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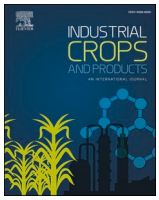

In vitro safety and ecotoxicity tests of suberin- and lignin-based nanoparticles

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ABSTRACT

Nanoparticles from biobased raw materials can be utilised to improve functional properties of textiles and fibre-based packaging materials. Safety of chemicals need to be assessed in the EU before entering to the market. This study investigates the bioactivity and potential ecotoxicity and toxicity of biobased nanoparticles from hydrolyzed suberin (SNPs), softwood kraft lignin (lignin nanoparticles, LNPs), and tall oil fatty acid esterified softwood kraft lignin (TOFA-lignin, TOFA-LNPs). Nanoparticles were assayed as concentrations sufficient for textile coating applications for cytotoxicity, skin sensitization, corrosion, and irritation *in vitro*. For ecotoxicity evaluation, algal inhibition, *Daphnia* sp. acute immobilization, *D. magna* reproduction, and toxicity to earthworms were tested. Antibacterial properties were examined using recombinant *Escherichia coli* and *Staphylococcus aureus* strains, while antioxidant activities were evaluated with *in vitro* assays. Results indicated that nanoparticles were safe at the studied concentrations according to the cytotoxicity and skin irritation or corrosion tests. However, LNPs at concentrations of 70.31 μg/mL and TOFA-LNPs at 125 μg/mL (w/v) showed skin sensitization, unlike SNPs. Ecotoxicity assays revealed that all aqueous nanoparticle dispersions exhibited effects on algae and daphnids but were harmless to earthworms at the concentrations tested. LNPs and TOFA-LNPs showed high antibacterial and antioxidant activities, which can likely be attributed to their high total phenolic content. With structurally more complex SNPs, both antioxidant and antibacterial activities were low. The results show that the investigated nanoparticles are potential alternatives to fossil-based and more harmful chemicals. Biobased, safe-assessed alternatives for coating of textiles could be a way to increase the environmentally sustainability.

1. Introduction

Bio-based, protective, and water-repellent coatings are emerging as green alternatives for surfaces of clothing and packaging to replace fossil-based chemicals. These innovations are important in addressing the current challenges related to global warming and pollution through new methods for manufacturing (Yadav et al., 2020; Harman-Ware et al., 2021). Bio-based renewable materials are needed to replace fossil-based products (United Nations, 2024). However, it is important to recognize that bio-based materials obtained from renewable biomass are not necessarily more environmentally sustainable than synthetic chemicals (Yadav et al., 2024). As such, the environmental impacts of the production of bio-based materials must be carefully evaluated using transparent and creditable methods to determine if any real environmental advantages can be achieved. An important element of sustainability efforts is to reduce chemical impacts on ecosystem health, as

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presented recently by Owsianiak and coworkers (Owsianiak et al., 2023) about the ecotoxicity characterization of chemicals in global recommendations.

The EU Chemical Strategy aims to ensure the safe and sustainable use of chemicals (EC, 2020). In addition, all substances in the EU market need to be tested to guarantee their safety for humans and the environment in accordance with EU's chemical legislations (REACH [Registration, Evaluation, Authorization and restriction of Chemicals]: EC (2006), and CLP [Classification, Labelling and Packaging of substances and mixtures]: EC, 2008). While there are some exceptions to naturally occurring substances in the legislation (Annex V in EC, 2006), products must pass comprehensive risk assessments to ensure safety.

Bio-based products can be used in various applications, such as on surfaces of clothing and packaging to increase water resistance, antimicrobial activity, or insect-repelling performance. Forestry industry side-streams, such as bark, are a rich feedstock containing various compounds with a high added value potential (e.g., lignin, suberin, betulin, tannins; (Li et al., 2016; Routa et al., n.d; Rasi et al., 2019). Lignin is a phenolic polymer, which is present in the cell walls of water-conducting and support-providing cells of all vascular plants (Boerjan et al., 2003; Barros et al., 2015). In woody plants, lignin constitutes 20 %–35 % of the dry weight of wood, making it the second-most abundant terrestrial biopolymer after cellulose. In lignocellulosic biomass utilization, such as pulping and bio-fuel production, lignin needs to be removed, making it a widely abundant industrial side-stream (Österberg et al., 2020; Tardy et al., 2023). Currently, most industry-derived lignin is combusted for energy, although several value-added applications of lignin have been demonstrated (Österberg et al., 2020; Tardy et al., 2023; Morena and Tzanov, 2022; Dixon et al., 2024). Suberin is another cell wall polymer and contains a polyaliphatic domain composed of long-chain fatty acids and glycerol as well as a polyphenolic domain composed of hydroxycinnamic acids (Kolattukudy, 2001; Bernards, 2002). It is present in the periderm of barks and in a few subterranean plant parts, such as the epidermis, exodermis, and endodermis of roots, where it forms a protective barrier (Kolattukudy, 2001; Bernards, 2002; Graça, 2015). In the outer bark of silver birch (*Betula pendula*), suberin content is approximately 45–50 % by weight (Li et al., 2016; Pinto et al., 2009). Birch bark, widely available in kraft pulp mills, is also mostly incinerated for energy (Routa et al., n.d; Pinto et al., 2009).

Nanoparticles prepared of lignin and suberin have a high surface-to-volume ratio and can be functionalized with other substances; both of these properties are advantageous for many applications (e.g. Jezo, 2024; Freitas et al., 2020). Lignin nanoparticles (LNPs) have been shown to possess antibacterial, antioxidant, and UV-absorbing properties, as summarized by Morena and Tzanov (2022). They can be used, for example, in composites, as dispersants, in biomedicine as drug carriers or constituents of biomedical materials, as UV absorbents in sunscreens, and as antioxidant and antibacterial agents in food packaging materials (reviewed by Österberg et al., 2020; Morena and Tzanov, 2022). Synthesis method and process parameters influence the size and morphology of nanoparticles, and modification of lignin is often needed to improve its properties and is essential for utilization in certain applications (Österberg et al., 2020; Setälä et al., 2020).

LNPs and hybrid LNPs have recently been utilized to coat cotton fabric and introduce advantageous properties (Babaeipour et al., 2024). Incorporation of tall oil fatty acid (TOFA) into LNPs (TOFA-LNPs) significantly enhances hydrophobicity of the coated textiles compared to coating with unmodified LNPs while maintaining high air breathability. Furthermore, coating with LNPs or TOFA-LNPs provide efficient UV light blocking properties to the material (Babaeipour et al., 2024). TOFA-LNPs have also been used to functionalize cellulose fibers in earlier studies (Setälä et al., 2020). In dispersions or coatings, TOFA-LNPs exhibit stronger antimicrobial activity against gram-positive bacteria (*Staphylococcus aureus*) compared to gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* (Setälä et al., 2020; Babaeipour et al., 2024). The advantages of both unmodified and modified LNPs make them promising tools for developing water-repellent (Henn et al., 2021), UV-shielding (Petkovska et al., 2022), and antimicrobial surfaces (Sunthornvarabhas et al., 2019).

Suberin nanoparticles (SNPs) have also demonstrated good antimicrobial properties against *S. aureus*, *P. aeruginosa*, and the yeast *Candida albicans,* and SNPs can be used as a carrier of anticancer drugs (Liakos et al., 2019). The properties of suberin-based polymers as a surface modifier of cellulosic substrates have been extensively studied (Li et al., 2016, 2015a, 2015b). Furthermore, betulin and betulin-based polymers have been investigated as more environmentally friendly alternatives in water-repellent textiles (Huang et al., 2019). Recent life cycle assessments (LCAs) indicate that suberin and betulin production via a cascade process from birch bark would have a lower environmental impact if efficient recycling methods for ethanol, which is necessary for the process, could be developed (Yadav et al., 2024).

Since LNPs and SNPs have been shown to be promising agents for the creation of sustainable and protective hydrophobic surfaces on cellulose fibers to replace harmful fossil-based compounds, this study aims to investigate their toxicity and ecotoxicity with standardized tests as well as their antibacterial and antioxidant properties. Hydrolyzed suberin (SNPs), softwood kraft lignin (lignin nanoparticles, LNPs), and tall oil fatty acid esterified softwood kraft lignin (TOFA-lignin, TOFA-LNPs) were chosen for their multifunctionality and potential as aqueous bio-based dispersion coating for textile and packaging. SNPs have been demonstrated to provide hydrophobic and antibacterial properties (Farooq et al., 2025). LNPs are well known for their intrinsic UV-blocking, antioxidant, and antibacterial functionalities (Babaeipour et al., 2024). TOFA-LNPs combine lignins properties with enhanced hydrophobicity, further broadening their suitability for packaging and textile applications, as shown in recent study (Babaeipour et al., 2025). They are furthermore all sourced from industrial side streams that are currently burned. The data obtained from these evaluations can be used for REACH and CLP assessments of these substances, ensuring their safety for human health and the environment. Additionally, in the future this data can contribute to the environmental impact assessment of the products' lifecycle, aiding in the long-term development of more sustainable materials.

2. Material and methods

In this study, we prepared nanoparticles from hydrolyzed suberin (SNPs), softwood kraft lignin (lignin nanoparticles, or LNPs), and tall oil fatty acid esterified softwood kraft lignin (TOFA-lignin, i.e., TOFA-LNPs), and studied their toxicity and ecotoxicity with standardized tests as well as their antibacterial and antioxidant properties as described in the following sections.

2.1. Materials

The kraft lignin BioPiva 395™ was obtained from UPM (Finland). Tall oil fatty acid as a distilled fraction with 98 % C18 fatty acid purity was sourced from Forchem (Rauma, Finland). Oxalyl chloride (ReagentPlus® > 99 %) was sourced from Sigma-Aldrich. The solvents pyridine (99.8 %), N,N-dimethylformamide (DMF, 99.8 %), and tetrahydrofuran (THF, 99.0 %) were from Sigma-Aldrich, and the ethanol (99.9 %) was from Altia, Finland.

2.2. Isolation of suberin

Suberin in the form of suberin hydrolysate (a mixture of suberin fatty acids) was obtained from outer birch bark as described by Yadav et al. (2024). The outer bark of freshly harvested silver birch (*Betula pendula* Roth.) stems, with diameters between 20–30 cm, from Punkaharju, Finland, was manually removed and air-dried at room temperature (ca. 20 °C). The bark was milled to particles using a Pulverisette cutting mill

(Fritsch) with a 4 × 4 mm sieve cassette, then freeze-dried and stored in a polyethylene bag at room temperature. The bark was ethanol-extracted followed by alkaline ethanolic hydrolysis. One hundred fifty (150) g bark calculated as dry matter was placed in a 2.0-liter stirred autoclave (büchiglasuster) with 1500 mL of ethanol/water (9:1 v/v) mixture, achieving a 10 kg/L solvent-to-bark ratio. This was heated to 90 °C for 15 min and extracted for 30 min at 90 °C with stirring (150 rpm). The liquid after the extraction was discharged and vacuum-evaporated using a Hei-VAP Precision vacuum rotary evaporator (Heidolph); the residue after the evaporation was further dried in a vacuum oven (Heraeus Vacutherm) at 40 °C for 20 min, resulting in a betulin-rich residue of roughly 21 % (w/w) of the original bark.

