https://doi.org/10.31925/farmacia.2024.3.6 **ORIGINAL ARTICLE**

THE ANTI-AGEING POTENTIAL OF *LITSEA OPPOSITIFOLIA* STEM EXTRACT: EVIDENCE FROM *IN VITRO* AND *EX VIVO* STUDY ON SKIN CELL LINES

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Abstract

Skin ageing, a natural process caused by prolonged exposure to stressors, affects skin function. Reactive oxygen species (ROS) play a key role in this process by inducing oxidative stress. To counteract skin aging, we evaluated *Litsea oppositifolia* Gibbs, a little-studied Indonesian plant, by characterizing the phytochemical constituents and antioxidant activities of the extract *in vitro* and *ex vivo* on skin cell lines. Its protective effects against H_2O_2-induced oxidative stress on 3T3 and HaCaT cells were evaluated at different concentrations. The results indicated significant antioxidant activity, with an IC_{50} value of 8.307 ± 0.04 µg/mL and an FeEAC value of 661.25 ± 1.13 µmol/g. The extract showed a phenolic content of 352.744 ± 1.199 mg GAE/g and a flavonoid content of 3.87 ± 0.013 mg QE/g. Protective effects on HaCaT cells were observed at concentrations of 12.5 ppm and 25 ppm, while for 3T3 cells, the optimal concentration was determined to be 6.25 ppm. LC-MS analysis identified the presence of eugenol in the extract. The results suggest the potential of *Litsea oppositifolia* Gibbs as an antioxidant agent for mitigating skin aging, highlighting its promising application in skin care interventions.

Rezumat

Îmbătrânirea pielii, un proces natural determinat de expunerea prelungită la factorii de stres, afectează fiziologia acesteia. Studiul a evaluat potențialul antioxidant al extractelor etanolice de *Litsea oppositifolia* Gibbs asupra unor linii celulare cutanate, și anume fibroblaști murini 3T3 și celule HaCaT. De asemenea, extractele au fost caracterizate din punct de vedere fitochimic. Rezultatele au indicat o activitate antioxidantă semnificativă, cu o valoare CI_{50} de 8,310 ± 0,04 µg/mL și o valoare FeEAC de 613,803 µmol/g. Extractul a prezentat un conținut fenolic de 352,744 mg GAE/g și un conținut de flavonoide de 3,87 ± 0,013 mg QE/g. Au fost identificate efecte protectoare asupra celulelor HaCaT la concentrații de 12,5 ppm și 25 ppm, în timp ce pentru celulele 3T3, concentrația optimă a fost determinată la 6,25 ppm. Analiza *LC-MS* a identificat prezența eugenolului în extract. Rezultatele au demonstrat potențialul antioxidant al extractelor de *Litsea oppositifolia* Gibbs, sugerând aplicabilitatea acestora în produsele dermatocosmetice.

Keywords: antioxidant, *Litsea oppositifolia* Gibbs, oxidative stress, skin ageing, skincare

Introduction

As the largest organ in the human body, the skin is essential for maintaining internal homeostasis and protecting against external stressors such as UV radiation, pollution and microbial and viral infections [1, 2]. Continuous exposure to these stressors can lead to a cascade of biological events that reduce skin function and impair its ability to repair. Due to these processes, the skin loses its natural elasticity and ability to retain moisture [3]. Physically, wrinkling and atypical pigmentation were visible indications of skin ageing [4].

The process of ageing involves numerous biological mechanisms, including the generation of reactive oxygen species (ROS) within the skin. ROS are generated as a natural component of the body's response to inflammation and function as a defensive mechanism against microbial invaders, and they are continuously generated within the skin at minimal levels [5]. Therefore, the body also undergoes anti-oxidant processes to prevent excessive oxidative damage, as ROS accumulation has been linked to skin ageing and other skin diseases [5, 6]. Furthermore, external influences such as ultraviolet (UV) radiation, pollutants and chemical exposure can trigger ROS generation. Hence, creating antioxidant products is necessary to counteract or prevent ROS generation within the body [7].

