dermal phototoxicity study was conducted to evaluate whether a test article would induce photoirritation in the EpiDerm™ model assay. MatTek Corporation's reconstructed human epidermal model is becoming a standard in determining the phototoxicity potential of a test substance. This assay is able to discriminate between photoirritants and non-photoirritants at varying concentrations.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; UVA-vis Irradiation Equipment; UVA meter; Pipettes
C. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM) based medium; Dulbecco's Phosphate-Buffered Saline (DPBS); sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, and 6-well tissue culture plates
F. Reagents:	MTT (*3-4,5-dimethyl thiazole 2-yl*) (1.0mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1%)
G. Other:	Wash bottle; sterile disposable pipette tips; Parafilm; forceps

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™ consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis. This model consists of organized basal, spinous, and granular layers, and contains a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile deionized water is used as the negative controls for the EpiDerm™ Phototoxicity assay.

C. Positive Control

Concentrations of chloropromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.

D. Data Interpretation Procedure

A photoirritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance and 60 minutes of 6 J/cm^2 is reduced by 20% compared to the non-irradiated control tissues.

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Phototoxicity Assay Analysis

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and tissue insert dosing begins.

B. Test Substance Exposure

50µL of the diluted test substance in their respective concentrations are applied to 2 tissue inserts and allowed to incubate for overnight in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Irradiation

Tissue inserts in their 6-well plates are UVA-irradiated for 60 minutes with 6 J/cm^2 at room temperature. The non-irradiated tissue inserts are incubated at room temperature in the dark.

D. Tissue Washing and Post Incubation

After UVA-irradiation and dark incubation is complete the tissue inserts are washed using sterile DPBS and transferred to fresh 6-well plates and media for overnight incubation at 37°C, 5% CO_2, 95% RH.

E. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 0.8.

B. Positive Control

The assay meets the acceptance criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.

C. Standard Deviation

Since the phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

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Phototoxicity Assay Analysis

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ assay kit were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. Cell viability is calculated for each tissue as a percentage of the corresponding vehicle control either irradiated or non-irradiated. Tissue viability was not reduced by 20% in the presence of the test substance and UVA-irradiation at concentrations of 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

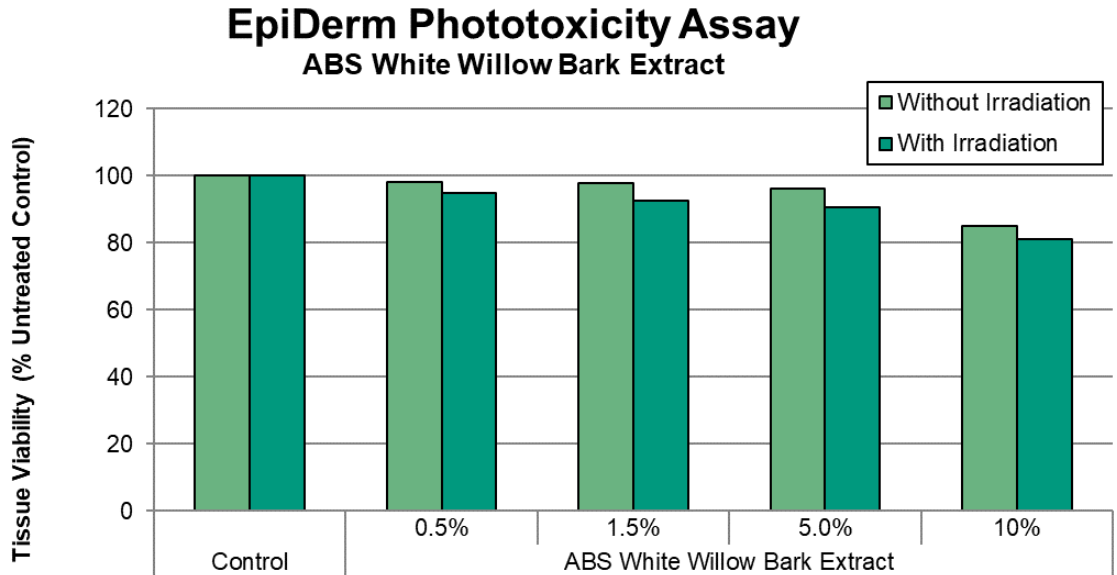
C. Test Validity

The data obtained from this study met criteria for a valid assay. The negative and positive controls performed as anticipated.

VII. Conclusion

Phototoxicity (photoirritation) is defined as an acute toxic response that is elicited after exposure of the skin to certain chemicals and subsequent exposure to light. Under the conditions of this assay, the test article substance was considered to be non-phototoxic at concentrations of 0.5%, 1.5%, 5.0%, and 10.0%.The negative and positive controls performed as anticipated.

There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that **ABS White Willow Bark Extract** is not a photoirritant when used at the suggested use levels of 1.0% - 10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	98.1	97.9	96.2	85.1
With Irradiation	100	94.8	92.7	90.6	81.2

Figure 1: EpiDerm Phototoxicity Graph

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ACB White Willow Bark Extract 20%
Code: 20199

Compositional Breakdown:

Ingredient	%
Salix Alba (Willow) Bark Extract	100.00

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the potential fragrance allergens listed below are present in our finished product or as an intentional component in the raw materials used to manufacture this product. We do not routinely analyze our product for the substances listed below:

ALLERGENS listed in Annex III of Commission Regulation (EU) 2023/1545 Amending Regulation (EC) No. 1223/2009	
Chemical/INCI NAME	CAS NUMBER
3-Propylidenephthalide	17369-59-4
6-Methyl Coumarin	92-48-8
Acetyl Cedrene	32388-55-9
Alpha-Damascone; Cis-Rose Ketone 1; Trans-Rose Ketone 1; Rose Ketone 4 (Damascone); Rose Ketone 3 (Delta-Damascone); Trans-Rose Ketone 3; Cis-Rose Ketone 2 (Cis-Beta-Damascone); Trans-Rose Ketone 2 (Trans-Beta Damascone)	43052-87-5; 23726-94-5; 23696-85-7; 57378-68-4; 71048-82-3; 23726-92-3; 23726-91-2
Alpha-Isomethyl Ionone	127-51-5
Alpha-Terpinene	99-86-5
Amyl Cinnamal	122-40-7
Amyl Salicylate	2050-08-0
Amylcinnamyl Alcohol	101-85-9
Anethole	104-46-1; 4180-23-8
Anise Alcohol	105-13-5
Benzaldehyde	100-52-7
Benzyl Alcohol	100-51-6
Benzyl Benzoate	120-51-4
Benzyl Cinnamate	103-41-3
Benzyl Salicylate	118-58-1
Beta-Caryophyllene	87-44-5
Camphor	76-22-2; 21368-68-3; 464-49-3; 464-48-2
Cananga Odorata Flower Oil and Extract	83863-30-3; 8006-81-3; 68606-83-7; 93686-30-7
Carvone	99-49-0; 6485-40-1; 2244-16-8
Cedrus Atlantica Bark or Wood Oil; Cedrus Atlantica Bark, Leaf or Wood Extract; Cedrus Atlantica Bark Water	92201-55-3; 8023-85-6
Cinnamal	104-55-2
Cinnamomum Cassia Leaf Oil	84961-46-6; 8007-80-5
Cinnamomum Zeylanicum Bark Oil	84649-98-9; 8015-91-6
Cinnamyl Alcohol	104-54-1
Citral	5392-40-5
Citronellol	106-22-9; 26489-01-0; 1117-61-9; 7540-51-4
Citrus Aurantium Amara and Dulcis Flower Oil	72968-50-4; 8028-48-6; 8016-38-4
Citrus Aurantium Amara and Dulcis Peel Oil	68916-04-1; 72968-50-4; 97766-30-8; 8028-48-6; 8008-57-9