The extracted bark then underwent hydrolysis with an ethanol/water mixture (9:1 v/v) containing 20 % (w/w) NaOH, calculated from dry extracted bark. A solvent-to-bark ratio of 10 kg/L was maintained in the hydrolysis stage. Hydrolysis was conducted in the same reactor at 90 °C for 60 min with stirring (150 rpm). After hydrolysis, the solution was discharged, and the residual bark was washed with an ethanol/water solution at 80 °C for 15 min. The combined hydrolysate and the wash solutions were evaporated. Boiling ultrapure water was then added to the residue, precipitating the second betulin fraction. The betulin was collected by a LAB-10T pressure filter (ErterAlsop), washed, and freeze-dried. This second betulin fraction was approximately 15 % (w/w) of the original bark. The flow-through filtrate, containing suberin fatty acids, was acidified to pH 4 with 2 M sulfuric acid, causing the fatty acids to precipitate. The precipitate was collected by filtration, washed with ultrapure water, and freeze-dried, yielding a suberin fatty acid fraction of about 26 % of the original outer bark.

2.3. Esterification of lignin with tall oil fatty acids (TOFAs)

TOFA chloride (TOFA-Cl) was synthesized by refluxing 10 g of TOFA (30 mmol, assuming an average fatty acid composition of C18) with 8 g of oxalyl chloride (40 mmol) overnight. The excess oxalyl chloride was removed through microdistillation. Lignin esterification was carried out following the method described in Babaeipour et al. (2024). In summary, 5 g of lignin was dissolved in a mixture of dry solvents, 30 mL THF, 4 mL pyridine, and 8 mL DMF and reacted with 3 mL TOFA-Cl at 60 °C under a nitrogen atmosphere for 48 h. Isolation and purification of TOFA-esterified lignin was done before production of the nanoparticles.

2.4. Preparation of lignin nanoparticles (LNPs, TOFA-LNPs)

LNPs were prepared by dissolving 30 g of softwood kraft lignin (BioPiva™ 395) received from UPM (Finland) in a 600 g acetone-water mixture (7:3 by mass) for 3 h. The solution was filtered using Whatman paper filters (pore size 0.7 μm) to remove insoluble residues. The lignin particles self-assembled into spherical nanoparticles when the filtered solution was rapidly poured into 2500 g of deionized water under vigorous stirring. Acetone was removed from the resulting colloidal LNP dispersion using rotary evaporation at 40 °C under reduced pressure, yielding a final concentration of 0.9 % (w/w). Esterified LNPs (i.e., TOFA-LNPs) were prepared by following the same procedure as for unmodified kraft lignin, using esterified lignin as the starting material (Babaeipour et al., 2024). Rotary evaporation was again used to remove the solvent and concentrate the colloidal TOFA-LNPs dispersion to a final concentration of 0.2 % (w/w).

2.5. Preparation of suberin nanoparticles (SNPs)

A solution of suberin hydrolysate was prepared by dissolving 20 g of hydrolyzed suberin in 2000 mL of acetone. The mixture was stirred at 600 rpm for 15 min at 85 °C, followed by continuous stirring at room temperature (c. 20 °C) for 12 h. After dissolution, the solution was centrifuged at 10,000 rpm for 30 min to remove any undissolved material. The self-assembly of nanoparticles was initiated by pouring the mixture into deionized water (10 L) at a 1:5 (v/v) ratio. Acetone was removed from the resulting suberin dispersion using rotary evaporation at 40 °C under reduced pressure, yielding a final concentration of 0.8 % (w/w).

2.6. Analysis of nanoparticles

The hydrodynamic size of the nanoparticles in their aqueous dispersions was analyzed using a Malvern Zetasizer Nano (Malvern Instruments, UK) dynamic light scattering device with a dip cell to measure the particles' hydrodynamic diameter and electrophoretic mobility. The surface morphology of the particles was studied using a field emission scanning electron microscope (FESEM, Zeiss Sigma VP, Germany). Prior to FESEM imaging the nanoparticle samples were applied on silica wafers that were further attached to aluminum SEM stubs with carbon tape, followed by sputter-coating using a Leica Microsystems EM ACE600 coating system (Leica Microsystems, Wetzlar, Germany) to coat a 5-nm layer of gold-platinum on the substrate.

2.7. Testing of the nanoparticles

The samples for ecotoxicity and toxicity and antibacterial and antioxidant testing were LNPs at 0.9 % (w/w, aqueous dispersion), TOFA-LNPs at 0.2 % (w/w, aqueous dispersion), and SNPs at 0.8 % (w/w, aqueous dispersion). The choice of nanoparticle concentrations was based on the established laboratory protocols of our laboratory (Babaeipour et al., 2024) that have demonstrated that these concentrations are sufficient for textile coating applications. For this study, 2.7 L of each particle dispersion was prepared.

2.7.1. In vitro cytotoxicity test

Cytotoxic potential was tested according to the Organisation for Economic Co-operation and Development (OECD) 129 guidelines "Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests" (OECD, 2010). This guidance describes methods to determine the *in vitro* basal cytotoxicity of test substances using a neutral red (NR) uptake assay. The NR uptake assay is performed in a dose-response format to determine the concentration that reduces NR uptake by 50 % compared to the controls (i.e., the IC50).

A murine fibroblast cell line BABL 3T3 clone A31 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, or IZSLR) was used as a study material. A cell suspension was prepared in Dulbecco's Modified Eagle Medium (DMEM; supplemented with 4.5 g/L glucose, 5 % (v/v) fetal bovine serum, and 4 mM L-glutamine, but without phenol red) and dispensed into the wells of a 96-well plate. After 24-h incubation at 37 °C with 90 % relative humidity (RH) and 5 % CO_2, the medium was removed and the nanoparticle samples (50 μL) at four dilutions in DMEM supplemented with 4 mM L-glutamine, 200 IU/mL penicillin, and 200 μg/mL streptomycin were added and six replicates were performed. Sodium lauryl sulphate (50 μL) and DMEM (50 μL) served as positive and negative controls, respectively. After the additions, the plates were incubated at 37 °C, 90 % RH, 5 % CO_2 for 48 h. NR uptake was analyzed by adding 250 μL of NR (25 μg/mL, Sigma-Aldrich) to all wells, followed by incubation at 37 °C, 90 % RH, 5 % CO_2 for 3 h. The NR medium was removed, and the cells were carefully rinsed with pre-warmed Dulbecco's phosphate-buffered saline (DPBS, 250 μL/well). The wash solution was removed, and 100 μL NR desorb solution (freshly prepared mixture of water–ethanol–glacial acetic acid, 49:50:1, v/v/v) was added to all wells to extract the dye. The absorbance/OD measured in a multiwell plate reader at 540 nm (Chromate-4300, Awareness Technology). Cell viability was calculated using DMEM as a blank. The tested compound was determined to be cytotoxic if it reduced cell vitality by 50 % compared to the controls.

2.7.2. In vitro skin sensitization test

The *in vitro* skin sensitization test was conducted according to OECD 442D guidelines for the ARE-Nrf2 luciferase KeratinoSens™ test method (OECD, 2024). KeratinoSens™ cells (acCELLerate GmbH, Germany) harboring a luciferase reporter gene under the control of the antioxidant response element of a human AKR1C2 gene were cultured in a growth medium (DMEM) containing GlutaMAX) and supplemented with 9.1 % (v/v) fetal bovine serum (FBS, Gibco) and 500 μg/mL geneticin (G418, Gibco) until passage 4 (OECD, 2024). Then, cells were seeded at a density of 10,000 cells/well in white 96-well plates for the luciferase activity assay or transparent 96-well plates for the cell viability analysis and cultivated at 37 ºC with 5 % CO_2. After 24 h, the medium was replaced with a fresh growth medium supplemented with each nanoparticle sample in 12 dilutions or cinnamic aldehyde (Sigma; 64, 32, 16, 8, 4 μM) as a positive control. All dilutions were prepared with the growth medium supplemented with 1 % FBS, 1 % dimethyl sulfoxide (DMSO), but without geneticin. The growth medium supplemented with 9.1 % FBS and 500 μg/mL geneticin and 1 % DMSO was used as a solvent control. After 48-h incubation at 37 °C with 5 % CO_2, cells were processed for luciferase activity or cell viability assays. For luciferase activity analysis, the supernatants were discarded, and cells were washed once with phosphate-buffered saline (PBS, Gibco). Lysis buffer (20 μL, Promega) was added to each well, and cells were incubated for 20 min at room temperature (c. 20 °C). After that, 50 μL of luciferase substrate (Promega) was added to each well, and luciferase activity was measured by measuring luminescence (BioTek Synergy H1 multimode microplate reader).

Cell viability determination is based on mitochondrial dehydrogenase activity, measured as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and conversion into a blue formazan salt that is quantitatively measured after extraction from the tissues. For the cell viability analysis, after 48 h of incubation with nanoparticles, the growth medium was replaced with 200 μL fresh medium containing 5 mg/mL MTT (Sigma), and cells were incubated for 4 h at 37 °C in the presence of 5 % CO_2. The medium was removed, and 50 μL isopropanol (Sigma) was added. After shaking for 30 min, the absorbance was measured at 570 nm with a multimode microplate reader (BioTek Synergy H1).

Three technical replicates per sample dilution for the luciferase assay and two technical replicates for the cell viability analysis were prepared. Additionally, two independent runs were performed. Data outliers were identified with a ROUT method (Q = 5 %) and excluded from the analysis. Raw data were statistically analyzed by an ordinary one-way analysis of variance (ANOVA) test and a Dunnet's post-hoc multiple comparisons test. Statistical significance was set at $p < 0.05$, 95 % confidence.

2.7.3. In vitro skin irritation test

The *in vitro* skin irritation test was conducted using the OECD Guidelines for Testing of Chemicals, No. 439 (OECD, 2021). The method included a reconstructed three-dimensional human epidermis EpiDerm™ model comprising the organized basal, spinous and granular layers and a multilayered stratum corneum (MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia, Batch No.:40022). The assay medium (Base medium: DMEM; Antibiotics: gentamicin, Anti-fungal agent: Fungizone; pH indicator: phenol red [except for the PRF medium]) was left under a laminar flow hood to reach room temperature before use. The 6-well assay plate were filled with the assay medium (0.9 mL per well). The epidermis units were placed in an empty sterile 24-well plate for visual inspection. Their surface was dried with a cotton swab, and the tissues were transferred to a 6-well plate pre-filled with the medium (avoiding bubbles) and incubated for 60 min at 37 °C in an incubator with 5 % CO_2 and 95 % RH. The inserts were transferred to new wells with 0.9 mL of assay medium and incubated in the incubator for 18 ± 3 h. Each nanoparticle sample (30 μL) or the control solution (30 μL) was applied to the epidermis model, and the tissues incubated for 60 min: first 35 min at 37 °C with 5 % CO_2 and 95 % RH, and then at room temperature (ca. 20 °C). DPBS and 5 % (w/v) aqueous sodium dodecyl sulphate (SDS) were used as negative and positive controls, respectively.

After incubation, the EpiDerm™ units were removed and rinsed 15 times with DPBS to remove all residual test material from the epidermal surfaces. To remove the rest of the test material, the inserts were submerged 3 times in DPBS and shaken. The excess of DPBS was removed by gently shaking the inserts and drying them on a blotting paper. The inserts were transferred to new wells containing 0.9 mL of fresh assay medium and incubated at 37 °C in an incubator with 5 % CO_2, 95 % RH for 24 ± 2 h. Then the inserts were moved to new wells with fresh medium and incubated for additional 18 ± 2 h.