Although ageing is an inevitable, normal biological process, age-related changes in human physiology are unappealing and can affect people's self-esteem. As a result, many individuals invest substantially in acquiring skincare items to delay the skin's ageing process. Consistent with the growing public awareness of the importance of skin health, there is an increasing

demand for skin care products that serve as anti-ageing [8]. This trend has made the global skincare products market one of the most profitable sectors of the world economy [9].

Along with the high market demand, exploratory work is required to identify innovative, safe and effective product ingredients as more consumers seek healthier alternatives. Naturally sourced components have been used for skincare purposes for centuries. Reports indicate that the demand for new effective natural active ingredients for skincare has increased sharply in recent years [10]. Plants are one of the most essential natural ingredients in cosmetics, including skincare. Some of the highlights of their skincare benefits include the antioxidant properties of plants, and their ability to reduce skin damage triggered by harmful environmental factors such as ultraviolet radiation (UVR) and pollution [11].

Many plants have been investigated for their anti-ageing properties, including those from the *Litsea* genus. This genus comprises perennial trees that grow in tropical and subtropical regions of North America and Asia. Previous studies have established the anti-oxidant properties of some *Litsea* species, including *Litsea petiolata*, *Litsea elliptica* and *Litsea resinosa* [12, 13]. Nevertheless, the antioxidant potential of certain *Litsea* species, like *Litsea oppositifolia* indigenous to Borneo, remains unexplored. Further investigations are necessary to determine the antioxidant properties of these species. Therefore, this study aims to examine the antioxidant properties of the stem extract derived from *L. oppositifolia* through *in vitro* analysis by conducting antioxidant activity assays using DPPH and FRAP methods. Additionally, the antioxidant effects of the extract on cellular skin cell models (HaCaT and 3T3) were confirmed by performing the cytoprotective assessments.

Materials and Methods

Chemicals

Ethanol (Bratachem, Indonesia), ethanol pro analysis, methanol pro analysis, sodium carbonate, sodium acetate, aluminium chloride ammonium ferro sulfat, Folin-Ciocâlteu reagent, formic acid (Merck, Germany), 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich D9132, USA), Gallic acid (Sigma-Aldrich G7384, USA), quercetin (Sigma-Aldrich Q4951, USA), 2,4,6-Tris(2-Pyridyl)-S-Triazine (Sigma-Aldrich T1253, USA), ascorbic acid (Sigma-Aldrich A5928, USA).

Cell lines

3T3 mouse fibroblasts were purchased and authenticated by VCPRC FK-UI while HaCaT human keratinocytes cells were purchased from Addexbio (catalogue number T0020001). Both sets of cells were grown in a complete Dulbecco's modified eagle medium (cDMEM) (Gibco, USA) containing 10% foetal bovine serum (FBS) (Gibco, USA) and 100 µg/mL penicillin-streptomycin (Gibco, USA) at 37°C supplied with 5% CO_2 incubator (LabServ, Pakistan). For the cyto-toxic and cytoprotective assay, 3T3 cells were seeded with 4 x 10^4 cells density *per* well while HaCaT were seeded with 1 x 10^4 cells *per* well followed by 24 h incubation prior to treatment in a 5% CO_2 incubator.

Plant material and extraction procedure

The stem of *Litsea oppositifolia* was harvested from Bogor Botanical Garden, West Java, Indonesia and its identities were verified at the Research Centre for Plant Conservation and Botanic Gardens, BRIN Cibinong, Indonesia (B-1612/IPH.3/KS/V/2019). Collected stems were then cleaned, dried at 30°C with 70% humidity for a week, ground into fine powder filtered with an 80-mesh sieve and extracted by maceration with 70% ethanol as a solvent, then evaporated using a rotary evaporator (Buchi, Switzerland) to obtain the plant extract. This process was repeated three times.

Phytochemical screening

The qualitative phytochemical content tested were flavonoid, tannin, phenolic, alkaloid, anthraquinone, saponin and glycoside [14, 15].