Compositional Breakdown

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Chemical/INCI NAME	CAS NUMBER
Citrus Aurantium Bergamia Peel Oil	8007-75-8; 89957-91-5; 68648-33-9; 8007-75-8; 85049-52-1
Citrus Limon Peel Oil	84929-31-7; 8008-56-8
Coumarin	91-64-5
Cymbopogon Schoenanthus, Flexuosus and Citratus (Lemongrass) Oil	8007-02-1; 89998-16-3; 91844-92-7; 91844-92-7
Delta-Damascone	57378-68-4
Dimethyl Phenethyl Acetate	151-05-3
Eucalyptus Globulus Leaf or Leaf/Twig Oil	97926-40-4; 8000-48-4
Eugenia Caryophyllus Leaf, Flower, Stem or Bud Oil	8000-34-8; 8015-97-2; 84961-50-2
Eugenol	97-53-0
Eugenyl Acetate	93-28-7
Evernia Furfuracea (Treemoss) Extract	90028-67-4
Evernia Prunastri (Oakmoss) Extract	90028-68-5
Farnesol	4602-84-0
Geranial	141-27-5
Geraniol	106-24-1
Geranyl Acetate	105-87-3
Hexadecanolactone	109-29-5
Hexamethylindanopyran	1222-05-5
Hexyl Cinnamal	101-86-0
Hydroxycitronellal	107-75-5
Isoegenyl Acetate	93-29-8
Isoeugenol	97-54-1; 5932-68-3; 5912-86-7
Jasminum Grandiflorum and Officinale Flower Oil and Extract	84776-64-7; 90045-94-6; 8022-96-6; 8024-43-9; 90045-94-6
Juniperus Virginiana Oil	8000-27-9; 85085-41-2
Laurus Nobilis Leaf Oil	8002-41-3; 8007-48-5; 84603-73-6
Lavandula Hybrida Oil and Extract; Lavandula Intermedia or Angustifolia Flower, Leaf, or Stem Oil and Extract	91722-69-9; 8022-15-9; 93455-96-0; 93455-97-1; 92623-76-2; 84776-65-8; 8000-28-0; 90063-37-9
Limonene	5989-27-5; 138-86-3; 5989-54-8; 7705-14-8
Linalool	78-70-6
Linalyl Acetate	115-95-7
Lippia Citriodora Absolute	8024-12-2; 85116-63-8
Mentha Piperita Oil	8006-90-4; 84082-70-2
Mentha Viridis Leaf Oil	8008-79-5; 84696-51-5
Menthol	89-78-1; 1490-04-6; 2216-51-5; 15356-60-2
Methyl 2-Octynoate	111-12-6
Methyl N-methylanthranilate	85-91-6

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Chemical/INCI NAME	CAS NUMBER
Methyl Salicylate	119-36-8
Myroxylon Balsamum Pereirae Balsam Oil, Resin and Extract	8007-00-9
Narcissus Poeticus, Jonquilla and Tazetta Extract; Narcissus Pseudonarcissus Flower Extract	90064-26-9; 68917-12-4; 90064-25-8; 90064-27-0
Neral	106-26-3
Pelargonium Graveolens Flower Oil	90082-51-2; 8000-46-2
Pinene	80-56-8; 7785-70-8; 127-91-3; 18172-67-3
Pinus Mugo Leaf or Twig Oil and Extract	90082-72-7
Pinus Pumila Twig Leaf Oil and Extract	97676-05-6
Pogostemon Cablin Oil	8014-09-3; 84238-39-1
Rosa Damascena, Alba or Centrifolia Flower Oil and Extract; Rosa Canina, Gallica, Moschata or Rugosa Flower Oil	8007-01-0; 90106-38-0; 93334-48-6; 84604-12-6; 84696-47-9; 84604-13-7; 92347-25-6
Salicylaldehyde	90-02-8
Santalol	115-71-9; 11031-45-1; 77-42-9
Santalum Album Oil	8006-87-9; 84787-70-2
Sclareol	515-03-7
Terpineol	8000-41-7; 98-55-5; 138-87-4; 586-81-2
Terpinolene	586-62-9
Tetramethyl Acetyloctahydronaphthalenes	54464-57-2; 54464-59-4; 68155-66-8; 68155-67-9
Trimethylbenzenepropanol	103694-68-4
Trimethylcyclopentenyl Methylisopentenol/Ebanol	67801-20-1
Turpentine (Gum, Oil, Steam Distilled)	9005-90-7; 8006-64-2; 8052-14-0
Vanillin	121-33-5

*Any ingredient at > 0.001% in leave-on product must be included on the Finished Product ingredients list.
*Any ingredient at > 0.01% in rinse-off product must be included on the Finished Product ingredients list.

Compositional Breakdown

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Active Concepts hereby confirms that to the best of our knowledge, none of the pesticides listed below are present in our finished product or as an intentional component in the raw material used to manufacture this product. We do not routinely analyze our product for the substances listed below:

INCI NAME	CAS Number
Alachlor	15972-60-8
Aldrin	309-00-2
Azinphos-methyl	86-50-0
Bromopropylate	18181-80-1
Chlordane (cis and trans)	57-74-9
Chlorfenvinphos	470-90-6
Chlorpyrifos	2921-88-2
Chlorpyrifos-methyl	5598-13-0
Cypermethrin	52315-07-8
DDT	50-29-3
Deltamethrin	52918-63-5
Diazinon	333-41-5
Dichlorvos	62-73-7
Dieldrin	50-57-1
Dithiocarbamates	142-84-7
Endosulfan	115-29-7
Endrin	72-20-8
Ethion	563-12-2
Fenitrothion	122-14-5
Fenvalerate	51630-58-1
Fonofos	944-22-9
Heptachlor	76-44-8
Hexachlorobenzene	118-74-1
Hexachlorocyclohexane	608-73-1
Lindane	58-89-9
Malathion	121-75-5
Methidathion	950-37-8
Parathion	56-38-2
Parathion-methyl	298-00-0
Permethrin	52645-53-1
Phosalone	2310-17-0
Piperonyl butoxide	51-03-6
Pirimiphos-methyl	29232-93-7
Pyrethrins	8003-34-7
Quintozene (sum of 3 items)	82-68-8

Manufacturing Flow Chart

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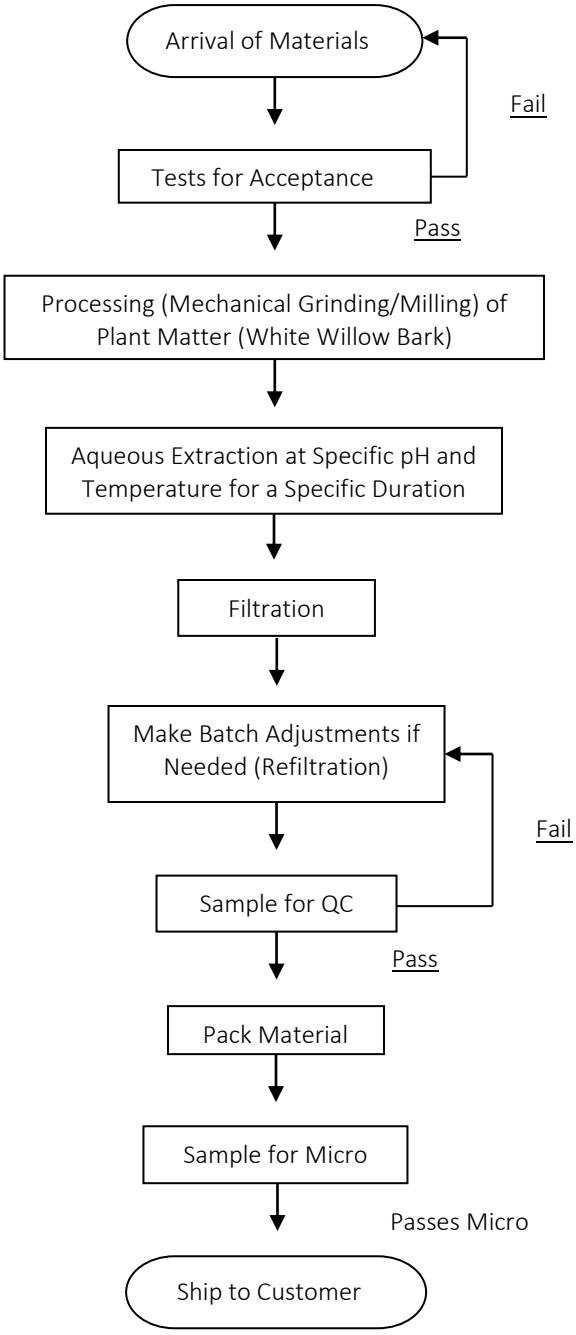
ACB White Willow Bark Extract 20% (20199)



Version#2/07.27.10/Form#245

Product Specification

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Product Name: ACB White Willow Bark Extract 20%
Code Number: 20199
CAS #'s: 84082-82-6
EINECS #'s: 282-029-0
INCI Name: Salix Alba (Willow) Bark Extract
Status: Approved

Specification	Parameter
Appearance	Clear Colorless to Light Amber Liquid
Odor	Characteristic
pH (direct)	4.0 – 6.5
NVM (1g, 105°C, 1hr)	> 25%
Ash (600°)	0.50% Maximum
Salicyns (Salicylic Acid (UV-vis))	18.0 – 22.0%
Identification (FTIR)	TMS
Heavy Metals	< 20 ppm
Lead	< 10 ppm
Arsenic	< 2 ppm
Cadmium	< 1 ppm
Microbial Content Yeast & Mold Gram Negative Bacteria	< 100 CFU/g; No pathogens < 100 CFU/g 0 CFU/g

May Sediment upon Standing; Mix Well Prior to Use
DO NOT FREEZE; Store at or Near Room Temperature

**Note: Product may change appearance if exposed to cold temperatures during shipment or storage. If this happens, please gently warm to 45-50°C and mix until normal appearance is restored.