The EpiDermTM units were transferred into a 24-well plate prefilled with MTT solution (1 mg/mL MTT, 0.3 mL per well) and incubated for 3 h at 37 °C with 5 % CO_2, 95 % RH in the dark. After incubation, the formazan that formed was extracted. The MTT medium was aspirated from the wells, the tissues were washed three times with DPBS, and the tissues were then transferred to new 24-well plates. The inserts containing epidermal tissues were immersed by addition of 2 mL isopropanol, and the plates were sealed with Parafilm to avoid evaporation and gently shaken at 120 rpm for 2 h at room temperature After this, all inserts were pierced with a pipette tip and discarded. Two 200 μL aliquots from each well were pipetted into a 96-well plate and the OD (absorbance / optical density, OD) at 570 nm was measured in a 96-well plate reader (Biotek PowerWave XS microplate spectrophotometer). Isopropanol was used as a blank with 6 replicates.

The percentage of reduction in viability is used to predict skin irritation potential. The reduction of cell viability in treated tissues was compared to that of negative controls. Three replicates for each sample and negative and positive controls were used. One valid experiment was performed.

2.7.4. In vitro skin corrosion test

The *in vitro* skin corrosion test was conducted using the OECD Guidelines for Testing of Chemicals, No. 431 (OECD, 2019). The method included a reconstructed three-dimensional human epidermis EpiDerm™ model comprising the organized basal, spinous, and granular layers and a multilayered stratum corneum (MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia, Batch No.: 40022/40023). Prior to starting the experiment, the assay medium was left to reach room temperature. The epidermis units were placed in a sterile 24-well plate for visual inspection. For both application times (3 min and 1 h), the appropriate number of wells of the assay plate were filled with the assay medium (Base medium: DMEM; Antibiotics: gentamicin; Anti-fungal agent: Fungizone; pH indicator: phenol red (except for PRF medium]), with 0.9 mL per well. The EpiDerm™ units were dried with a sterile cotton-tip swab and transferred to a 6-well plate pre-filled with the medium to avoid bubbles, then incubated for 60 ± 5 min at 37 °C in an incubator with 5 % CO_2.

During the pre-incubation time, two 24-well plates to be used as a "holding plate" (one for each application time) were prepared. Each well was filled with 0.3 mL of assay medium. In addition, two 24-well plates for the MTT assay were prepared and filled with 0.3 mL of MTT solution (1 mg/mL). The plates were incubated at 37 °C with 5 % CO_2 until use.

In the 1 h experiment, 50 μL of each nanoparticle sample or control solution (DPBS as a negative control, 8 N KOH as a positive control) was applied at one-minute intervals to a 3D reconstructed human epidermis model. The tissues were then incubated for 60 min at 37 °C with 5 % CO_2 and 95 % RH. After this, the EpiDermTM units were removed and rinsed thoroughly (20 times in a 60-sec timeframe) with DPBS to remove all residual test substance from the epidermal surface. The excess of DPBS was removed by gently shaking the inserts and drying them on blotting paper. After each rinsing, the insert was placed into the holding plate pre-filled with 0.3 mL of assay medium until all samples were washed. Each insert's surface was dried with a cotton swab. The viability of each

disk was assessed by incubating the tissues for 3 h with MTT solution at 37 °C, 5 % CO_2, and 95 % RH in the dark. Extraction of the formazan that formed was conducted as described above (cf. *In vitro* skin irritation test in Section 2.7.3), and the OD was measured at 570 nm in a 96-well plate reader (Biotek PowerWave XS microplate spectrophotometer). Isopropanol was used as a blank with 6 replicates.

The experiment with a 3-min application time was conducted as described above, except that after 3 min of incubation the plates were placed under the laminar flow hood for rinsing. Three replicates for each sample and the negative and positive controls were used for both application times. One valid experiment was performed.

2.7.5. Algae inhibition test

The algae inhibition test was based on DIN 38412–59 (DIN, 2022), OECD 201 (OECD, 2011) and ISO 8692 (ISO, 8692, 2012) standards. The aim of the test was to determine the ecotoxicity of the nanoparticles towards the green alga *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata*). An Altenburger medium was used as a test medium on multiwell plates (2.0 mL of test solution per 2.5-mL well) covered with a light-permeable foil. The test included 4 dilution levels of each nanoparticle sample with 3 replicates each. The test medium and potassium dichromate (0.8 mg/L) served as the negative and positive controls, respectively, with 3 replicates each. The static exposure (i.e., the test medium was not changed during the experiment) was conducted in microtiter plates over 72 h at 21–24 °C in a permanent light (60–120 μmol $m^{-2}s^{-1}$) with 100 ± 5 oscillations/min on a horizontal shaker. The number of algae cells of each test vessel was recorded daily via fluorescence intensity measurement (Infinite F Nano+ multiple reader, Tecan) for three days after the start of the exposure to calculate the growth rate. The device output (relative fluorescence units) was converted using a calibration curve (cells/mL). The data are presented as the inhibition of growth of algae cells (growth rate and yield) in relation to that of controls. Data were evaluated with Probit, Logit, and Weibull analysis using linear maximum likelihood regression, Williams' multiple *t*-test, and a multiple sequentially-rejective Welsh-*t*-test.

2.7.6. Daphnia sp. acute immobilization test

The *Daphnia* sp. acute immobilization test is based on OECD 202 (OECD, 2004) and ISO 6341 (ISO, 6341, 2012) standards and aims here to determine the acute toxicity of the nanoparticle samples to the water flea *Daphnia magna* (Straus). The test included five daphnids per test vessel. An Elendt M4 medium was used as a test medium with four dilution levels of each nanoparticle sample type, while the negative control consisted of the test medium only (40–50 mL /100 mL glass beaker, covered with a glass lid). The immobility of the daphnids was determined after 48 h of static exposure in a 16 h/8 h light/dark cycle at c. 20 °C (5.0–7.5 μmol $m^{-2}s^{-1}$). Data was evaluated by Probit and Weibull analyses using linear maximum likelihood regression, Fisher's exact binomial test, and the step-down Cochran–Armitage test procedure.

2.7.7. Daphnia magna reproduction test

The *Daphnia magna* reproduction test is based on the OECD 211 (OECD, 2012) standard with *D. magna* as a test species. This test was used to determine the chronic toxicity of the nanoparticle samples to *D. magna*. The Elendt M4 medium was used as a test medium for three dilution levels of each nanoparticle sample type with 5 replicates, and a negative control was performed using the test medium only with 10 replicates (70–90 mL /100 mL glass beaker, covered with a glass lid). One adult daphnid was inserted per beaker. The exposure of daphnids to nanoparticles was conducted over 21 days in a 16 h/8 h light/dark cycle (light intensity 11–17 μmol $m^{-2}s^{-1}$) at c. 20 °C in semi-static conditions. Daphnids were fed three times per week with a fresh algae suspension of *Desmodesmus subspicatus* (0.1–0.2 mg C/daphnia × d); the test medium was also renewed three times per week during exposure. Reproduction as assessed by fecundity (total number of living offspring [F1] per parental animal [F0]), whereas mortality/survival was assessed by immobility of parental (F0) daphnids. Data were evaluated with Probit and Weibull analyses using linear maximum likelihood regression, Fisher's exact binomial test, and the step-down Cochran–Armitage test procedure.

2.7.8. Toxicity test for earthworms

The toxicity test for earthworms was based on the OECD 207 (OECD, 1984) standard and was performed to determine the acute toxicity of the nanoparticle samples to the earthworm *Eisenia fetida*. To achieve this, a series of treated soils containing different portions of aqueous nanoparticle samples in relation to the maximum water content (soil moisture) was prepared. Three dilution levels of each nanoparticle sample and a negative control (test solution only) with 4 replicates each were analyzed. Adult earthworms (10 worms per 1000 mL Polystyrol beaker containing artificial soil according to ISO 11268–2 (ISO 11268–2, 2012); 644.8 g wet weight/beaker) were treated for 14 days with no feeding in permanent light (light intensity 5.0–7.5 μmol $m^{-2}s^{-1}$) at 20 ± 2 °C. The beakers were closed with a perforated transparent lid. Survival of worms in the treated test soil in relation to the untreated control soil was determined at the end of exposure.

2.7.9. Antibacterial analyses

The antibacterial properties of the nanoparticles were assessed using *Escherichia coli* K12 + pcGLS11 (gram-negative) and *Staphylococcus aureus* RN4220 +pAT19 (gram-positive) strains. The strains have been genetically modified to emit a continuous luminescent signal as a part of their normal metabolism (Vesterlund et al., 2004). When antibacterial substances are introduced to the strains, the measurable luminescent light signal decreases, with the reduction being dependent on the concentration of the antibacterial agent. For more information, the methodology has been reported in a previous study (Tienaho et al., 2021). In short, the strains were stored at −80 °C and revived through cultivation of approximately 16 h at 30 °C (for *E. coli*) and at 37 °C (for *S. aureus*) on lysogeny agar plates containing tryptone (10 g/L), yeast extract (5 g/L), NaCl (10 g/L), and agar (15 g/L). The plate supplementations for *E. coli* plates were 10 % (v/v) sterilized phosphate buffer (1 M, pH 7.0) and 100 μg/mL of ampicillin, versus 5 μg/mL erythromycin for the *S. aureus* plates. For stock solutions, a single colony was inoculated into lysogeny broth containing the same supplements and cultivated for 16 h at 300 rpm shaking at 30 °C (*E. coli*) and 37 °C (*S. aureus*). The nanoparticle samples were diluted in water to achieve nine V% concentrations of each sample: 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, and 50. Aliquots of 50 μL of the sample dilutions, positive controls (8.75 and 17.5 % [v/v] ethanol), and a negative control (double-distilled water) were added in triplicates to opaque white polystyrene microplates. The measurement was initiated with the addition of the bacterial inoculum (50 μL) to each well. The resulting luminescent signal was measured using a microplate reader (Varioskan Flash, Thermo Scientific) at c. 20 °C every 5 min up to 60 min, with the plate briefly being shaken before each measurement. Results are expressed as inhibition percentages (inhibition-%) against the w/v% of each nanoparticle sample dilution after 50 min of incubation. Inhibition percentages were calculated using the following formula:

$$\text{inhibition-\%} = (1\text{-}RLU_{sample}/RLU_{neg\_control}) \times 100\%,$$

where RLU represents relative light units obtained from the microplate reader.

2.7.10. Antioxidant analyses

Ferric-reducing antioxidant power (FRAP) method is based on the capacity of antioxidants to reduce chelated metal ions (Fe^{3+}) via the single electron transfer (SET) mechanism. In the presence of antioxidants, $[Fe(III)(TPTZ)_2]^{3+}$ is reduced and turns to a deep-blue colored compound. The used methodology was slightly modified from that

reported by Benzie and Strain (1996). Five dilutions of the sample nanoparticles in three technical replicates were investigated in a microplate format, as previously reported (Tienaho et al., 2021). An aliquot of 25 μL of the sample dilutions was added to a transparent flat-bottom 96-well plate with 50 μL of 300 mM acetate buffer (pH 3.6). The reaction was initiated by the addition of the reaction mixture containing 20 mM $FeCl_3$•6 H_2O and 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ; 100 μL/well). The formation of ferrous-tripyridyltriazine complex was then monitored in the dark by measuring the absorbance at 593 nm with a microplate reader (Varioskan Flash, Thermo Scientific). A dilution series of $FeSO_4$•7 H_2O was used to make a standard curve against which all the results were compared, and the sample color effect was minimized with blank subtraction. L(+)-ascorbic acid (150 μM and 800 μM; VWR Chemicals) was used as a positive control, and the results are expressed as μM Fe(II) equivalents per nanoparticle dispersion weight-%.