LC-MS

For this study, a solution was prepared by dissolving 0.01 g of *L. oppositifolia* extract in 10 mL of type 1 water for subsequent analysis via LC-MS, followed by filtration through a 0.01 nylon filter. The LC-MS analysis was carried out with a flow rate of 0.3 mL/min, using methanol and water with 0.1% formic acid as mobile phase with 50:50 ratio running for 30 minutes. Chromatograms and mass spectra were carefully examined and compared against blanks and compound libraries. The peaks that corresponded to the molecular weights of the compounds in the library were highlighted for closer examination. This was achieved by identifying the fragmentation patterns in the mass spectrum. Subsequently, the identification of a compound manifesting a consistent fragmentation pattern was indicative of its presence within the analysed *L. oppositifolia* extract.

Total Phenolic Content (TPC) Assay

Total Phenolic Content (TPC) was conducted using the Folin-Ciocâlteu reagent, slightly modified from the method described by [16]. Folin-Ciocâlteu reagent was made by diluting it with distilled water with a final concentration of 7.5%. Gallic acid served as the standard, diluted in ethanol to 3.75 - 10 µg/ml. Subsequently, 25 µL of both the sample solution, and standard were dispensed into a 96-well plate. Following this, 100 µL Folin-Ciocâlteu was added, shaken on the microplate reader, and let react for 4 minutes. Subsequently, 75 µL of 10% Na_2CO_3 was mixed with the solution, and incubated for 2 hours at 25°C, before absorbance measurement at 750 nm using a Versamax Microplate Reader (USA). The entire procedure was conducted three times, and the

outcomes were quantified as milligrams of gallic acid equivalent (GAE) *per* gram of the extract.

Total Flavonoid Content (TFC) Assay

The quantification of flavonoids was taken from [16] with several adjustments. A standard curve was made using quercetin with a concentration ranging from 3 - 9 µg/mL. Ethanol was used to dissolve the sample and standard. A volume of 0.5 mL of both the sample solution and a standard reference was individually dispensed into separate test tubes. Subsequently, 1.5 mL of ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of type III water were thoroughly mixed into each test tube. The resulting solution was allowed to incubate at room temperature for 30 minutes. Absorbance readings were taken with a UV-Vis spectrophotometer at 415 nm wavelength, with each experiment conducted three times. Total flavonoid levels were indicated as milligrams of Quercetin Equivalents (QE) *per* gram of plant extract.

2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay

Antioxidant activity with the DPPH method was conducted using [17] with minor modifications. DPPH and extract were dissolved in methanol, then 20 µL of sample, and 180 µL DPPH 150 µmol were added into 96-well, and mixed for 1 minute. The scavenging activity was calculated by measuring the absorbance reduction of the mixture incubated for 40 minutes in the dark at 515 nm using a microplate reader with the following equation:

$$\text{Inhibition } (\%) = [1 - \frac{Sample\ absorbance - Blank\ absorbance}{Control\ absorbance - Blank\ absrobance}] \text{ x } 100\%.$$

The mixture of methanol and DPPH were used as control, methanol as blank and ascorbic acid as the positive control. The analysis was done in triplicate. The IC_{50} value states the concentration of the sample when it can scavenge 50% of DPPH radical.

Ferric Reducing Antioxidant Power Assay (FRAP)

As much as 20 µL of the sample was pipetted into a 96-well, then 280 µL FRAP solution (acetate buffer: TPTZ:$FeCl_3 \times 6\ H_2O$ = 10:1:1) was added, shaken and incubated at 37°C in dark for 30 minutes. The absorbance of the sample was read at 593 nm using a microplate reader. This test was replicated three times. Antioxidant activity by FRAP method showed equivalent values of ferro (Fe^{2+}) [18]. This formula is used to calculate Ferrous Equivalent Antioxidant Capacity:

$$FeEAC\ (\mu mol/g) = \frac{\Delta A}{GRAD} \times \frac{Av}{Spv} \times D \times \frac{1}{Csampel} \times 10^5,$$

where, ΔA = absorbance correction, GRAD = gradient from AFS curve (M^{-1}), Av = total volume of solution, Spv = sample volume in total solution, Csample = sample concentration (g/L), D = 1.