Bacterial Reverse Mutation Test

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Test Article: ACB White Willow Bark Extract 20%
Code Number: 20199
CAS #: 84082-82-6

Sponsor:
Active Concepts, LLC
107 Technology Drive
Lincolnton, NC 28092

Study Director: *Maureen Danaher*
Principle Investigator: *Monica Beltran*

Test Performed:
Genotoxicity: Bacterial Reverse Mutation Test

Reference:
OECD471/ISO10993.Part3

Test Request Number: 3220

SUMMARY

A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study described by Ames *et al.* (1975) was conducted to evaluate whether a test article solution **ACB White Willow Bark Extract 20%** would cause mutagenic changes in the average number of reveratants for histidine-dependent *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535 and tryptophan-dependent *Escherichia coli* strain WP2*uvr*A in the presence and absence of Aroclor-induced rat liver S9. This study was conducted to satisfy, in part, the Genotoxicity requirement of the International Organization for Standardization: Biological Evaluation of Medical Devices, Part 3: Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.

The stock test article was tested at eight doses levels along with appropriate vehicle control and positive controls with overnight cultures of tester strains. The test article solution was found to be noninhibitory to growth of tester strain TA98, TA100, TA1537, TA1535 and WP2*uvr*A after Sport Inhibition Screen.

Separate tubes containing 2 ml of molten top agar at 45^0C supplemented with histidine-biotin solution for the *Salmonella typhimurium* strains and supplemented with tryptophan for *Escherichia coli* strain were inoculated with 100 µl of tester strains, 100 µl of vehicle or test article dilution were added and 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. After vortexing, the mixture was poured across the Minimal Glucose Agar (GMA) plates. Parallel testing was also conducted with positive control correspond to each strain, replacing the test article aliquot with 50µl aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for 48 hours at 37^0C. The mean numbers of revertants of the test plates were compared to the mean number of revertants of the negative control plates for each of the strains tested. The means obtained for the positive controls were used as points of reference.

Under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* tester strain WP2*uvr*A. The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

Bacterial Reverse Mutation Test

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I. Introduction

A. **Purpose**
A *Salmonella typhimurium/Escherichia coli* reverse mutation standard plate incorporation study was conducted to evaluate whether a test article solution would cause mutagenic changes in the average number of revertants for *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A in the presence and absences of the S9 metabolic activation. Bacterial reverse mutation tests have been widely used as rapid screening procedures for the determination of mutagenic and potential carcinogenic hazards.

II. Materials

A. **Storage Conditions:** Room temperature (23-25C).
B. **Vehicle:** Sterile DI Water.
C. **Preparation:** Eight different doses level were prepared immediately before use with sterile DI water.
D. **Solubility/Stability:** 100% Soluble and Stable.
E. **Toxicity:** No significant inhibition was observed.

III. Test System

A. Test System
Each *Salmonella typhimurium* and *Escherichia coli* tester strain contains a specific deep rough mutation (*rfa)*, the deletion of *uvr*B gene and the deletion in the *uvr*A gene that increase their ability to detect mutagens, respectively. These genetically altered *Salmonella typhimurium* strains (TA98, TA100, TA1537 and TA1535) and *Escherichia coli* strain (WP2*uvr*A) cannot grow in the absence of histidine and tryptophan, respectively. When placed in a histidine-tryptophan free medium, only those cells which mutate spontaneously back to their wild type states are able to form colonies. The spontaneous mutation rate (or reversion rate) for any one strain is relatively constant, but if a mutagen is added to the test system, the mutation rate is significantly increased.

Tester strain	Mutations/Genotypic Relevance
TA98	hisD3052, Dgal chlD bio *uvr*B *rfa* pKM101
TA100	hisG46, Dgal chlD BIO *uvr*B *rfa* pKM101
TA1537	hisC3076, *rfa*, Dgal chlD bio *uvrB*
TA 1535	hisG46, Dgal chlD bio *uvr*B *rfa*
WP2*uvr*A	trpE, *uvr*A

rfa	=	causes partial loss of the lip polysaccharide wall which increases permeability of the cell to large molecules.
uvrB	=	deficient DNA excision-repair system (i.e., ultraviolet sensitivity)
pKM101	=	plasmid confers ampicillin resistance (R-factor) and enhances sensitivity to mutagens.
*uvr*A	=	All possible transitions and transversions, small deletions.

B. Metabolic Activation
Aroclor induced rat liver (S9) homogenate was used as metabolic activation. The S9 homogenate is prepared from male Sprague Dawley rats. Material is supplied by MOLTOX, Molecular Toxicology, Inc.

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C. Preparation of Tester strains
Cultures of *Salmonella typhimurium* TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2uvrA were inoculated to individual flasks containing Oxoid broth No.2. The inoculated broth cultures were incubated at 37°C in an incubator shaker operating at 140-150 rpm for 12-16 hours.

D. Negative Control
Sterile DI water (vehicle without test material) was tested with each tester strain to determine the spontaneous reversion rate. Each strain was tested with and without S9 activation. These data represented a base rate to which the number of reveratants colonies that developed in each test plate were compared to determine whether the test material had significant mutagenic properties.

E.Positive Control
A known mutagen for each strain was used as a positive control to demonstrate that tester strains were sensitive to mutation to the wild type state. The positive controls are tested with and without the presence of S9 homogenate.

F. Titer of the Strain Cultures:
Fresh cultures of bacteria were grown up to the late exponential or early stationary phase of growth; to confirm this, serial dilutions from each strain were conducted, indicating that the initial population was in the range of 1 to $2x10^9$/ml.

IV. Method

A. Standard Plate Incorporation Assay:
Separate tubes containing 2 ml of molten top agar supplemented with histidine-biotin solution for the *Salmonella typhimurium* and tryptophan for *Escherichia coli* were inoculated with 100 µl of culture for each strain and 100 µl of testing solution or vehicle without test material. A 500 µl aliquot of S9 homogenate, simulating metabolic activation, was added when necessary. The mixture was poured across Minimal Glucose Agar plates labeled with strain number and S9 activation (+/-). When plating the positive controls, the test article aliquot was replaced by 50µl aliquot of appropriate positive control. The test was conducted per duplicate. The plates were incubated for 37°C for 2 days. Following the incubation period, the revertant colonies on each plate were recorded. The mean number of reveratnts was determined. The mean numbers of revertants of the test plates were compared to the mean number of reverants of the negative control of each strain used.

V. Evaluation

For the test solution to be evaluated as a test failure or “potential mutagen” there must have been a 2-fold or greater increase in the number of mean revertants over the means obtained from the negative control for any or all strains. Each positive control mean must have exhibited at least a 3-fold increase over the respective negative control mean of the *Salmonella* and *Escherichia coli* tester strain used.

VI. Results and Discussion

A. Solubility:
Water was used as a solvent. Solutions from the test article were made from 0.015 to 50mg/ml.

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B. Dose levels tested:
The maximum dose tested was 5000 µg per plate. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate.

C. Titer (Organisms/ml):
5 x 10^8 UFC/ml plate count indicates that the initial population was in the range of 1 to 2 x 10^9 UFC/ml.

C. Standard Plate Incorporation Assay
In no case was there a 2-fold or greater increase in the mean number of revertant testing strains TA98, TA100, TA1537, TA1535 and WP2*uvr*A in the presence of the test solution compared with the mean of vehicle control value. The positive controls mean exhibited at least a 3-fold increase over the respective mean of the *Salmonella typhimurium* and *Escherichia coli* tester strains used. The results are summarized in Appendix 2.

VII. Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that under the conditions of this assay, the test article solution was considered to be Non-Mutagenic to *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and *Escherichia coli* WP2*uvr*A.The negative and positive controls performed as anticipated. The results of this study should be evaluated in conjunction with other required tests as listed in ISO 100993, Part 3: Tests for Genotoxicity, Carcinogenicity, and Reproductive Toxicology.