Oxygen radical absorbance capacity ($ORAC_{FL}$) is based on the ability of antioxidants to prevent the peroxyl radical from harming a fluorescent molecule fluorescein. The method follows the hydrogen atom transfer (HAT) mechanism and follows a previously reported protocol (Huang et al., 2002; Prior et al., 2003). For more information, the detailed experimental setup has been published (Tienaho et al., 2020). In brief, all the samples were measured in five dilutions and with two technical replicates, and additional dilutions were prepared if needed. The reaction mixture consisted of 25 μL nanoparticle dilutions in 75 mM phosphate buffer (PB), pH 7.5 (Merck) (25 μL), and 0.0816 μM fluorescein (150 μL) as well as 0.153 mM peroxyl radical creator 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) (25 μL) in a black opaque flat-bottom 96-well plate. Fluorescence (emission 538 nm; excitation 485 nm with a bandwidth of 12 nm) decay was monitored with a microplate reader (Varioskan Flash, Thermo Scientific) every 2 min 21 times. Overall, the program takes 54 min from the injection of AAPH. The temperature was kept at 37 °C, and the plate was briefly shaken before each measurement. As a vitamin E analogue, Trolox ([±]-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) concentrations of 50, 25, 12.5, and 6.25 μM were used to draw the standard curve against which all the results were compared. Results are expressed as Trolox equivalents (TEs) per nanoparticle dispersion weight-% (μM TE /g).

2.8. Total phenolic contents by Folin-Ciocalteu

Nanoparticle samples were analyzed for their total phenolic content by the well-reported Folin-Ciocalteu method (Singleton and Rossi, 1965; Singleton et al., 1999; Ainsworth and Gillespie, 2007). In brief, all the samples were analyzed in four dilutions and with three technical replicates of 160 μL each and then mixed with 10 μL of the Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) in a transparent flat-bottom 96-well plate. Measurement was initiated by adding 30 μL of 20 % Na_2CO_3 (Merck KGaA, Darmstadt, Germany). After 45 min of incubation at 40 °C in the dark, absorbance was measured at 750 nm. The sample color effect was minimized with blank subtraction. The standard curve was prepared using gallic acid (0, 25, 100, 250, and 500 mg/L; stock solution 5 g/L [29.4 mM]). The results are expressed as gallic acid equivalents per nanoparticle dispersion weight-% (mg GAE /g).

3. Results

To study possible health and environmental effects, nanoparticles were prepared from lignin (LNPs), lignin esterified with tall oil fatty acid (TOFA-LNPs), or from suberin (SNPs). The surface morphology of the nanoparticles was investigated via scanning electron microscopy (Fig. 1). LNPs were slightly smaller than those prepared from TOFA-lignin with hydrodynamic diameters of 83 ± 1 and 105 ± 1 nm, respectively, whereas SNPs did not show a particle-like appearance and instead had irregular needle-like structures (Fig. 1; Supplementary materials Table S1). The surface charge of both nanoparticle types prepared from lignin were similar, with zeta potentials of −25 ± 1 mV, while suberin nanoparticles exhibited a zeta potential of −35 mV. The pH of all nanoparticle samples in aqueous solutions was acidic (4.3–4.9).

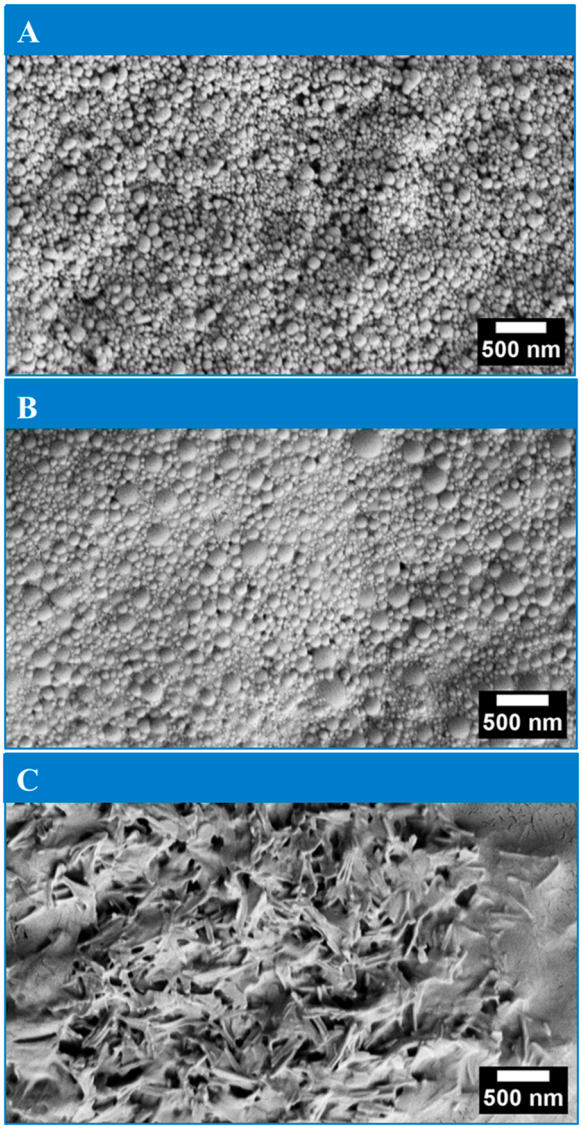

Fig. 1. Scanning electron microscopy images of nanoparticles. (A) Lignin nanoparticles, (B) TOFA-lignin nanoparticles, (C) suberin nanoparticles.

3.1. Toxicity studies in vitro

The results obtained from the toxicity tests using cell lines derived from murine fibroblasts and human keratinocytes, or by using the human epidermis model, indicated that the nanoparticles were safe at the studied concentrations according to the test of cytotoxicity and skin irritation or corrosion. Detailed results are shown in Tables S2–S4. For skin sensitization, on the other hand, LNPs at 70.31 μg/mL (w/v) and TOFA-LNPs at 125 μg/mL (w/v) were positive (showing sensitization for skin), whereas SNPs were negative for skin sensitization (Table 1; Fig. 2.).

3.2. Ecotoxicity tests

According to the ecotoxicity tests, all the nanoparticle types affected the aquatic test organisms, algae, and daphnids. These effects were

Table 1
Skin sensitization test. The maximal average fold induction of luciferase activity (Imax), cell viability (%), and EC1.5 values after treatment of KeratinoSens™ cells with nanoparticles prepared from lignin, TOFA-lignin, and suberin. EC1.5 is an interpolated concentration resulting in a 1.5-fold luciferase induction. There was a dose-dependent increase in luciferase induction in each sample. A sample is considered to be positive (i.e., shows sensitization for skin) if all the following four conditions are met, otherwise the sample is negative (i.e., not showing sensitization for skin): the Imax is ≥ 1.5-fold and statistically significantly different than the control; cellular viability is > 70 % at the lowest concentration, with induction of luciferase activity ≥ 1.5-fold; the EC1.5 value is < 200 μg/mL for test chemicals with no defined MW (molecular weight); there is a dose-dependent increase in luciferase induction.

Measured value		Concentration (μg/mL)		
	Experiment	LNP	TOFA-LNP	SNP
Imax	1	2.2	2.3	2.5
	2	5.7	6.1	5.8
Cell viability (%)	1	96.3	101.4	104.8
	2	91.5	97.1	105.2
EC1.5 (μg/mL)	1	22.50	145.80	1461.90
	2	81.00	80.80	1056.80
Ind. Dose-dep.	1	Yes	Yes	Yes
	2	Yes	Yes	Yes
Classification		Positive	Positive	Negative

measured in the algae inhibition test in terms of yield and growth rate (Table 2 and Table S5), in the *Daphnia* sp. immobilization test for acute toxicity (Table 3 and Table S6), and in the *Daphnia* reproduction test for chronic toxicity (Table 3 and Table S7).

All nanoparticles showed effects on the growth of algae with a similar range of concentrations. SNPs were the least toxic when comparing EC50 values in both tests (yield, growth rate) to other nanoparticle samples (i.e., the effects were induced by the highest concentrations in both tests; Table 2). However, SNPs had the lowest LOEC and NOEC concentrations in both tests from all samples, with the values varying between 0.64 and 3.2 mg/mL (Table 2).

LNPs were the least toxic to *Daphnia* in the acute immobilization test and in the chronic reproduction test (Table 3) as compared to the other nanoparticles. This means that the concentration needed to induce effect was the highest for LNPs compared to the TOFA-LNP and SNP. TOFA-LNPs were the most toxic in the *Daphnia* acute immobilization test, with LOEC, NOEC, and EC50 values of 200, 13.3, and 51.2 μg/mL, respectively (Table 3). The concentration of SNPs were two times higher in EC50 and LOEC values on the Daphnia immobilization test as compared to TOFA-LNPs. In contrast, in the chronic *Daphnia* reproduction test, SNPs were more toxic than TOFA-LNPs (Table 3), with EC50 and LOEC values of 5.4 and 5.0 μg/mL, respectively. The corresponding values for TOFA-LNPs were 12.2 and 10.0 μg/mL, respectively. However, TOFA-LNPs' NOEC values were the lowest of all in the *Daphnia* reproduction test (Table 3).

None of the nanoparticle samples showed toxicity on earthworms, meaning their mortality did not increase in any of the concentrations tested (Table S8). Thus, the EC50 and NOEC values were higher than the highest tested concentrations (9000, 2000, and 8000 μg/mL for LNPs, TOFA-LNPs, and SNPs, respectively).

3.3. Antibacterial activity

The antibacterial activity of each nanoparticle dispersion was investigated against recombinant bioluminescent indicator strains of *E. coli* K12 +pcGLS11 (gram-negative) and *S. aureus* RN4220 +pAT19 (gram-positive). Both strains are of epidemiological importance and have been shown to be the leading bacterial pathogens of healthcare-associated infections and bacteremia (Poolman and Anderson, 2018; Abban et al., 2023). TOFA-LNP samples showed the highest inhibitory activity against both bacterial strains in all concentrations tested, and the LNPs had also high activities (Fig. 2). However, the SNP samples showed low activity against *E. coli*, with only the highest concentration giving inhibition-% values above zero (Fig. 3**A**). Surprisingly, the inhibition value development of SNPs was not dose-dependent against *S. aureus*, and the highest inhibition was instead observed with 0.025 % (w/w; Fig. 3B). The reason for this is likely explained by SNPs containing a source of nutrition for the strain. This feature results into a phenomenon that higher amount of particles also contain more nutrition, which exceeds the amount of antibacterial activity caused by the particles.

3.4. Antioxidant and total phenolic content

In addition to the antibacterial effects, we were interested in the potential antioxidant activity and total phenolic content of the nanoparticle dispersions. Both LNPs and TOFA-LNPs showed high antioxidant activity in the FRAP test (Fig. 4A) as well as high total phenolic content (Fig. 4C). Similarly, the LNP dispersion showed high activity in the ORAC test, while the TOFA-LNPs activity was lower in a statistically significant manner (Fig. 4B). SNP activity was very low in all the tests.