Viability assay

3T3 mouse fibroblasts and HaCaT cells were used as cell models. Ascorbic acid was used as the standard and H_2O_2 as the free radical model. 3T3 cells were seeded with 4 x 10^4 cells density *per* well while HaCaT were seeded with 1 x 10^4 cells *per* well, then both were incubated for 24 hours. Cells were then treated in triplicate with the predetermined treatments of ascorbic acid, *L. oppositifolia* stem extracts and H_2O_2, followed by another 24 hours of incubation. Afterwards, cell viability was assessed using an MTT assay. After washing with 100 µL of DMEM *per* well, 10 µL of MTT reagent was introduced into each well. The plate was subsequently incubated for roughly 3 hours in an atmosphere containing 5% CO_2 at 37°C, and it was shielded with aluminium foil to maintain the stability of the MTT reagent. After the allotted incubation period, the reactions were terminated by adding isopropanol and 0.04 N HCl. Absorbance at 570 nm was then measured using a plate reader, and cell viability was determined as a percentage using the provided formula.

$$\text{Cell viability} = \frac{Sample\ absorbance - Blank\ absorbance}{Negative\ control\ absorbance - Blank\ absrobance} \times 100\%.$$

Cell cytoprotective assay

Each cell was seeded into individual wells and allowed to incubate for 24 hours. Subsequently, the cells were pre-treated with various concentrations (6.25 ppm, 12.5 ppm, 25 ppm, 50 ppm and 100 ppm) of *L. oppositifolia* extract and ascorbic acid for 1 hour. Following this, the pre-treatments were removed, and a new series of treatments with the same concentration range was introduced, along with 50 µM H_2O_2 to induce cellular stress. The plate was then subjected to an additional 24-hour incubation period. Post-incubation, 10 µL of MTT reagent was administered to each well, followed by another 3-hour incubation in an environment with 5% CO_2 at 37°C. To prevent MTT degradation, the plate was shielded with aluminium foil. The reactions were terminated by adding isopropanol and HCl and absorbance measurements were taken at 570 nm using a plate reader. Cytoprotective activity determined as a percentage using the same formula to calculate viability of cell.

Statistical analysis

GraphPad Prism version 9.3.1 software (GraphPad Software, USA) was used to process all the data obtained. Antioxidant activity, phenolic, flavonoid content and the MTT assay results were averaged and presented as a mean ± standard deviation (SD). The Saphiro-Wilk test was used to measure the normality of the data distribution. One-way ANOVA and Kruskal-Wallis tests were use to compare statistical differences of the normal and abnormal data respectively,

between experimental and control groups. The significance between samples were further assessed using Dunnet's multiple comparison test for the ordinary one-way ANOVA, while the post-hoc test for the Kruskal-Wallis test was done using Dunn's multiple comparison test method with a p value < 0.05 is considered as statistically significant.

Results and Discussion

Plant-derived pharmaceutical compounds have emerged as a promising source of bioactive agents. They have gained increased recognition as a significant category of skincare ingredients due to their remarkable efficacy in addressing various skin conditions [11]. Among these, *L. oppositifolia* is a plant species of particular interest for its potential anti-ageing properties. In this study, from 250 g of *L. oppositifolia* stem *simplicia*, 25.41 g of extract was obtained, constituting 10.16% of the total weight. We conducted preliminary phytochemical profiling of secondary metabolites present in *L. oppositifolia* stem extract to assess its anti-ageing potential further. Our analysis unveiled various categories of secondary metabolites, including alkaloids, phenolics, flavonoids, terpenoids, saponins and glycosides. However, steroids, tannins and anthraquinones were not detected (Table I). Phenolics, including flavonoids, are widely recognised for their ability to act as antioxidants. Phenolic compounds form a major group known for acting as primary antioxidants or free radical neutralisers, underscoring the importance of quantifying these compounds in the extract. Natural phenolics exert antioxidant activities by substituting hydroxyl groups in their aromatic phenolic rings. Our findings revealed that *L. oppositifolia* stem extract contains phenolics and flavonoids, indicating their promising potential as antioxidants.