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Bacterial Reverse Mutation Test

Appendix 2:

Bacterial Mutation Assay
Plate Incorporation Assay Results

	Concentration µg per Plate	*TA98*		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	18	22	20
	1500	24	33	29
	500	38	48	43
	150	30	57	44
	50	41	36	39
	15	39	56	48
	5.0	37	39	38
	1.5	29	46	38
Test Solution w/o S9	5000	9	19	14
	1500	38	29	34
	500	48	28	38
	150	46	44	45
	50	29	36	33
	15	42	36	39
	5.0	67	47	57
	1.5	47	33	40
DI Water w/S9		48	34	41
DI Water w/o S9		44	39	42
2-aminoanthracen w/ S9		380	347	364
2-nitrofluorene w/o S9		178	137	158
Historical Count Positive w/S9		**43-1893**		
Historical Count Positive w/o S9		**39-1871**		
Historical Count Negative w/S9		**4-69**		
Historical Count Negative w/o S9		**3-59**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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Bacterial Reverse Mutation Test

	Concentration µg per Plate	***TA100***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	28	27	28
	1500	105	107	106
	500	100	95	98
	150	104	105	105
	50	86	93	90
	15	81	84	83
	5.0	91	99	95
	1.5	105	124	115
Test Solution w/o S9	5000	11	10	11
	1500	48	69	59
	500	91	97	94
	150	94	102	98
	50	86	107	97
	15	100	84	92
	5.0	100	84	92
	1.5	102	89	96
DI Water w/S9		94	124	109
DI Water w/o S9		85	108	97
2-aminoanthracen w/ S9		812	813	813
Sodium azide w/o S9		688	634	661
Historical Count Positive w/S9		**224-3206**		
Historical Count Positive w/o S9		**226-1837**		
Historical Count Negative w/S9		**55-268**		
Historical Count Negative w/o S9		**47-250**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

Bacterial Reverse Mutation Test

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	Concentration µg per Plate	***TA1537***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	6	0	3
	1500	8	14	11
	500	10	4	7
	150	6	6	6
	50	11	5	8
	15	4	4	4
	5.0	8	6	7
	1.5	4	3	4
Test Solution w/o S9	5000	4	1	3
	1500	4	1	3
	500	1	10	6
	150	11	6	9
	50	1	6	4
	15	8	6	7
	5.0	5	5	5
	1.5	5	4	5
DI Water w/S9		4	6	5
DI Water w/o S9		5	5	5
2-aminoanthracen w/ S9		58	51	55
2-aminoacridine w/o S9		530	471	501
Historical Count Positive w/S9		**13-1934**		
Historical Count Positive w/o S9		**17-4814**		
Historical Count Negative w/S9		**0-41**		
Historical Count Negative w/o S9		**0-29**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

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	Concentration µg per Plate	***TA1535***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	3	3	3
	1500	19	18	19
	500	19	18	19
	150	11	14	13
	50	11	19	15
	15	9	10	10
	5.0	14	19	17
	1.5	10	20	15
Test Solution w/o S9	5000	8	0	4
	1500	0	4	2
	500	10	6	8
	150	11	20	16
	50	9	5	7
	15	5	14	10
	5.0	4	14	9
	1.5	5	6	6
DI Water w/S9		13	15	14
DI Water w/o S9		6	11	9
2-aminoanthracen w/ S9		86	95	91
Sodium azide w/o S9		541	601	571
Historical Count Positive w/S9		**22-1216**		
Historical Count Positive w/o S9		**47-1409**		
Historical Count Negative w/S9		**1-50**		
Historical Count Negative w/o S9		**1-45**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

Bacterial Reverse Mutation Test

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	Concentration µg per Plate	***WP2uvrA***		
		Revertants per plate (CFU)		Mean
Test Solution w/ S9	5000	27	30	29
	1500	41	29	35
	500	20	33	27
	150	48	33	41
	50	33	20	27
	15	32	32	32
	5.0	37	41	39
	1.5	37	23	30
Test Solution w/o S9	5000	19	32	26
	1500	18	36	27
	500	22	20	21
	150	22	27	25
	50	30	24	27
	15	29	19	24
	5.0	33	25	29
	1.5	28	33	31
DI Water w/S9		17	25	21
DI Water w/o S9		30	24	27
2-aminoanthracen w/ S9		130	11	121
Methylmethanesulfonate w/o S9		258	271	265
Historical Count Positive w/S9		**44-1118**		
Historical Count Positive w/o S9		**42-1796**		
Historical Count Negative w/S9		**8-80**		
Historical Count Negative w/o S9		**8-84**		

*CFU = Colony Forming Units
*Mean = Average of duplicate plates

Dermal and Ocular Irritation Tests

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Sample: ACB White Willow Bark Extract 20%

Code: 20199

CAS #: 84082-82-6

Test Request Form/Submission #: 3465

Lot #: 53186P

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Erica Segura*
Principle Investigator: *Maureen Danaher*

Test Performed:
In Vitro EpiDerm™ Dermal Irritation Test (EPI-200-SIT)
EpiOcular™ Eye Irritation Test (OCL-200-EIT)

SUMMARY

In vitro dermal and ocular irritation studies were conducted to evaluate whether **ACB White Willow Bark Extract 20%** would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays.

The product was tested according to the manufacture's protocol. The test article solution was found to be **non-irritating**. Reconstructed human epidermis and cornea epithelial model were incubated in growth media overnight to allow for tissue equilibration after shipping from MatTek Corporation, Ashland, MA. Test substances were applied to the tissue inserts and incubated for 60 minutes for liquid and solid substances in the EpiDerm™ assay and 30 minutes for liquid substances and 90 minutes for solid substances in the EpiOcular™ assay at 37°C, 5% CO_2, and 95% relative humidity (RH). Tissue inserts were thoroughly washed and transferred to fresh plates with growth media. After post substance dosing incubation is complete, the cell viability test begins. Cell viability is measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that is measured after extraction from the tissue. The irritation potential of the test chemical is dictated by the reduction in tissue viability of exposed tissues compared to the negative control.

Under the conditions of this assay, the test article was considered to be **non-irritant**. The negative and positive controls performed as anticipated.

Dermal and Ocular Irritation Tests

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I. Introduction

A. Purpose

In vitro dermal and ocular irritation studies were conducted to evaluate whether a test article would induce dermal or ocular irritation in the EpiDerm™ and EpiOcular™ model assays. MatTek Corporation's reconstructed human epidermal and human ocular models are becoming a standard in determining the irritancy potential of test substances. They are able to discriminate between irritants and non-irritants. The EpiDerm™ assay has accuracy for the prediction of UN GHS R38 skin irritating and no-label (non-skin irritating) test substances. The EpiOcular™ assay can differentiate chemicals that have been classified as R36 or R41 from the EU classifications based on Dangerous Substances Directive (DSD) or between the UN GHS Cat 1 and Cat 2 classifications.

II. Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Media/Buffers:	DMEM based medium; DPBS; sterile deionized H_2O
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, 12-well, and 6-well tissue culture plates
F. Reagents:	MTT (1.0mg/mL); Extraction Solution (Isopropanol); SDS (5%); Methyl Acetate
G. Other:	Nylon Mesh Circles (EPI-MESH); Cotton tip swabs; 1mL tuberculin syringes; Ted Pella micro-spatula; 220mL specimen containers; sterile disposable pipette tips; Parafilm

III. Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™, and cornea epithelial model, EpiOcular™, consist of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis and cornea epithelium. These models consist of organized basal, spinous, and granular layers, and the EpiDerm™ systems also contains a multilayer stratum corneum containing intercellular lamellar lipid layers that the EpiOcular™ system is lacking. Both the EpiDerm™ and EpiOcular™ tissues are cultured on specially prepared cell culture inserts.

B. Negative Control

Sterile DPBS and sterile deionized water are used as negative controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

C. Positive Control

Known dermal and eye irritants, 5% SDS solution and Methyl Acetate, were used as positive controls for the EpiDerm™ and EpiOcular™ assays, respectfully.

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D. Data Interpretation Procedure

a. EpiDerm™

An irritant is predicted if the mean relative tissue viability of the 3 tissues exposed to the test substance is reduced by 50% of the mean viability of the negative controls and a non-irritant's viability is > 50%.

b. EpiOcular™

An irritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance is reduced by 60% of the mean viability of the negative controls and a non-irritant's viability is > 40%.

IV. Method

A. Tissue Conditioning

Upon MatTek kit arrival at Active Concepts, LLC the tissue inserts are removed from their shipping medium and transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for 60 minutes. After those 60 minutes the inserts are transferred into fresh media and tissue culture plates and incubated at 37°C at 5% CO_2 and 95% relative humidity for an additional 18 to 21 hours.

B. Test Substance Exposure

a. EpiDerm™

30µL (liquid) or 25mg (solid) of the undiluted test substance is applied to 3 tissue inserts and allowed to incubate for 60 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

b. EpiOcular™

Each tissue is dosed with 20µL DPBS prior to test substance dosing. 50µL (liquid) or 50mg (solid) of the undiluted test substance is applied to 2 tissue inserts and allowed to incubate for 90 minutes in a humidified incubator (37°C, 5% CO_2, 95% RH).