4. Discussion

Safety of all chemicals need to be assessed in the EU before entering to the market. The data obtained from these evaluations can be used for REACH and CLP assessments of these substances, but also environmental impact assessment of the products' lifecycle. Based on our study, nanoparticles prepared from the hydrolyzed suberin (SNPs), and lignin (LNPs) were not cytotoxic, irritant, or corrosive to skin. However, LNPs at high concentrations sensitized skin, whereas SNPs did not. According to the ecotoxicity assays, all aqueous dispersions of nanoparticles exhibited clear effects on aquatic organisms, algae, and daphnids, whereas they were harmless to earthworms in the studied concentrations. Both LNP and TOFA-LNP showed high antibacterial and antioxidant activities. According to our knowledge, this is the first report investigating the toxicity of TOFA-LNPs and ecotoxicity of both TOFA-LNPs and SNPs, in addition to skin sensitization, corrosion, and irritation effects of LNPs. However, some studies have been performed on isolated suberin and lignin, as well as for LNPs as described in more detailed in the following paragraphs.

Lignin (CAS 8068–05–1) has not been registered in REACH, and thus the European Chemicals Agency (ECHA) does not have any toxicological or ecotoxicological data. However, lignin has been signaled to the Classification and Labelling (C&L) inventory database (ECHA (European Chemical Agency), 2024), where it is classified as skin-irritative, skin--sensitive, and eye-irritative and as potentially causing respiratory irritation. According to our results, LNPs, TOFA-LNPs, and SNPs were not corrosive or irritative for skin at the studied concentrations. In line with this, Carriço et al. (2019) also determined that suberin is not a skin irritant. In our study, LNPs and TOFA-LNPs sensitized skin at 70.31 μg/mL and 125 μg/mL, respectively. SNPs, on the other hand, did not sensitize skin. The choice of the nanoparticle concentrations was based on the established protocols of our laboratory (Babaeipour et al., 2024). These concentrations have proven to be suitable for both spray coating and layer-by-layer deposition on textile. In real-world applications, the nanoparticles-based coatings adhere to the surface with limited mobility and significantly reduced contact area compared to their dispersed form. As a result, the potential for direct skin exposure and sensitization under normal use conditions is expected to be much lower than that observed *in vitro*. Furthermore, the textile coatings are suggested for protective outdoor clothing, that typically contain inner lining where no exposure for direct contact is not expected. Unlike many commercial textile coatings that are several micrometers thick, the multifunctional coating based on lignin nanoparticles has a thickness of less than approximately 300 nm. It has been demonstrated that the majority of these nanoparticles remain on the fabric even after washing (Babaeipour et al., 2024), and the small amounts that may be gradually released would be highly diluted in the water system, rendering the

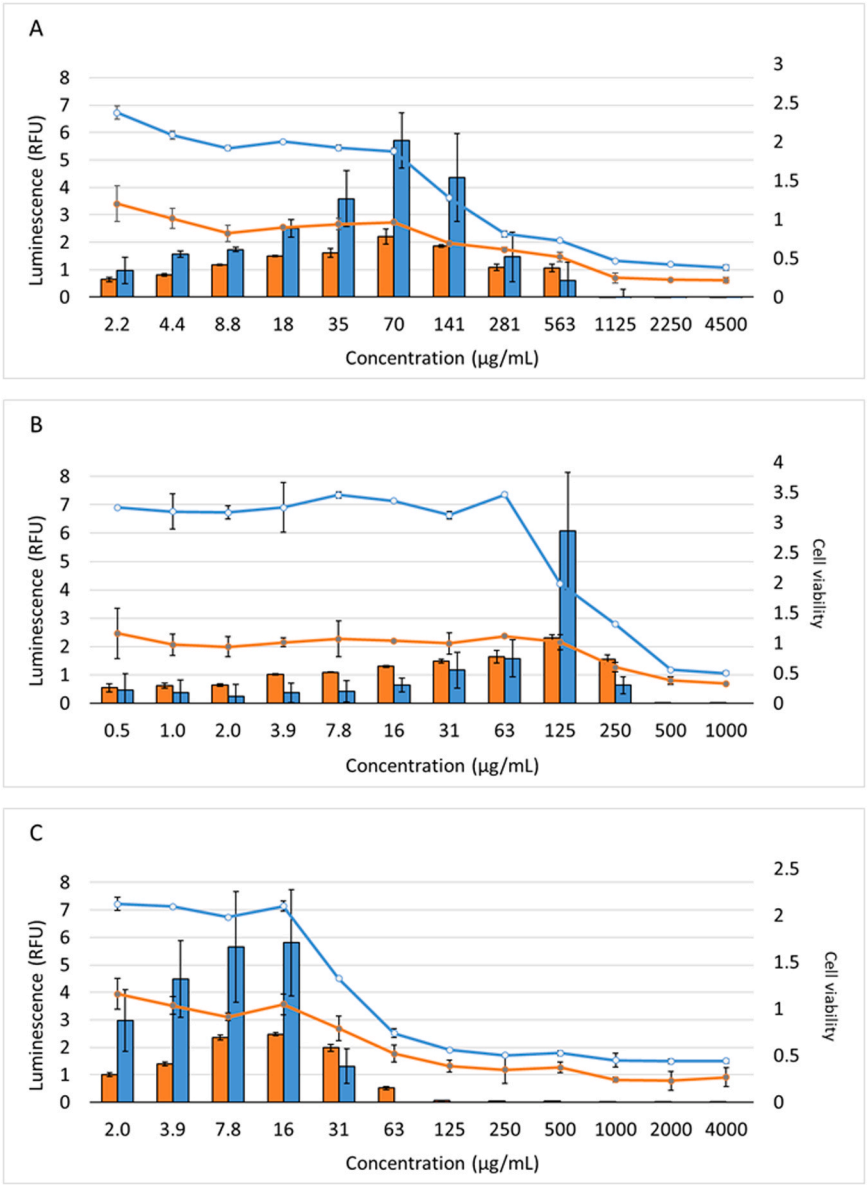

Fig. 2. Skin sensitization test. Luciferase activity (bars), and cell viability (lines) values after treatment of KeratinoSens™ cells with nanoparticles prepared from lignin, TOFA-lignin, and suberin (A, B, and C, respectively) in two independent experiments shown by light and dark gray color. Data are presented as mean ± SD, and they were normalized to solvent control levels (1.0).

Table 2
EC, LOEC, and NOEC values for the yield and growth rate of algae in the algae inhibition test. LNPs = lignin nanoparticles, TOFA-LNPs = TOFA-lignin nanoparticles, SNPs = suberin nanoparticles. EC = effective concentration; NOEC = No Observed Effect Concentration; LOEC = Lowest Observed Effect Concentration.

Measured value		Concentration (µg/mL)		
		LNP	TOFA-LNP	SNP
Yield of Algae	EC10	2.9	5.5	1.3
	EC20	3.9	6.6	3.2
	EC50	6.5	9.4	12.5
	LOEC	3.6	20.0	3.2
	NOEC	0.7	4.0	0.6
Growth Rate of Algae	EC10	2.3	9.8	12.0
	EC20	6.3	11.9	19.3
	EC50	34.9	17.4	39.7
	LOEC	18.0	20.0	3.2
	NOEC	3.6	4.0	0.6

Table 3
EC, LOEC, and NOEC values obtained from the *Daphnia* immobilization (acute toxicity) and *Daphnia* reproduction (chronic toxicity) tests. LNPs = lignin nanoparticles, TOFA-LNPs = TOFA-lignin nanoparticles, SNPs = suberin nanoparticles. EC = effective concentration; NOEC = No Observed Effect Concentration; LOEC = Lowest Observed Effect Concentration.

Test	Measured value	Concentration (µg/mL)		
		LNP	TOFA-LNP	SNP
Daphnia Acute	EC50	1242.0	51.2	74.4
	LOEC	900.0	200.0	400.0
	NOEC	450.0	13.3	26.6
Daphnia Chronic	EC50	402.3	12.2	5.4
	LOEC	900.0	10.0	< 5.0
	NOEC	180.0	2.5	≤ 5.0

environmental impact negligible. Moreover, hardwood or softwood lignin and suberin are natural components already present in ecosystems and aquatic environments. The lignin that may be released during

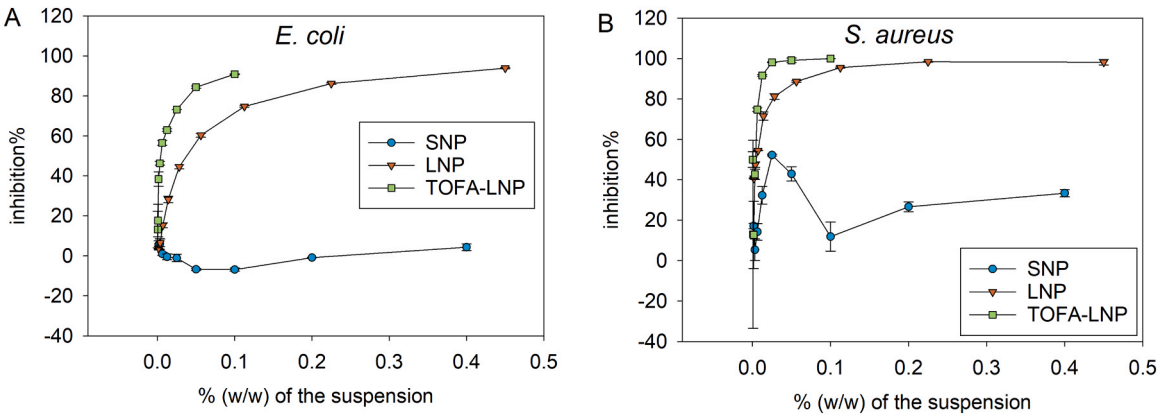

Fig. 3. Antibacterial effects of suberin, lignin, and TOFA-lignin nanoparticles (SNPs, LNPs, and TOFA-LNPs, respectively) against *E. coli* (A) and *S. aureus* (B). Results are expressed as averages of three dilution replicates ± standard deviation and normalized against % (w/w) of the dispersions.