Table I

Phytochemical screening results of *Litsea oppositifolia* Gibbs stem extract

Chemical Compounds	Tests	Observation	Result
Terpenoid	Liebermann-Burchard	Red purple colour is formed	Positive (+)
Saponin	Foam test	Foam with 1 - 2 cm height is formed and maintain after adding HCl	Positive (+)
Glycoside	Molisch test	Purple ring is formed	Positive (+)
Phenol	$FeCl_3$ 3%	Blue black colour is formed	Positive (+)
Flavonoid	Shinoda test	Orange to red colour is formed	Positive (+)
	Zn-HCl test	Orange to red colour is formed	Positive (+)
	$AlCl_3$	Yellow fluorescence is apparent at 366 nm UV	Positive (+)
Steroid	Liebermann-Burchard	Blue green colour does not appear	Negative (-)
Tannin	Gelatine precipitation	A white precipitate is not formed	Negative (-)
Anthraquinone	Borntrager's test	Pink to red colour is not formed in water layer	Negative (-)

Total flavonoid content (TFC) and total phenolic content (TPC) assessments were performed on the ethanolic extract of *L. oppositifolia* stem to quantify both compound groups. The Folin–Ciocâlteu method is a colorimetric method based on the chemical reduction of the Folin reagent by phenols in solution [19]. This method stands out for its ability to react with any phenol type. This method stands out for its ability to interact with various phenols. When phenolic compounds combine with the Folin reagent, which is a mixture of sodium tungstate and sodium molybdate in an acidic environment, a yellow complex is formed [20]. This complex can then be measured using a spectrophotometric approach at a wavelength of 765 nm [21]. The TPC in the plant extract was determined to be 352.744 ± 1.199 mg GAE/g extract (Table III), which surpassed some other studied *Litsea spp*. Studies have reported that *Litsea cubeba* stem extract contains phenolics with a total content of 282.93 ± 0.33 mg GAE/g extract, while *Litsea glutinosa* has a TPC value of 151.73 mg GAE/g extract [22, 23].

Furthermore, total flavonoid content was also examined, as flavonoids represent a widely occurring class of polyphenolic compounds within natural products [24]. The determination of TFC is often conducted using the aluminium chloride ($AlCl_3$) colourimetric assay. This methodology involves the formation of stable complexes between aluminium chloride and the C-4 keto group, along with either the C-3 or C-5 hydroxyl group that exists in flavones and flavonols. Additionally, this assay generates acid-labile complexes with ortho-dihydroxyl groups located in the A- or B-ring of flavonoids [25]. The outcome showed that *L. oppositifolia* stem extract has a TFC value of 3.87 ± 0.013 mg QE/g extract (Table III), notably lower than the TPC value. This disparity is attributed to flavonoids being just one type of phenolic, suggesting that other phenolic contents may be more dominant than flavonoids [26].

The present study also reports on an LC-MS analysis of the extract to identify the plant's detailed phytochemical constituents. In-depth the phytochemical identification was done by comparing the Mass Spectrometry (MS) data with established standards or referencing a compound library. However, the analysis was conducted using a compiled compound library due to lacking a representative sample standard. The results showed that 30 compounds exhibiting 20 distinct molecular weights were identified within the chromatogram of the extract. Among these, 9

indicated the presence of more than one compound with molecular weights of 256, 314, 326, 328, 339, 342, 343, 356 and 433 (Table II). Relying solely on one or two peaks in MS spectra for compound identification is insufficient, given the genus-based nature of the compound library. Therefore, a detailed analysis of fragmentation peaks was conducted. The putative compounds were refined by matching MS fragmentation patterns to structural profiles, with probable compounds designated in Table II (denoted by an asterisk (*)). Notably, only one molecule correlated with four fragments of its structure, ultimately identified as eugenol within the extract. Eugenol (4-allyl-2-methoxyphenol) is a volatile phenolic compound with a molecular weight of 164.2 g/mol, presenting as a pale-yellow oil with a pungent aroma [27]. This molecule is a weak acid that can be dissolved in organic solvents [28]. Numerous studies have highlighted the potent antioxidant effects of eugenol and its esters. These properties include inhibiting free radical formation, repairing oxidative damage and eliminating damaged particles [29-32].