C. Tissue Washing and Post Incubation

a. EpiDerm™

All tissue inserts are washed with DPBS, dried with cotton tipped swab, and transferred to fresh media and culture plates. After 24 hours the inserts are again transferred into fresh media and culture plates for an additional 18 to 20 hours.

b. EpiOcular™

Tissue inserts are washed with DPBS and immediately transferred into 5mL of assay medium for 12 to 14 minutes. After this soak the inserts are transferred into fresh media and tissue culture plates for 120 minutes for liquid substances and 18 hours for solid substances.

D. MTT Assay

Tissue inserts are transferred into 300µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Inserts are then removed from the MTT medium and placed in 2mL of the extraction solution. The plate is sealed and incubated at room temperature in the dark for 24 hours. After extraction is complete the tissue inserts are pierced with forceps and 2 x 200µL aliquots of the blue formazan solution is transferred into a 96 well plate for Optical Density reading. The spectrophotometer reads the 96-well plate using a wavelength of 570 nm.

V. Acceptance Criterion

A. Negative Control

The results of this assay are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 1.0 and ≤ 2.5 (EpiDerm™) or ≥ 1.0 and ≤ 2.3 (EpiOcular™).

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Dermal and Ocular Irritation Tests

B. Positive Control

a. EpiDerm™

The assay meets the acceptance criterion if the mean viability of positive control tissues expressed as a % of the negative control is ≤ 20%.

b. EpiOcular™

The assay meets the acceptance criterion if the mean viability of positive control tissues is < 60% of control viability.

C. Standard Deviation

Since each irritancy potential is predicted from the mean viability of 3 tissues for EpiDerm™ and 2 tissues for EpiOcular™, the variability of the replicates should be < 18% for EpiDerm™ and < 20% EpiOcular™.

VI. Results

A. Tissue Characteristics

The tissue inserts included in the MatTek EpiDerm™ and EpiOcular™ assay kits were in good condition, intact, and viable.

B. Tissue Viability Assay

The results are summarized in Figure 1. In no case was the tissue viability ≤ 50% for EpiDerm™ or ≤ 60% for EpiOcular™ in the presence of the test substance. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited substantial loss of tissue viability and cell death.

C. Test Validity

The data obtained from this study met criteria for a valid assay.

VII. Conclusion

Under the conditions of this assay, the test article substance was considered to be **non-irritating**. The negative and positive controls performed as anticipated.

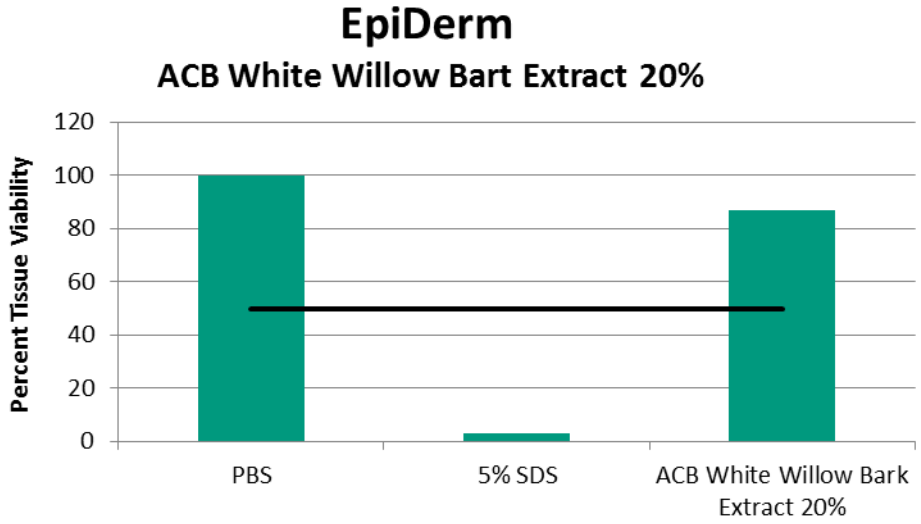

Figure 1: EpiDerm tissue viability

Dermal and Ocular Irritation Tests

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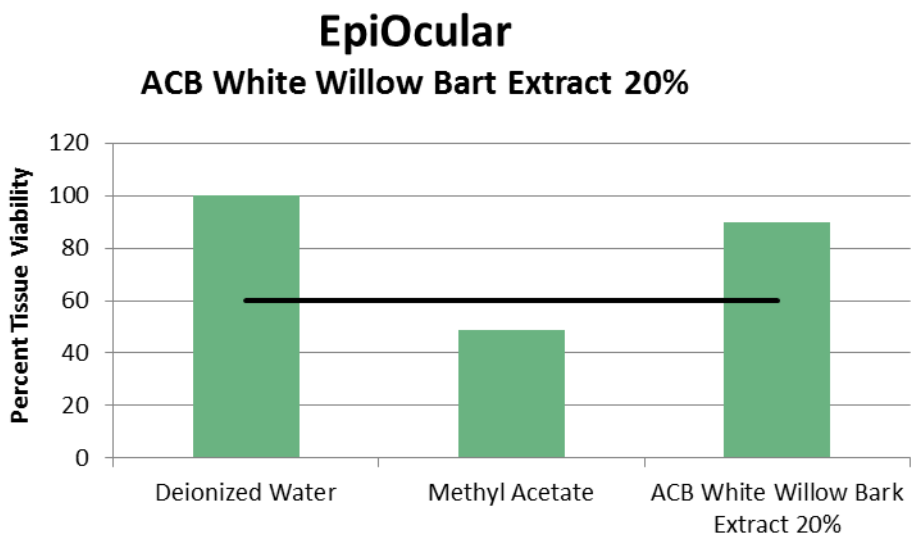

Figure 2: EpiOcular tissue viability

OECD 442C: *In Chemico* Skin Sensitization

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Tradename: ACB White Willow Bark Extract 20%

Code: 20199

CAS #: 84082-82-6

Test Request Form #: 11224

Lot #: 9397051

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:

OECD TG 442C: *In Chemico* Skin Sensitization – Direct Peptide Reactivity Assay (DPRA)

Introduction

A skin sensitizer is a substance that will lead to an allergic response following skin contact[1]. Haptenation is the covalent binding of a hapten, or low-molecular weight substance or chemical, to proteins in the skin. This is considered the prominent mechanism which defines a chemical as a sensitizer. Haptenation is described as a "molecular initiating event" in the OECD Adverse Outcome Pathway (AOP) for skin sensitization which summarizes the key events known to be involved in chemically-induced allergic contact dermatitis[2]. The direct peptide reactivity assay (DPRA) is designed to mimic the covalent binding of electrophilic chemicals to nucleophilic centers in skin proteins by quantifying the reactivity of chemicals towards the model synthetic peptides containing cysteine and lysine. The DPRA is able to distinguish sensitizers from non-sensitizer with 82% accuracy (sensitivity of 76%; specificity of 92%)[3].

This assay was conducted to determine skin sensitization hazard of **ACB White Willow Bark Extract 20%** in accordance with European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) and OECD Test Guideline 442C.

Assay Principle

The DPRA is an *in chemico* method which addresses peptide reactivity by measuring depletion of synthetic heptapeptides containing either cysteine or lysine following 24 hours incubation with the test substance. The peptide is a custom material containing phenylalanine to aid in detection. Depletion of the peptide in the reaction mixture is measured by HPLC with gradient elution and UV detection at 220 nm. Cysteine and lysine peptide percent depletion values are then calculated and used in a prediction model which allows assigning the test chemical to one of four reactivity classes used to support the discrimination between sensitizers and non-sensitizers.

1. United Nations Economic Commission (UNECE) (2013) Global Harmonized System of Classification and Labelling of Chemicals (GHS) 5th Revised Edition
2. OECD (2012). The Adverse Outcome Pathway for Skin Sensitization Initiated by Covalent Binding to Proteins. Part 1: Scientific Evidence. Series on Testing and Assessment No. 168
3. EC EURL ECVAM (2012) Direct peptide reactivity assay (DPRA) validation study report; pp 1 -74.