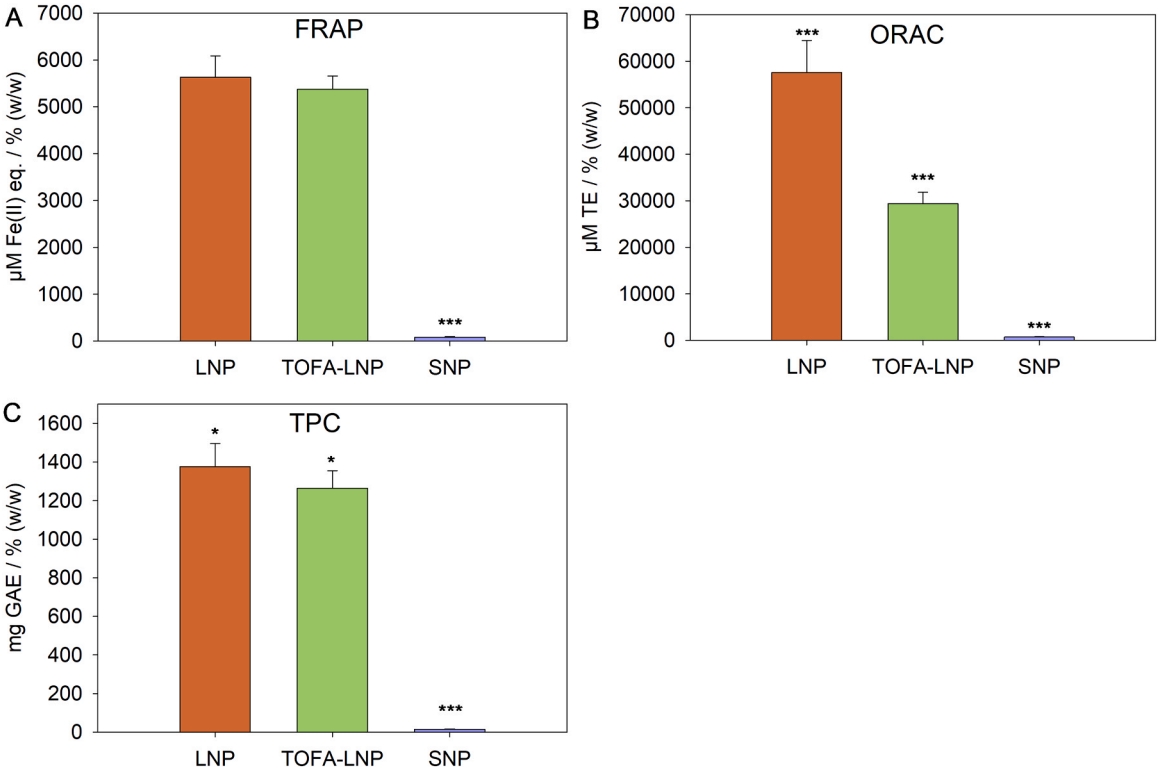

Fig. 4. Antioxidant activities in ferric-reducing antioxidant power (FRAP) (A) and oxygen radical absorbance capacity (ORAC) (B) tests as well as the total phenolic content (TPC) measured via the Folin-Ciocalteu method (C) of the nanoparticle dispersions. Results are expressed as averages of four to 10 concentration replicates ± standard deviation and normalized against % (w/w) of the dispersions. The statistical differences are indicated with asterisks: *** = $p < 0.0005$; ** = $p < 0.005$, * = $p < 0.05$. LNPs = lignin nanoparticles, TOFA-LNPs = TOFA-lignin nanoparticles, SNPs = suberin nanoparticles.

washing retains similar aromatic molecular structure as that which naturally occurs through biomass degradation, assuming posing no additional environmental risk. Furthermore, we have shown that the stability of the coating can be improved by oxidative crosslinking to prevent migration or leaching in packaging materials (Babaeipour et al., 2025).

Based on our results, the nanoparticles were not cytotoxic to the murine fibroblast cell line. Pure LNPs together with modified LNPs (at ≤ 100 µg/mL) have shown good cytocompatibility with several cancer cell lines, making them promising candidates for drug delivery applications (Figueiredo et al., 2017; Imlimthan et al., 2019). Similarly, LNPs were not cytotoxic at concentrations below 100 µg/mL after 24 h of incubation with human Caco-2 cells (Freitas et al., 2020). Additionally, the mouse fibroblast NIH-3T3 cell viabilities decreased below 90 % in 72-h incubation when the LNP concentrations were over 500 and 200 µg/mL, respectively, depending on the method of the LNPs' preparation (Chen et al., 2018).

According to the ecotoxicity tests, the effects of different nanoparticles varied in the acute and chronic tests. In addition, the hazardous nature of the substances varied across the different tests. All nanoparticles in aqueous dispersions showed clear effects on the aquatic test organisms, the alga *Pseudokirchneriella subcapitata*, and the daphnid *Daphnia* sp. Both the yields (EC50 values 6.5–12.5 µg/mL) and growth rate (EC50 values 17.4–39.7 µg/mL) of algae were inhibited in the 72-h algal test. The nanoparticles also restricted the mobility of daphnids in the *Daphnia* acute immobilization test (EC50 values 51.2–1242 µg/mL)

and in the chronic reproduction tests (EC50 values 5.4–402.3 μg/mL). Rivière et al. (2021) obtained similar results: EC50 values in the acute *Daphnia magna* 24-h and 48-h tests for anionic and cationic colloidal lignin particles were over 500 μg/mL. In addition, the EC50 values of the colloidal LNPs in the algae 72-h inhibition test varied between 15.9–43.2 μg/mL (Rivière et al., 2021). However, LNPs at 1.5 mg/mL were found not to be toxic to the microalga *Chlamydomonas reinhardtii* or the yeast (*Saccharomyces cerevisiae*) in a 1-h treatment (Frangville et al., 2012).

In the algae inhibition test with increased proportions of the aqueous dispersions of LNPs and TOFA-LNPs, the test solutions turned brownish, starting with 18.0 μg/mL and 20.0 μg/mL of LNP and TOFA-LNP samples, respectively. The browning may have affected the light conditions of the algae at the 72-h treatment, leading to growth-delaying effects at and above these concentrations. In the *Daphnia* immobilization test, addition of the LNP dispersion to the growth solution of *Daphnia* led to a decrease in pH from 7.8 (in controls) by 0.7, 1.0, and 1.5 units when the LNP concentration was 450, 900, or 2250 μg/mL, respectively. The pH was still in a suitable range to cultivate *D. magna* (pH 6–9; OECD, 2004). Conversely, SNPs formed precipitates in the *Daphnia* reproduction test at all dilution levels, with higher amounts at higher concentrations. The observed effects of SNPs may be caused by a physical stress due to precipitates formed in the experimental solutions. In our study, the nanoparticles were not toxic to terrestrial organisms (earthworms) at the studied concentrations (2000–9000 μg/mL). We did not find any other results based on similar test methods.

In the antibacterial tests, TOFA-LNP samples showed the highest inhibitory activity against both bacterial strains. Previous research has demonstrated that TOFA-LNP coatings on cotton textiles exhibit antimicrobial properties, with TOFA-LNPs achieving slightly higher bacterial inhibition (51 %) than pure LNPs (46 %) against *Staphylococcus aureus (*Babaeipour et al., 2024*)*. This enhanced effect is due to the inhibitory role of the unsaturated fatty acids in TOFA, which hinder bacterial growth. The higher bacterial inhibition observed in the current study can be explained by the increased surface area of the particle dispersion, which enhances interactions with bacterial cells compared to coated textiles with particles. Because of the way the TOFA-LNPs were produced, only traces of resin acids are left as residuals, which means that their role should be insignificant in the detected activities. Several other studies have also reported LNP dispersions to be strong inhibitors of bacteria (reviewed by Morena and Tzanov, 2022). While multiple mechanisms of action have been suggested for lignin and LNPs, systematic studies on penetration and membrane-disturbing capabilities are needed to elucidate the varying efficacies against gram-positive and gram-negative bacteria. The low inhibition of SNPs in the antibacterial tests (Fig. 2) are likely caused by the combination of the larger particle size and consequently reduced reactive surface area of the particle suspension as well as suberin's structure, which contains both poly-aromatic and poly-aliphatic domains (Kolattukudy, 2001; Bernards, 2002). The SNPs were produced from hydrolysed suberin and not suberin as such. During the hydrolysis, the long chain fatty acids are cleaved from the polymer and isolated as a mixture of free acids. The suberin hydrolysate fraction contains high amounts of free fatty acids and low amounts of free aromatic residues (Hu et al., 2024). While even low amounts of aromatic structures can be effective against bacteria, the aliphatic part can be a source of nutrition to the bacterial strains. Thus, the concentration needs to be balanced for the maximum effect. It has been suggested that the complex structure of suberin makes it resistant to microbial degradation and the presence of ferulic acid and betulin derivative traces can enhance the antimicrobial effects (Qasim et al., 2024; Xie et al., 2023). In a yet to be peer-reviewed manuscript by Correia et al. (2025), it was found that suberin interacts with cell membranes of both *E. coli* and *S. aureus* bacteria leading to cell death. They also indicated that activity was altered by hydrolyzation of suberin cross-linking, which decreased the activity against gram-negative strains and increased activity against gram-positive ones (Correia et al., 2025). However, their conclusions state that the bactericidal mechanism of suberin seems to be atypical for membrane-targeting antimicrobial polymers and is not aligned with the current understanding of them. Thus, further studies are needed to fully understand the reasons behind antimicrobial effects of suberin and suberin based materials.

Similar to our findings, also previous literature has concluded that lignin and LNPs have high antioxidant and radical scavenging activities (Morena and Tzanov, 2022; Ali et al., 2024) Antioxidant activity is caused by the complex structure of lignin, containing aromatic rings with hydroxy and methoxy functional groups (Ali et al., 2024). Both LNPs and TOFA-LNPs had high total phenolic content, which is surprising in the case of the TOFA-LNP sample. It is possible that TOFA reacts mainly with aliphatic hydroxyls, but on the other hand, esterification also significantly reduces the fraction of lignin in the sample concentrations. The ^{31}P NMR analysis of synthesized TOFA-lignin by Babaeipour et al. (2024) revealed that the phenolic content of TOFA-lignin was 2.13 mmol/g and lignin was 3.41 mmol/g, meaning that around 60 % of phenols were free compared to non-derivatized lignin. Here, the values are almost equal. However, these measurements were conducted in dispersions, where free hydroxyls may participate in self-assembly.

It is also possible that the high antioxidant activity observed in the FRAP test is due to the antioxidant properties of lignin, which have previously been attributed to phenolic hydroxyl groups (Li et al., 2023). Similarly, the LNP dispersion showed high activity in the ORAC test, whereas the activity of TOFA-LNPs was significantly lower. Because of the different mechanisms of the FRAP and ORAC methods (Benzie and Strain, 1996; Huang et al., 2002; Prior et al., 2003), it is not unusual for the tests to yield different results even though they both assess antioxidant properties. TOFA-LNPs therefore exhibit lower peroxyl radical absorption compared to LNPs without the addition of TOFA.

Bio-based compounds, as green alternatives, need to be developed for surfaces of clothing and packaging to replace fossil-based compounds, and to make recycling easier and to avoid harmful chemicals. For example, extreme persistent fluoropolymers, which are a group of polymers within the class of per- and polyfluoroalkyl substances (PFAS; Henry et al., 2017), can cause serious effects on humans and the environment (e.g., Lohmann et al., 2020) and have considerably higher global warming potential than bio-based alternatives (Yadav et al., 2024). Similarly to lignin, polytetrafluoroethylene (CAS 9002–84–0) has not been registered in REACH, but its data has been notified to the C&L inventory (ECHA (European Chemical Agency), 2024). It has been classified as skin irritative and eye irritative, and it may cause respiratory irritation and damage to organs through prolonged and repeated exposure. It may also cause long-lasting harmful effects to aquatic life (ECHA (European Chemical Agency), 2024). Suberin biopolymers are readily decomposed (Angst et al., 2016). Lignin as well as lignin nanoparticles are biodegradable (Schneider et al., 2021). To our knowledge, biodegradability of suberin- and TOFA-lignin nanoparticles has not yet been studied. Thus, further studies on the fate of nanoparticles, such as nanoparticle release, migration, and transformation in practical applications, and effects on humans and the environment are needed to conclude safety of these substances before applications are ready for market. However, these bio-based nanoparticles provide a potential alternative to fossil-based compounds.

We selected some in vitro tests as a starting point for investigating the safety of the nanoparticles. For human toxicity, basic cytotoxicity was investigated. In addition, because of the properties of our chemicals for clothing, we chose the tests for skin in vitro: skin sensitization, skin corrosion, and skin irritation. For the ecotoxicity, basic tests were chosen for three different organism level: acute test for algae, acute and chronic test for Daphnia magna, and acute test for earthworms. Any in vivo tests, including tests on fish, were not yet performed. This is a clear limitation of our study. Future investigations are needed to investigate the biodegradability of nanovesicles, and effects on other organisms, including ecotoxicity on marine organisms and vertebrates, such as fish.