Table II

LC-MS result for *Litsea oppositifolia* Gibbs extract

Molecular Weight	Compound Class	Possible Compound (S)
164	Phenol	Eugenol
200	Fatty Acid	Lauric Acid
220	Sesquiterpene	Humulene oxide
256	Fatty Acid	Pinocembrin, Pinocembrin Chalcone Palmitic acid*
282	Fatty Acid	Oleic acid
286	Flavonoid	Kaempferol
295	Alkaloid	Ushinsunine
296	Diterpene	Trans-phytol
314	Alkaloid	(-)-Litcubinine (-)-Magnocurarine (-)-Oblongine
326	Lignan	Dehydrodieugenol* Dehydrodiisoeugenol
328	Alkaloid	(-)-Litcubine (-)-8-O-Methyloblongine
337	Alkaloid	Atheroline
339	Alkaloid	Dicentrine Litebamine
342	Alkaloid	Magnoflorine* Phyllocoumarin
343	Diterpenes	N-Feruloyl-3-methoxytyramine* Glaucine
356	Lignan	Balanophonin B* Xanthoplanine
371	Alkaloid	(+)-N-(Methoxycarbonyl)-N-norboldine
386	Lignans	(+)-Eudesmin
433	Flavonoid	Pelargonidin 3-glucoside Pelargonidin 5-glucoside

Comparative analysis with established antioxidants such as butylated hydroxytoluene (BHT), trolox and α-tocopherol revealed eugenol's exceptional efficacy in reducing oxidative stress across various assays, including DPPH, ABTS and DMPD. Notably, its potency exceeded that of Trolox, a widely recognized antioxidant standard, by fivefold [33].

Further investigation revealed eugenol's anti-ageing potential in normal human dermal fibroblasts (NHDFs) subjected to UVB irradiation. Eugenol effectively suppressed matrix metalloproteinase-1 and matrix metalloproteinase-3 (MMP-1 and MMP-3) activities, preventing photodamage-induced skin alterations. Eugenol also stimulated Nrf2/ARE signalling and reduced NFATc1, establishing it as a well-established anti-ageing agent for skin care [34].

Table III

The percentage yield, total phenolic and flavonoid content of *Litsea oppositifolia* Gibbs stem extract

Sample	Yield (% w/w)	Total phenolic (GAE mg/g)	Total Flavonoid (QE mg/g)
Litsea oppositifolia Gibbs	10,16	352,744 ± 1,199	3,87 ± 0,013

Data are expressed as mean ± SD (n = 3)