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Materials

A. Equipment:	HPLC-UV (Waters Breeze - Waters 2998 Photodiode Array Detector); Pipettes; Analytical balance
B. HPLC/Guard Columns:	Agilent Zorbax SB-C18 2.1 mm x 100 mm x 3.5 µm; Phenomenex Security Guard C18 4 mm x 2 mm
C. Chemicals:	Trifluoroacetic acid; Ammonium acetate; Ammonium hydroxide; Acetonitrile; Cysteine peptide (Ac-RFAA**C**AA-COOH); Lysine peptide (Ac-RFAA**K**AA-COOH); Cinnamic aldehyde
D. Reagents/Buffers:	Sodium phosphate buffer (100 mM); Ammonium acetate buffer (100 mM)
E. Other:	Sterile disposable pipette tips

Methods

A. Solution Preparation

a. 0.667 mM Cysteine Peptide in 100 mM Phosphate Buffer (pH 7.5)
b. 0.667 mM Lysine Peptide in 100 mM Ammonium Acetate Buffer (pH 10.2)
c. 100 mM Cinnamic Aldehyde in Acetonitrile
d. 100 mM* **ACB White Willow Bark Extract 20%** in Acetonitrile

*For mixtures and multi-constituent substances of known composition such as **ACB White Willow Bark Extract 20%**, a single purity should be determined by the sum of the proportion of its constituents (excluding water), and a single apparent molecular weight determined by considering the individual molecular weights of each component in the mixture (excluding water) and their individual proportions. The resulting purity and apparent molecular weight can then be used to calculate the weight of test chemical necessary to prepare a 100 mM solution.

B. Reference Controls

a. Reference Control A: For calibration curve accuracy
b. Reference Control B: For peptide stability over analysis time of experiment
c. Reference Control C: For verification that the solvent does not impact percent peptide depletion

C. Sample, Reference Control, and Co-Elution Control Preparation

a. Once these solutions have been made they should be incubated at room temperature, protected from light, for 24±2 hours before running HPLC analysis.
b. Each chemical should be analyzed in triplicate.

1:10 Ratio, Cysteine Peptide 0.5 mM Peptide, 5 mM Test Chemical	1:50 Ratio, Lysine Peptide 0.5 mM Peptide, 25 mM Test Chemical

• 750 µL Cysteine Peptide Solution
  ○ *Or 100mM Phosphate Buffer, pH 7.5, for Co-Elution Controls*
• 200 µL Acetonitrile
• 50 µL Test Chemical Solution
  ○ *Or Acetonitrile for Reference Controls*
	• 750 µL Lysine Peptide Solution
	  ○ *Or 100 mM Ammonium Acetate Buffer, pH 10.2, for Co-Elution Controls*
	• 250 µL Test Chemical Solution
	  ○ *Or Acetonitrile for Reference Controls*

D. Calibration Curve

a. Standards are prepared in a solution of 20% Acetonitrile:Buffer
  i. For the Cysteine peptide using the phosphate buffer, pH 7.5
  ii. For the Lysine peptide using the ammonium acetate buffer, pH 10.2

	Standard 1	*Standard 2*	*Standard 3*	*Standard 4*	*Standard 5*	*Standard 6*	*Standard 7*
mM Peptide	0.534	0.267	0.1335	0.0667	0.0334	0.0167	0.000

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E. HPLC Analysis
   a. HPLC-UV system should be equilibrated at 30°C with 50% Mobile Phase A (0.1% (v/v) trifluoroacetic acid in water) and 50% Mobile Phase B (0.085% (v/v) trifluoroacetic acid in acetonitrile) for 2 hours
   b. Absorbance is measured at 220 nm
   c. Flow Conditions:

Time (minutes)	*Flow (mL/min)*	*% A*	*% B*
0	0.35	90	10
10	0.35	75	25
11	0.35	10	90
13	0.35	10	90
13.5	0.35	90	10
20	End Run		

F. Calculations
   a. Percent peptide depletion is determined by the following equation:

$$Percent\ Peptide\ Depletion = \left[1 - \left(\frac{Peptide\ Peak\ Area\ in\ Replicate\ Injection}{Mean\ Peptide\ Peak\ Area\ in\ Reference\ Controls\ C}\right)\right] \times 100$$

Data and Reporting

A. Acceptance Criteria
   a. The following criteria must be met for a run to be considered valid:
      i. Standard calibration curve should have an $r^2 > 0.99$.
      ii. Mean percent peptide depletion values of three replicates for the positive control cinnamic aldehyde should be between 60.8% and 100% for the cysteine peptide and between 40.2% and 69% for the lysine peptide and the maximum standard deviation should be < 14.9 for the percent cysteine depletion and < 11.6 for the percent lysine depletion.
      iii. Mean peptide concentration of reference controls A should be 0.50 ± 0.05 mM and the coefficient of variable of the peptide peak areas for reference B and C in acetonitrile should be < 15.0%.
   b. The following criteria must be met for a test chemical's results to be considered valid:
      i. Maximum standard deviation should be <1 4.9 for percent cysteine depletion and < 11.6 for percent lysine depletion.
      ii. Mean peptide concentration of the three reference control C should be 0.50 ± 0.05 mM.

B. Prediction Model
   a. Cysteine 1:10/Lysine 1:50 Prediction Model

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
0% < Mean % Depletion < 6.38%	Minimal Reactivity	Non-sensitizer
6.38% < Mean % Depletion < 22.62%	Low Reactivity	Sensitizer
22.62% < Mean % Depletion < 42.47%	Moderate Reactivity	Sensitizer
42.47% < Mean % Depletion < 100%	High Reactivity	Sensitizer

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b. If co-elution occurs with the lysine peptide, than the cysteine 1:10 prediction model can be used:

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
0% < Cys % Depletion < 13.89%	Minimal Reactivity	Non-sensitizer
13.89% < Cys % Depletion < 23.09%	Low Reactivity	Sensitizer
23.09% < Cys % Depletion < 98.24%	Moderate Reactivity	Sensitizer
98.24% < Cys % Depletion < 100%	High Reactivity	Sensitizer

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. The measured values of percent depletion, reactivity class, and prediction for each assay are outlined in Table 1 and Table 2.

Table 1. Cysteine 1:10/Lysine 1:50 Prediction Model.

Cysteine 1:10/Lysine 1:50 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
3.15	Minimal Reactivity	Non-sensitizer
3.19	Minimal Reactivity	Non-sensitizer
3.13	Minimal Reactivity	Non-sensitizer

Table 2. Cysteine 1:10 Prediction Model.

Cysteine 1:10 Prediction Model		
Mean of Cysteine and Lysine % Depletion	*Reactivity Class*	*Prediction*
3.11	Minimal Reactivity	Non-sensitizer
3.09	Minimal Reactivity	Non-sensitizer
3.08	Minimal Reactivity	Non-sensitizer

Discussion

Based on HPLC-UV analysis of **ACB White Willow Bark Extract 20% (20199)** we can determine this product is not classified as a sensitizer and is not predicted to cause allergic contact dermatitis. The Mean Percent Depletion of Cysteine and Lysine was 3.13% causing minimal reactivity in the assay giving us the prediction of a non-sensitizer.

OECD 442D: *In Vitro* Skin Sensitization

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Tradename: ACB White Willow Bark Extract 20%

Code: 20199

CAS #: 84082-82-6

Test Request Form #: 11226

Lot #: 9397051

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:

OECD TG 442D: In Vitro Skin Sensitization *ARE-Nrf2 Luciferase Test Method*

Introduction

Skin sensitization refers to an allergic response following skin contact with the tested chemical, as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals[1]. Substances are classified as skin sensitizers if there is evidence in humans that the substance can lead to sensitization by skin contact or positive results from appropriate tests, both *in vivo* and *in vitro*. Utilization of the KeratinoSens™ cell line allows for valid *in vitro* testing for skin sensitization.

This assay was conducted to determine skin sensitization potential of **ACB White Willow Bark Extract 20%** in accordance with the UN GHS.

Assay Principle

The ARE-Nrf2 luciferase test method addresses the induction of genes that are regulated by antioxidant response elements (ARE) by skin sensitizers. The Keap1-Nrf2-ARE pathways have been shown to be major regulator of cytoprotective responses to oxidative stress or electrophilic compounds. These pathways are also known to be involved in the cellular processes in skin sensitization. Small electrophilic substances such as skin sensitizers can act on the sensor protein Keap1 (Kelch-like ECH-associated protein 1), by covalent modification of its cysteine residue, resulting in its dissociation from the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2). The dissociated Nrf2 can then activate ARE-dependent genes such as those coding for phase II detoxifying enzymes.

The skin sensitization assay utilizes the KeratinoSens™ method which uses an immortalized adherent human keratinocyte cell line (HaCaT cell line) that has been transfected with a selectable plasmid to quantify luciferase gene induction as a measure of activation of Keap1-Nrf2-antioxidant/electrophile response element (ARE). This test method has been validated by independent peer review by the EURL-ECVAM. The addition of a luciferin containing reagent to the cells will react with the luciferase produced in the cell resulting in luminescence which can be quantified with a luminometer.