In addition, more toxicity tests to study effects on humans are needed. These aspects should also be studied in the future to provide a more comprehensive picture of (environmental) safety assessment and ecological relevance of the studied chemicals, and to complement the requirements of the EU's chemical legislation for marketing purposes, which was not in the scope of our study.

EU's REACH and CLP regulations aim to ensure a high level of protection of human health and the environment as well as the free movement of chemical substances, mixtures and certain specific articles. The regulations include safety testing of chemicals for health hazards (toxicological acute and chronic tests), physical hazards (physical and chemical properties) and environmental hazards (ecotoxicological acute and chronic tests). Our findings are preliminary and represent only the early stages of safety evaluation. For these materials to be considered viable for commercial adoption, further research is essential, particularly long-term (eco)toxicity assessment, and environmental fate and large-scale production assessments. In this context we have conducted and published an LCA of suberin and betulin production from birch bark (Yadav et al., 2024), providing insight into environmental performance.

5. Conclusions

Bio-based nanoparticles offer a promising tool to coat surfaces of textiles or paper board and offer alternatives to long-lasting, fossil-based chemicals. However, before bio-based nanoparticles can be put on the market, it is important to study their effects on humans and the environment. In the present study, nanoparticles produced from kraft lignin, tall oil fatty acid ester of lignin, and suberin were observed to be non-cytotoxic, non-corrosive, and non-irritative for skin as well as non-toxic to earthworms. LNPs and TOFA-LNPs, however, sensitized skin, and all aqueous nanoparticle dispersions affected aquatic test organisms, algae, and daphnids. High antioxidant and antibacterial activity among LNPs and TOFA-LNPs is likely attributable to their high total phenolic content, whereas the complexity of the SNP structure resulted in lower activities in both tests. While our results are in line with previous studies, they also enhance knowledge of the safety of these nanoparticles. The results highlight the need for thorough assessment and tailored risk management strategies to ensure the safe and sustainable use of lignin and suberin nanoparticles in various applications. This information is crucial for the future applications of these nanoparticles.

To fully consider the risks associated with the studied nanoparticles, more information on their bioactivity and potential adverse effects on humans, such as genotoxicity and mutagenicity, is required. Additionally, further studies on their fate and effects on the environment, including biodegradation and impacts on marine organisms and vertebrate species such as fish, are necessary. Moreover, comprehensive toxicity and ecotoxicity studies with different organisms to investigate both acute and chronic responses are essential to determine the safety of these substances. Our results show that the investigated nanoparticles are potential alternatives to fossil-based and more harmful chemicals. The present investigation supports the ongoing effort to develop green alternatives for coating of textiles that meet safety standards and ultimately promote ecological sustainability.

CRediT authorship contribution statement

Pekka Saranpää: Writing – review & editing, Resources, Project administration, Funding acquisition. **Monika Österberg:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Pooja Yadav:** Writing – review & editing. **Risto Korpinen:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Jenni Tienaho:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Kärkönen:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kati Räsänen:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tarmo Räty:** Writing – review & editing. **Sahar Babaeipour:** Writing – review & editing, Methodology, Investigation. **Muhammad Farooq:** Writing – review & editing, Methodology, Investigation, Data curation. **Paula Nousiainen:** Writing – review & editing, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.122226.

Data availability

Data will be made available on request.

References

Abban, M.K., Ayerakwa, E.A., Mosi, L., Isawumi, A., 2023. The burden of hospital acquired infections and antimicrobial resistance. Heliyon 9 (10), e20561. https://doi.org/10.1016/j.heliyon.2023.e20561.

Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin–Ciocalteu reagent. Nat. Protoc. 2, 875–877. https://doi.org/10.1038/nprot.2007.102.

Ali, M.A.S., Abdel-Moein, N.M., Owis, A.S., Ahmed, S.E., Hanafy, E.A., 2024. Eco-friendly lignin nanoparticles as antioxidant and antimicrobial material for enhanced textile production. Sci. Rep. 14 (1), 17470. https://doi.org/10.1038/s41598-024-67449-0.

Angst, G., Heinrich, L., Kögel-Knabner, I., Mueller, C.W., 2016. The fate of cutin and suberin of decaying leaves, needles and roots – Inferences from the initial decomposition of bound fatty acids. Org. Geochem. 95, 81–92. https://doi.org/10.1016/j.orggeochem.2016.02.006.

Babaeipour, S., Nousiainen, P., Kimiaei, E., Tienaho, J., Kohlhuber, N., Korpinen, R., Kaipanen, K., Österberg, M., 2024. Thin multifunctional coatings for textiles based on the layer-by-layer application of polyaromatic hybrid nanoparticles. Mater. Adv. 5, 6114–6131. https://doi.org/10.1039/d4ma00085d.

Babaeipour, S., Nousiainen, P., Garcia, E.M., Mohammadi, P., Vuoriluoto, M., Kimiaei, E., Koivula, H., Österberg, M., 2025. Oxidative crosslinking for the development of barrier coatings utilizing lignin-containing cellulose nanofibrils and lignin nanoparticles. Food Packag. Shelf Life 49, 101538. https://doi.org/10.1016/j.fpsl.2025.101538.

Barros, J., Serk, H., Granlund, I., Pesquet, E., 2015. The cell biology of lignification in higher plants. Ann. Bot. 115, 1053–1074. https://doi.org/10.1093/aob/mcv046.

Benzie, I.F.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. 239, 70–76. https://doi.org/10.1006/abio.1996.0292.

Bernards, M.A., 2002. Demystifying suberin. Can. J. Bot. 80, 227–240. https://doi.org/10.1139/b02-017.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519–546. https://doi.org/10.1146/annurev.arplant.54.031902.134938.
Carriço, C., Pinto, P., Graça, A., Gonçalves, L.M., Ribeiro, H.M., Marto, J., 2019. Design and characterization of a new quercus suber-based pickering emulsion for topical application. Pharmaceutics 11, 131. https://doi.org/10.3390/pharmaceutics11030131.
Chen, L., Zhou, X., Shi, Y., Gao, B., Wu, J., Kirk, T.B., Xu, J., Xue, W., 2018. Green synthesis of lignin nanoparticle in aqueous hydrotropic solution toward broadening the window for its processing and application. Chem. Eng. J. 346, 217–225. https://doi.org/10.1016/j.cej.2018.04.020.
Correia, V.G., Nunes, F., Rodrigues, R., Cairrão, A., Domingos, P., Tomé, A., Barras, R., Oliva, A., Pereira, C.S., Melo, M.N., 2025. Hydrophobic polymeric particles of the plant polyester suberin destroy bacteria by interacting with their membranes. A Prepr. ChemRxiv 2025. https://doi.org/10.26434/chemrxiv-2025-t6n9l.
DIN, 2022. German standard methods for the examination of water, waste water and sludge - Test methods using water organisms (group L) - Part 59: Algal growth inhibition test on microplate with unicellular green fresh water algae (L 59). DIN 38412–38459.
Dixon, R.A., Puente-Urbina, A., Beckham, G.T., Román-Leshkov, Y., 2024. Enabling lignin valorization through integrated advances in plant biology and biorefining. Annu. Rev. Plant Biol. 75, 239–263. https://doi.org/10.1146/annurev-arplant-062923-022602.
EC, 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. ⟨https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32006R1907⟩.
EC, 2008. Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006 (Text with EEA relevance). ⟨https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32008R1272⟩.
EC, 2020. Communication from the commission to the European parliament, the council, the European economic and social committee and the committee of the regions. Chemicals strategy for sustainability towards a toxic-free environment. ⟨https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A52020DC0667⟩.
ECHA (European Chemical Agency). 2024. Search for chemicals. ⟨https://echa.europa.eu/information-on-chemicals⟩.
Farooq, M., Zborowski, C., Nousiainen, P.A., Tienaho, J., Korpinen, R., Österberg, M., 2025. Supramolecular architectures based on the self-assembly of suberin hydrolysate, betulin, and their hybrids. Langmuir 41, 19156–19172. https://doi.org/10.1021/acs.langmuir.5c01278.
Figueiredo, P., Lintinen, K., Kiriazis, A., Hynninen, V., Liu, Z., Bauleth-Ramos, T., Rahikkala, A., Correia, A., Kohout, T., Sarmento, B., Yli-Kauhaluoma, J., Hirvonen, J., Ikkala, O., Kostiainen, M.A., Santos, H.A., 2017. In vitro evaluation of biodegradable lignin-based nanoparticles for drug delivery and enhanced antiproliferation effect in cancer cells. Biomaterials 121, 97–108. https://doi.org/10.1016/j.biomaterials.2016.12.034.
Frangville, C., Rutkevičius, M., Richter, A.P., Velev, O.D., Stoyanov, S.D., Paunov, V.N., 2012. Fabrication of environmentally biodegradable lignin nanoparticles. Chem. Phys. Chem. 13, 4235–4243. https://doi.org/10.1002/cphc.201200537.
Freitas, F.M.C., Cerqueira, M.A., Gonçalves, C., Azinheiro, S., Garrido-Maestu, A., Vicente, A.A., Pastrana, L.M., Teixeira, J.A., Michelin, M., 2020. Green synthesis of lignin nano- and micro-particles: physicochemical characterization, bioactive properties and cytotoxicity assessment. Int. J. Biol. Macromol. 163, 1798–1809. https://doi.org/10.1016/j.ijbiomac.2020.09.110.
Graça, J., 2015. Suberin: the biopolyester at the frontier of plants. Front. Chem. 3, 62. https://doi.org/10.3389/fchem.2015.00062.
Harman-Ware, A.E., Sparks, S., Addison, B., Kalluri, U.C., 2021. Importance of suberin biopolymer in plant function, contributions to soil organic carbon and in the production of bio-derived energy and materials. Biotechnol. Biofuels 14, 1–21. https://doi.org/10.1186/s13068-021-01892-3.
Henn, K.A., Forsman, N., Zou, T., Österberg, M., 2021. Colloidal lignin particles and epoxies for bio-based, durable, and multiresistant nanostructured coatings. ACS Appl. Mater. Interfaces 13, 34793–34806. https://doi.org/10.1021/acsami.1c06087.
Henry, B.J., Carlin, J.P., Hammerschmidt, J.A., Buck, R.C., Buxton, L.W., Fiedler, H., Seed, J., Hernandez, O., 2017. A critical review of the application of polymer of low concern and regulatory criteria to fluoropolymers. Integr. Environ. Assess. Manag 14, 316–334. https://doi.org/10.1002/ieam.4035.
Hu, L., Koppolu, R., Hämäläinen, R., Kanerva, H., Nick, T., Toivakka, M., Korpinen, R., Saranpää, P., Qasim, U., Liimatainen, H., Xu, C., Anghelescu-Hakala, A., 2024. Suberin-based aqueous dispersions for barrier packaging applications. ACS Sust. Chem. Eng. 12, 8902–8912. https://doi.org/10.1021/acssuschemeng.4c02244.
Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A., Prior, R.L., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50, 4437–4444. https://doi.org/10.1021/jf0201529.
Huang, T., Chen, C., Li, D., Ek, M., 2019. Hydrophobic and antibacterial textile fibres prepared by covalently attaching betulin to cellulose. Cellulose 26, 665–677. https://doi.org/10.1007/s10570-019-02265-8.
Imlimthan, S., Correia, A., Figueiredo, P., Lintinen, K., Balasubramanian, V., Airaksinen, A.J., Kostiainen, M.A., Santos, H.A., Sarparanta, M., 2019. Systematic in vitro biocompatibility studies of multimodal cellulose nanocrystal and lignin nanoparticles. J. Biomed. Mater. Res 108A, 770–783. https://doi.org/10.1002/jbm.a.36856.
ISO 11268-2:2012. Soil quality — Effects of pollutants on earthworms — Part 2: Determination of effects on reproduction of Eisenia fetida/Eisenia andrei.
ISO 6341:2012. Water quality — Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) — Acute toxicity test. Published (Edition 4, 2012).
ISO 8692:2012. Water quality — Fresh water algal growth inhibition test with unicellular green algae. Published (Edition 3, 2012).
Jeżo, A., 2024. Valorization of tree bark-derived suberin in applications for the bio-based composites industry–a recent review. J. Renew. Mater. 12, 1029–1042. https://doi.org/10.32604/jrm.2024.051330.
Kolattukudy, P.E., 2001. Polyesters in higher plants. Adv. Biochem. Eng. Biotechnol. 71, 1–49. https://doi.org/10.1007/3-540-40021-4_1.
Li, D., Iversen, T., Ek, M., 2015a. Hydrophobic materials based on cotton linter cellulose and an epoxy-activated polyester derived from a suberin monomer. Holzforschung 69, 721–730. https://doi.org/10.1515/hf-2014-0261.
Li, D., Iversen, T., Ek, M., 2015b. Treatment of a cellulose fiber surface with a suberin monomer-derived polymer. Polym. Renew. Resour. 6, 75–90. https://doi.org/10.1177/204124791500600.
Li, D., Moriana, R., Ek, M., 2016. From forest residues to hydrophobic nanocomposites with high oxygen-barrier properties. Nord. Pulp Pap. Res. J. 31, 261–269. https://doi.org/10.3183/npprj-2016-31-02-p261-269.
Li, K., Zhong, W., Li, P., Ren, J., Jiang, K., Wu, W., 2023. Recent advances in lignin antioxidant: antioxidant mechanism, evaluation methods, influence factors and various applications. Int. J. Biol. Macromol. 251, 125992. https://doi.org/10.1016/j.ijbiomac.2023.125992.
Liakos, I.L., Menager, C., Guigo, N., Holban, A.M., Iordache, F., Pignatelli, F., Grumezescu, A.M., Mazzolai, B., Sbirrazzuoli, N., 2019. Suberin/trans-cinnamaldehyde oil nanoparticles with antimicrobial activity and anticancer properties when loaded with paclitaxel. ACS Appl. Bio Mater. 2, 3484–3497. https://doi.org/10.1021/acsabm.9b00408.
Lohmann, R., Cousins, I.T., DeWitt, J.C., Glüge, J., Goldenman, G., Herzke, D., Lindstrom, A.B., Miller, M.F., Ng, C.A., Patton, S., Scheringer, M., Trier, X., Wang, Z., 2020. Are fluoropolymers really of low concern for human and environmental health and separate from other PFAS? Environ. Sci. Technol. 54, 12820–12828. https://doi.org/10.1021/acs.est.0c03244.
Morena, A.G., Tzanov, T., 2022. Antibacterial lignin-based nanoparticles and their use in composite materials. Nanoscale Adv. 4, 4447–4469. https://doi.org/10.1039/d2na00423b.
OECD, 1984. Test No. 207: Earthworm, acute toxicity tests, OECD guidelines for the testing of chemicals, Section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264070042-en.
OECD, 2004. Test No. 202: Daphnia sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264069947-en.
OECD, 2010. Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systematic Toxicity Tests. OECD Publishing, Paris. https://doi.org/10.1787/d77a7e39-en.
OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264069923-en.
OECD, 2012. Test No. 211: Daphnia Magna Reproduction Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264185203-en.
OECD, 2019. Test No. 431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RHE) Test Method, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264264618-en.
OECD, 2021. Test No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264242845-en.
OECD, 2024. Test No. 442D: In Vitro Skin Sensitisation: Assays Addressing the Adverse Outcome Pathway Key Event on Keratinocyte Activation, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264229822-en.
Österberg, M., Sipponen, M.H., Mattos, B.D., Rojas, O.J., 2020. Spherical lignin particles: a review on their sustainability and applications. Green. Chem. 22, 2712–2733. https://doi.org/10.1039/d0gc00096e.
Owsianiak, M., Hauschild, M.Z., Posthuma, L., Saouter, E., Vijver, M.G., Backhaus, T., Douziech, M., Schlekat, T., Fantke, P., 2023. Ecotoxicity characterization of chemicals: global recommendations and implementation in USEtox. Chemosphere 310, 136807. https://doi.org/10.1016/j.chemosphere.2022.136807.
Petkovska, J., Mladenovic, N., Markovic, D., Radoičić, M., Vest, N.A., Palen, B., Radetić, M., Grunlan, J.C., Jordanov, I., 2022. Flame-retardant, antimicrobial, and uv-protective lignin-based multilayer nanocoating. ACS Appl. Polym. Mater. 4, 4528–4537. https://doi.org/10.1021/acsapm.2c00520.
Pinto, P.C.R.O., Sousa, A.F., Silvestre, A.J.D., Neto, C.P., Gandini, A., Eckerman, C., Holmbom, B., 2009. Quercus suber and Betula pendula outer barks as renewable sources of oleochemicals: a comparative study. Ind. Crops Prod. 29, 126–132. https://doi.org/10.1016/j.indcrop.2008.04.015.
Poolman, J.T., Anderson, A.S., 2018. Escherichia coli and Staphylococcus aureus: leading bacterial pathogens of healthcare associated infections and bacteremia in older-age