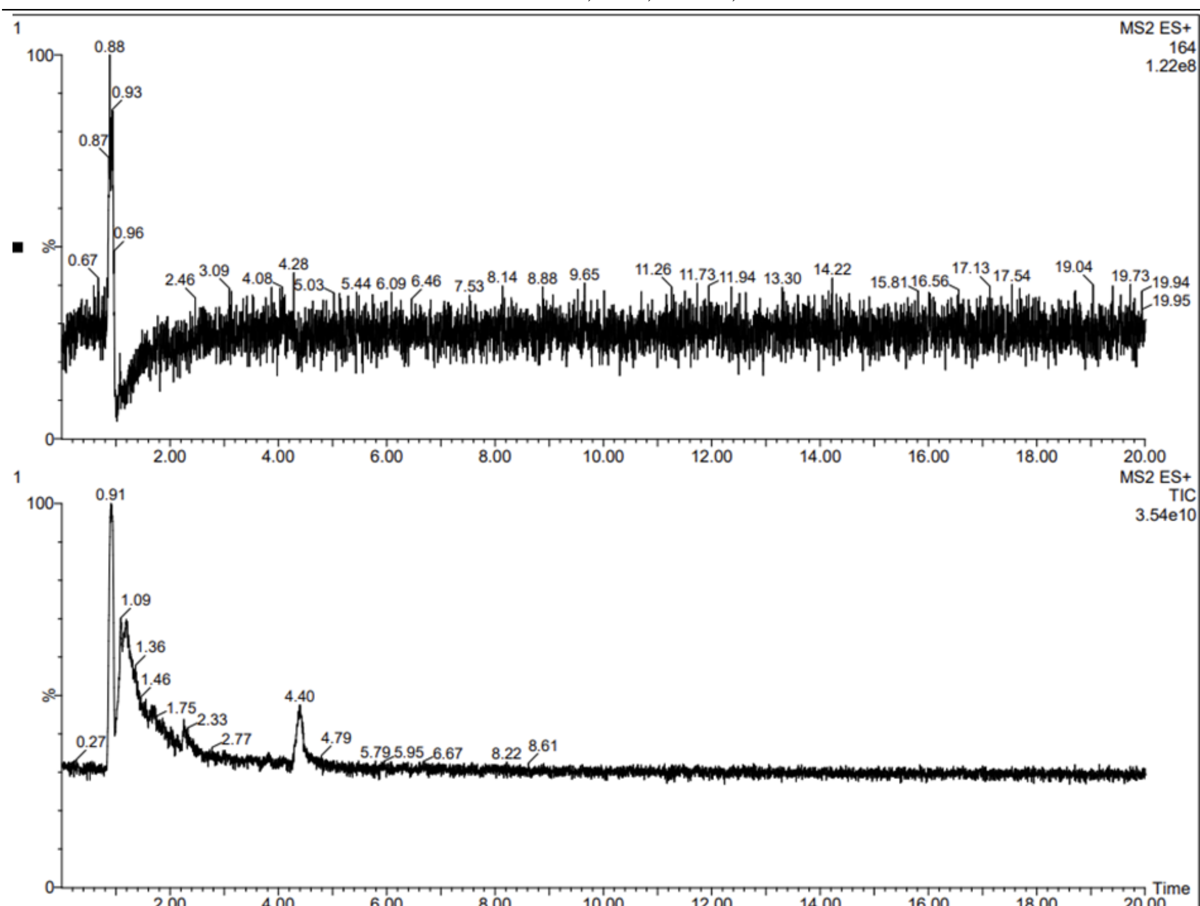

Figure 1.

Chromatogram of *Litsea oppositifolia* Gibbs extract (upper) and type III water as blank (lower)

Additionally, current research underscores the significant lipophilicity of eugenol and its esters, highlighting their role as effective absorption enhancers [35]. Studies have demonstrated that eugenol facilitates permeation through rat skin, comparable to the commercial permeation enhancer DMSO [36].

However, eugenol's low the chemical stability and the susceptibility to oxidation and other chemical reactions pose a significant challenge, making it imperative to explore strategies to address this limitation. To mitigate these stability concerns, incorporating eugenol into complexes or microemulsions offers a promising avenue to enhance its thermal stability and achieve a more controlled release profile [37]. Further studies are necessary to explore the potential of eugenol as delivery agents across diverse applications. Despite its chemical stability concerns, the widespread availability, cost-effectiveness and diverse medical applications of eugenol make it a promising candidate for transdermal delivery systems [31, 36, 38]. This holds particular relevance in skincare regimens, especially concerning anti-ageing interventions.

Based on the phytochemical profiling results above, we then analysed the antioxidant activity of the extract. The extract is expected to exhibit the antioxidative properties owing to its abundance of the phenolic compounds, primarily eugenol and flavonoids. Phenolic and flavonoid molecules exert their antioxidant effects through multiple mechanisms, including scavenging radical species such as ROS and RNS, inhibiting the production of ROS/RNS by blocking specific enzymes or chelating trace metals involved in the free radical generation, and strengthening or protecting the anti-oxidant defence system [39]. It should be noted that antioxidant action is a complex phenomenon, and multiple intricate mechanisms are involved. Each approach is selective for certain antioxidant components and reactions, and none can accurately estimate the capacity of all antioxidants [40].

The activity of antioxidants in plant extracts can be measured using various methods. Using at least two different methods is recommended to get an accurate measure [41]. These methods can be divided into two categories depending on the chemical processes involved: hydrogen atom transfer (HAT) reaction-based methods and single electron transfer (SET) reaction-based methods [42]. HAT assays are designed to evaluate an antioxidant's capacity to neutralize free radicals by donating hydrogen atoms. On the other hand, SET assays determine the antioxidant's ability to transfer a single electron to reduce metallic ions, carbonyl groups and free radicals [43]. The antioxidant properties of this extract were measured using DPPH and FRAP. FRAP measures antioxidant activity through SET,