1. United Nations (UN) (2013). Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Fifth revised edition, UN New York and Geneva, 2013

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Materials

A. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes
C. Cell Line:	KeratinoSens™ by Givaudan Schweiz AG
D. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM); Phosphate Buffered Saline (PBS)
E. Culture Plate:	Flat bottom 96-well tissue culture treated plates
F. Reagents:	Dimethyl Sulfoxide (DMSO); Cinnamic Aldehyde; ONE-Glo Reagent; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); sodium lauryl sulfate (SLS)
G. Other:	Sterile disposable pipette tips; wash bottles

Methods

KeratinoSens™ were into seeded four 96-well tissue culture plates and allowed to grow to 80 – 90% confluency in DMEM. Twelve test concentrations of **ACB White Willow Bark Extract 20%** were prepared in DMSO with a concentration range from 0.98 – 2000 µM. These 12 concentrations were assayed in triplicate in 2 independently performed experiments. The positive control was cinnamic aldehyde for which a series of 5 concentrations prepared in DMSO had final test concentrations of 4 – 64 µM. The negative control was a 1.0% test concentration of DMSO.

24 hours post KeratinoSens™ seeding, the culture media was removed and replaced with fresh media containing 10% FBS without G418 geneticin. 50 µL of the above described test concentrations was added to the appropriate wells. The treated plates were then incubated for 48 hours at 37°C in the presence of 5% CO_2 and 95% relative humidity. After treatment incubation was complete the media was removed and the wells were washed with PBS 3 times.

One of the four plates was used for a cytotoxicity endpoint, where MTT was added to the wells and incubated for 4 hours at 37°C in the presence of 5% CO_2. SLS was then added to the wells and incubated overnight at room temperature. A spectrometer measured the absorbance at 570 nm. The absorbance values (optical density) were then used to determine the viability of each well by comparing the optical density of each test material treated well to that of the solvent control wells to determine the IC_{50} and IC_{30} values.

The remaining 3 plates were used in the luciferase induction endpoint of the assay. 100 µL of Promega's ONE-Glo Reagent was added to 100 µL of fresh media containing 10% FBS without geneticin. Cells were incubated for 5 minutes to induce cell lysis and release luciferin into the media. Plates were read with a luminometer and $EC_{1.5}$ and maximum response (I_{max}) values were obtained.

Data and Reporting

A. Acceptance Criteria

a. Gene induction obtained with the positive control, cinnamic aldehyde, should be statistically significant above the threshold of 1.5 in at least one of the tested concentrations (from 4 to 64 µM).
b. The $EC_{1.5}$ value should be within two standard deviations of the historical mean and the average induction in the three replicates for cinnamic aldehyde at 64 µM should be between 2 and 8.
c. The average coefficient of variability of the luminescence reading for the negative (solvent) control DMSO should be below 20% in each experiment.

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B. A KeratinoSens™ prediction is considered positive if the following conditions are met:
   a. The I_{max} is higher than 1.5-fold and statistically significantly higher as compared to the solvent (negative) control
   b. The cellular viability is higher than 70% at the lowest concentration with a gene induction above 1.5 fold (i.e., at the $EC_{1.5}$ determining concentration)
   c. The $EC_{1.5}$ value is less than 1000 µM (or < 200 µg/ml for test chemicals with no defined MW)
   d. There is an apparent overall dose-response for luciferase induction

Results

Table 1. Overview of KeratinoSens™ Assay Results (I_{max} equals the average induction values in Figure 1).

Compound	*Classification*	*$EC_{1.5}$ (µM)*	*IC_{50}*	*I_{max}*
Cinnamic aldehyde	Sensitizer	19	289.19 µM	31.58
DMSO	Non-Sensitizer	No Induction	243.24 µM	0.19
ACB White Willow Bark Extract 20%	Non-Sensitizer	No Induction	> 1000 µM	0.40

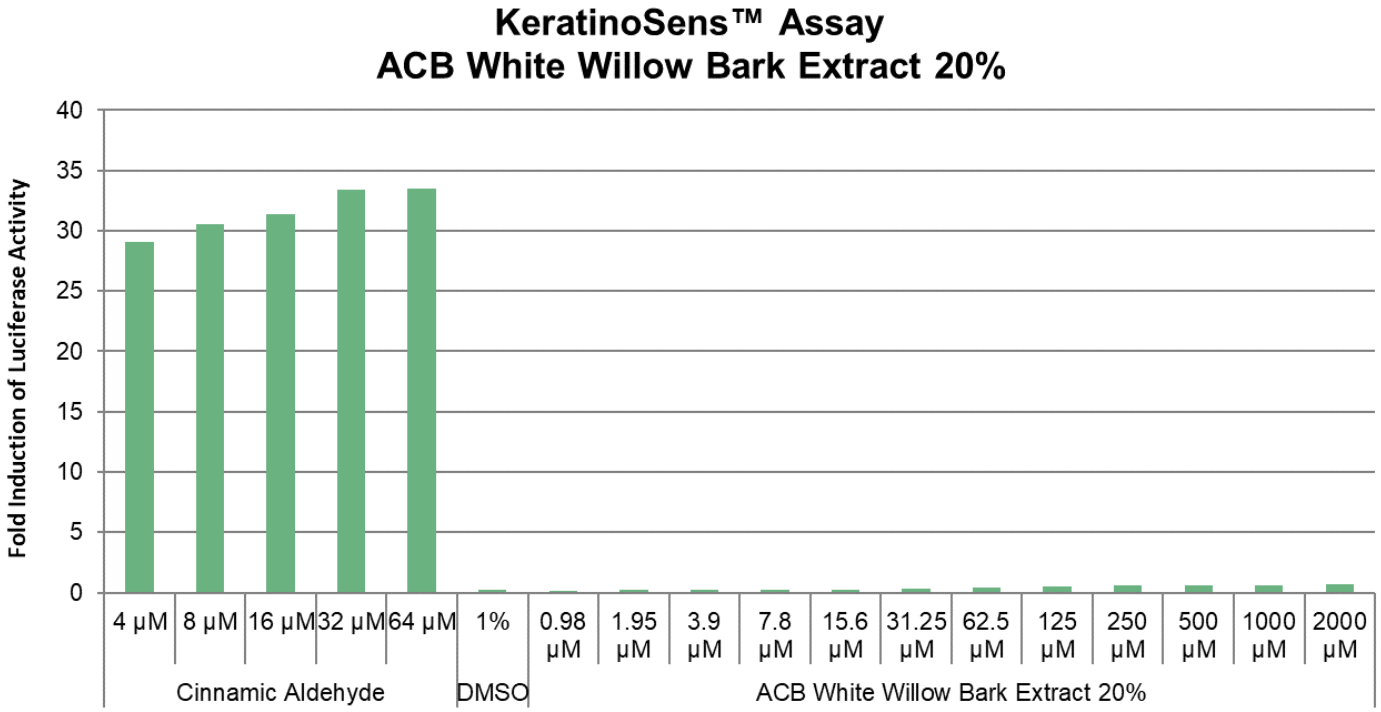

Figure 1. Effect of Test Conditions on Luciferase Fold Induction.

Discussion

As shown in the results, **ACB White Willow Bark Extract 20% (20199)** was not predicted to be a skin sensitizer based on the KeratinoSens™ ARE-Nrf2 Luciferase Test Method as there was not a significant increase in luciferase expression. It can be concluded that **ACB White Willow Bark Extract 20%** can be safely used in cosmetics and personal care products at typical use levels.

Phototoxicity Assay Analysis

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Tradename: ACB White Willow Bark Extract 20%

Code: 20199

CAS #: 84082-82-6

Test Request Form #: 11227

Lot #: 9397051

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
In Vitro EpiDerm™ Model (EPI-200-SIT) Phototoxicity

SUMMARY

In vitro phototoxicity irritation studies were conducted to evaluate whether **ACB White Willow Bark Extract 20%** would induce phototoxic irritation in the EpiDerm™ model assay.

The was performed according to the manufacturer's protocol. The test article solution was found to be a non-photoirritant at 0.5%, 1.5%, 5.0% and 10.0% concentrations. Reconstructed human epidermis were incubated in growth media for one hour to allow for tissue equilibration. The test substance was applied to the tissue inserts in four varying concentrations and incubated overnight in a humidified incubator. The following day, the appropriate tissue inserts were irradiated (UVA) for 60 minutes with 1.7 mW/cm^2 (=6 J/cm^2). After substance incubation, irradiation, and washing was completed, cell viability was assessed. Cell viability was measured by dehydrogenase conversion of MTT [(*3-4,5-dimethyl thiazole 2-yl*)], present in the cell mitochondria, into blue formazan salt that was measured after extraction from the tissue. The photoirritation potential of the test chemical was dictated by the reduction in tissue viability of UVA exposed tissues compared to non-UVA exposed tissues.