populations. Expert Rev. Vaccin. 17, 607–618. https://doi.org/10.1080/14760584.2018.1488590.

Prior, R.L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R., 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC FL) of plasma and other biological and food samples. J. Agric. Food Chem. 51, 3273–3279. https://doi.org/10.1021/jf0262256.

Qasim, U., Sirviö, J.A., Suopajärvi, T., Hu, L., Pratiwi, F.W., Lin, M.K.T.H., Anghelescu-Hakala, A., Ronkainen, V.-P., Xu, C., Liimatainen, H., 2024. A multifunctional biogenic films and coatings from synergistic aqueous dispersion of wood-derived suberin and cellulose nanofibers. Carbohydr. Polym. 338, 122218. https://doi.org/10.1016/j.carbpol.2024.122218.

Rasi, S., Kilpeläinen, P., Rasa, K., Korpinen, R., Raitanen, J.-E., Vainio, M., Kitunen, V., Pulkkinen, H., Jyske, T., 2019. Cascade processing of softwood bark with hot water extraction, pyrolysis and anaerobic digestion. Bioresour. Technol. 292, 121893. https://doi.org/10.1016/j.biortech.2019.121893.

Rivière, G.N., Pion, F., Farooq, M., Sipponen, M.H., Koivula, H., Jayabalan, T., Pandard, P., Marlair, G., Liao, X., Baumberger, S., Österberg, M., 2021. Toward waste valorization by converting bioethanol production residues into nanoparticles and nanocomposite films. Sustain. Mater. Technol. 28, e00269. https://doi.org/10.1016/j.susmat.2021.e00269.

J. Routa, H. Brännström, P. Anttila, M. Mäkinen, J. Jänis, A. Asikainen. (n.d)Wood extractives of Finnish pine, spruce and birch – availability and optimal sources of compounds: A literature review. Natural resources and bioeconomy studies 73/2017. Natural Resources Institute Finland, Helsinki. 55 p. ⟨http://urn.fi/URN:ISBN:978-952-326-495-3⟩.

Schneider, W.D.H., Dillon, A.J.P., Camassola, M., 2021. Lignin nanoparticles enter the scene: A promising versatile green tool for multiple applications. Biotechnol. Adv. 47, 107685. https://doi.org/10.1016/j.biotechadv.2020.107685.

Setälä, H., Alakomi, H.-L., Paananen, A., Szilvay, G.R., Kellock, M., Lievonen, M., Liljestrom, V., Hult, E.-L., Lintinen, K., Österberg, M., Kostiainen, M., 2020. Lignin nanoparticles modified with tall oil fatty acid for cellulose functionalization. Cellulose 27, 273–284. https://doi.org/10.1007/s10570-019-02771-9.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158. https://doi.org/10.5344/ajev.1965.16.3.144.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1.

Sunthornvarabhas, J., Liengprayoon, S., Lerksamran, T., Buratcharin, C., Suwonsichon, T., Vanichsriratana, W., Sriroth, K., 2019. Utilization of lignin extracts from sugarcane bagasse as bio-based antimicrobial fabrics. Sugar Technol. 21, 355–363. https://doi.org/10.1007/s12355-018-0683-2.

Tardy, B.L., Lizundia, E., Guizani, C., Hakkarainen, M., Sipponen, M.H., 2023. Prospects for the integration of lignin materials into the circular economy. Mater. Today 65, 122–132. https://doi.org/10.1016/j.mattod.2023.04.001.

Tienaho, J., Karonen, M., Muilu-Mäkelä, R., Kaseva, J., de Pedro, N., Vicente, F., Genilloud, O., Aapola, U., Uusitalo, H., Vuolteenaho, K., Franzén, R., Wähälä, K., Karp, M., Santala, V., Sarjala, T., 2020. Bioactive properties of the aqueous extracts of endophytic fungi associated with Scots pine (Pinus sylvestris) roots. Planta Med 86, 1009–1024. https://doi.org/10.1055/a-1185-4437.

Tienaho, J., Reshamwala, D., Sarjala, T., Kilpeläinen, P., Liimatainen, J., Dou, J., Viherä-Aarnio, A., Linnakoski, R., Marjomäki, V., Jyske, T., 2021. Salix spp. Bark hot water extracts show antiviral, antibacterial, and antioxidant activities—the bioactive properties of 16 clones. Front. Bioeng. Biotechnol. 9, 797939. https://doi.org/10.3389/fbioe.2021.797939.

United Nations. Sustainable development—The 17 goals. https://sdgs.un.org/goals, 2024 (accessed 18 December 2024.

Vesterlund, S., Paltta, J., Lauková, A., Karp, M., Ouwehand, A., 2004. Rapid screening method for the detection of antimicrobial substances. J. Microbiol. Methods (1), 23–31. https://doi.org/10.1016/j.mimet.2003.11.014.

Xie, P., Yang, Y., Oyom, W., Su, T., Tang, Y., Wang, Y., Li, Y., Prusky, D., Bi, Y., 2023. Chitooligosaccharide accelerated wound healing in potato tubers by promoting the deposition of suberin polyphenols and lignin at wounds. Plant Physiol. Biochem. 199, 107714. https://doi.org/10.1016/j.plaphy.2023.107714.

Yadav, P., Athanassiadis, D., Yacout, D.M., Tysklind, M., Upadhyayula, V.K., 2020. Environmental impact and environmental cost assessment of methanol production from wood biomass. Environ. Pollut. 265, 114990. https://doi.org/10.1016/j.envpol.2020.114990.

Yadav, P., Korpinen, R., Räty, T., Korkalo, P., Räsänen, K., Tienaho, J., Saranpää, P., 2024. Life cycle assessment of suberin and betulin production from birch bark. J. Clean. Prod. 474, 143570. https://doi.org/10.1016/j.jclepro.2024.143570.