Under the conditions of this assay, the test article was considered to be non-phototoxic at concentrations of 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated.

Introduction

A. Purpose

An i*n vitro* dermal phototoxicity study was conducted to evaluate whether a test article would induce photoirritation in the EpiDerm™ model assay. MatTek Corporation's reconstructed human epidermal model is becoming a standard in determining the phototoxicity potential of a test substance. This assay is able to discriminate between photoirritants and non-photoirritants at varying concentrations.

Phototoxicity Assay Analysis

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Materials

A. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; UVA-vis Irradiation Equipment; UVA meter; Pipettes
C. Media/Buffers:	Dulbecco's Modified Eagle Medium (DMEM) based medium; sterile Dulbecco's phosphate-buffered saline (DPBS); sterile deionized water H_2O (diH_2O)
D. Preparation:	Pre-incubate (37°C) tissue inserts in assay medium; Place assay medium and MTT diluent at 4°C, MTT concentrate at -20°C, and record lot numbers of kit components
E. Tissue Culture Plates:	Falcon flat bottom 96-well, 24-well, and 6-well tissue culture plates
F. Reagents:	MTT (*3-4,5-dimethyl thiazole 2-yl*) (1.0 mg/mL); Extraction Solution (Isopropanol); Chlorpromazine; Triton X-100 (1.0%)
G. Other:	Wash bottle; sterile disposable pipette tips; Parafilm; forceps

Test Assay

A. Test System

The reconstructed human epidermal model, EpiDerm™ consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayer, highly differentiated model of the human epidermis. This model consists of organized basal, spinous, and granular layers, and contains a multilayer stratum corneum containing intercellular lamellar lipid layers. The EpiDerm™ tissues are cultured on specially prepared cell culture inserts. The EpiDerm™ tissue inserts from MatTek were in good condition, intact, and viable upon arrival.

B. Positive and Negative Controls

a. Positive Control: Concentrations of chlorpromazine, ranging from 0.001% to 0.1%, were used as positive controls for the EpiDerm™ Phototoxicity assay.
b. Negative Control: diH_2O was used as the negative control for the EpiDerm™ Photoxicity assay.

C. Data Interpretation Procedure

A photoirritant is predicted if the mean relative tissue viability of the 2 tissues exposed to the test substance and 60 minutes of 6 J/cm^2 is reduced by 20% compared to the non-irradiated control tissues.

Method

A. Tissue Conditioning

Upon arrival, the EpiDerm™ tissue inserts were removed from their shipping medium and transferred into new culture plates with fresh media and incubated at 37°C, 5% CO_2, and 95% RH for 60 minutes. After the 60 minute incubation, the inserts were transferred into new culture plates with fresh media and dosing commenced.

B. Test Substance Exposure and Tissue Washing

a. Exposure: 50 µL of the diluted test substance in their respective concentrations were applied to 2 tissue inserts and allowed to incubate for overnight at 37°C, 5% CO_2, and 95% RH.
b. Washing: After UVA-irradiation and dark incubation, the tissue inserts were washed using DPBS and transferred to new culture plates with fresh media for an overnight incubation at 37°C, 5% CO_2, 95% RH.

C. MTT Assay

Tissue inserts were transferred into 300 µL MTT media in pre-filled plates and incubated for 3 hours at 37°C, 5% CO_2, and 95% RH. Next, inserts were placed into sealed culture plates with 2 mL of the extraction solution and incubated in the dark for 24 hours at room temperature. Following this incubation, extraction was complete and the tissue inserts were pierced with forceps. Duplicate 200 µL aliquots of the blue formazan solution from each tissue insert was transferred into a 96-well plate for Optical Density reading at a wavelength of 570 nm (OD_{570}) with a spectrophotometer.

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Acceptance Criterion

A. Positive and Negative Control
   a. Positive Control: The assay meets acceptable criterion if a dose dependent reduction in cell viability in the UVA-irradiated tissues is between 0.00316% and 0.0316%.
   b. Negative Control: The results are acceptable if the mean negative control Optical Density (OD_{570}) is ≥ 0.8.

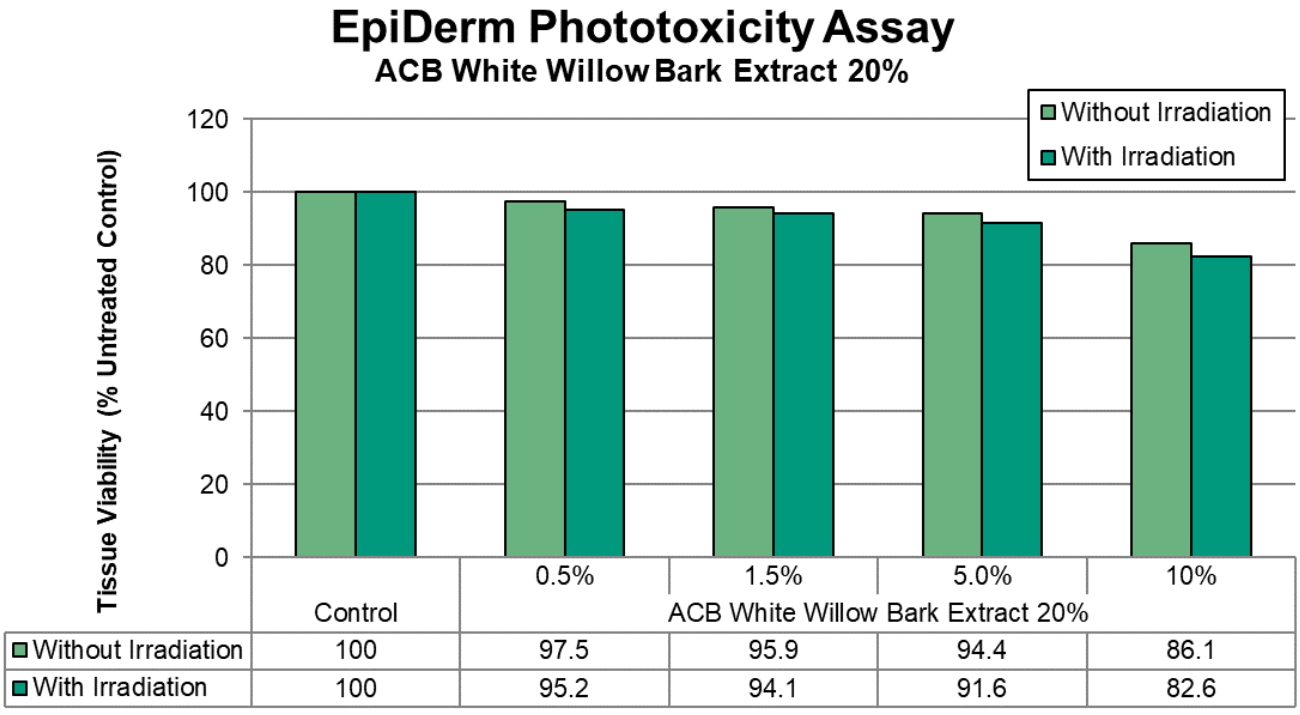
B. Standard Deviation
   Provided phototoxicity potential is predicted from the mean viability of 2 tissues for the EpiDerm™ Phototoxicity Protocol, the variability of the replicates should not exceed 30%.

Results

A. Test Validity and Tissue Viability Assay
   The data obtained from this study met criteria for a valid assay. The results are summarized in Figure 1. Cell viability was calculated for each tissue as a percentage of the corresponding vehicle control either irradiated or non-irradiated. Tissue viability was not reduced by 20% in the presence of the test substance and UVA-irradiation at concentrations of 0.5%, 1.5%, and 5.0%. The negative control mean exhibited acceptable relative tissue viability while the positive control exhibited dose dependent loss of tissue viability and cell death.

Conclusion

Phototoxicity (photoirritation) is defined as an acute toxic response that is elicited after exposure of the skin to certain chemicals and subsequent exposure to light. Under the conditions of this assay, the test article substance was considered to be non-phototoxic at concentrations of 0.5%, 1.5%, 5.0%, and 10.0%. The negative and positive controls performed as anticipated. There is a slight decrease in viability at the 10% concentration but viability does not decrease more than the acceptable 20%. We can safely say that **ACB White Willow Bark Extract 20%** is not a photoirritant when used at the suggested use levels of 1.0% - 10.0%.



	Control	0.5%	1.5%	5.0%	10%
Without Irradiation	100	97.5	95.9	94.4	86.1
With Irradiation	100	95.2	94.1	91.6	82.6

Figure 1. EpiDerm™ Phototoxicity Graph.