whereas DPPH is measured *via* both mechanisms [44]. The antioxidant strength is measured as IC_{50} values, representing the tested substance's concentration that will inhibit 50% of free radicals. The lower the IC_{50} value, the greater the antioxidant activity [45].
DPPH is a commonly utilised free radical for *in vitro* antioxidant studies. This method involves the utilization of DPPH radicals to assess the efficacy of antioxidants in counteracting free radicals. The radical form of DPPH exhibits an absorption band at λmax 515 - 517 nm [46]. Upon reaction with antioxidants, a change in the solution colour from purple to pale yellow is [47]. The reduction in absorption correlates directly with the concentration of antioxidants [48]. In the present study, ascorbic acid was utilised as a positive control since it has significant antioxidant activity and is effective against radical chemicals such as superoxide radical ions, hydrogen peroxide and hydroxyl radicals [49]. According to the DPPH method, the ethanol extract of *L. oppositifolia* stem exhibited very strong antioxidant activity (IC_{50} < 50 µg/mL), as indicated by an IC_{50} value of 8.307 ± 0.05 µg/mL (Table IV).
Additionally, we conducted the FRAP method to assess the antioxidant capabilities of the extract by reducing the ferric complex with Fe^{3+}-TPTZ to Fe^{2+}. The resulting reduced complex displayed an intense blue colour, and the antioxidant activity was quantified by absorbance at 593 nm [50]. The FRAP method indicated that the extract possessed an antioxidant activity with an IC_{50} value of 661.25 ± 1.13 µmol/g (Table IV). Taken together, these findings underscore the considerable antioxidant potential of the extract.

Table IV

Antioxidant activity of *Litsea oppositifolia* Gibbs stem extract determined by DPPH and FRAP method

Sample	**Scavenging ability on DPPH radicals (µg/mL)**	**Scavenging ability on FRAP radicals (µmol/g)**
Litsea oppositifolia Gibbs stem extract	8.307 ± 0.04	661.25 ± 1.13
Ascorbic acid	2.71 ± 0.14	2028.89 ± 4.87

Data are expressed as mean ± SD (n = 3). Each treatment is significantly different according to ANOVA ($p < 0.05$)

To evaluate the potential of *L. oppositifolia* stem extract as an anti-ageing agent for skin cells, we conducted *ex vivo* examinations to measure its cytotoxicity and cytoprotective effects on HaCaT and 3T3 cells. The MTT assay was used to determine cell viability in HaCaT cells as a representative model for human keratinocytes and 3T3 cells as a model for dermal fibroblasts. These cells are regularly exposed to oxidative agents, leading to elevated intracellular hydrogen peroxide levels [51]. It is crucial to understand the impact of antioxidant agents, such as *L. oppositifolia* stem extract, on HaCaT cells due to their vulnerability to pro-oxidative agents. Cytotoxicity studies are essential to assess the toxicity of candidates, mainly plant extracts or bioactive compounds, intended for use in pharmaceuticals and cosmetics, where minimal toxicity is preferred [52]. In this study, the MTT assay was employed to measure cell viability, where the reduction of MTT reagent to formazan products is proportional to the number of viable cells [53].
For our cell viability and cytoprotective assays, we utilized concentrations of ascorbic acid and *L. oppositifolia* extract ranging from 6.25 ppm to 100 ppm. The selection of these concentrations for our experimental treatments was guided by a comprehensive review of the literature, preliminary investigations and practical considerations. Our findings are consistent with prior research, which suggests a maximum inhibitory concentration of 100 ppm for the ascorbic acid, beyond which cytotoxic effects are observed. Confirmation of this threshold through subsequent tests at 200 ppm led us to limit ascorbic acid concentrations to 100 ppm to maintain experimental integrity. Regarding minimal concentration, we chose 6.25 ppm based on our previous cytoprotective assays utilizing the MTT assay [54]. These assays revealed a decline in cell viability at this concentration, indicating insufficient antioxidant activity. To ensure consistency and comparability, we maintained a concentration range of 6.25 ppm to 100 ppm for both ascorbic acid and *L. oppositifolia* extract. This approach facilitates meaningful comparative analysis between treatments and strengthens the validity of our experimental results.
The viability assay results indicated that *L. oppositifolia* stem extract did not induce cytotoxicity in HaCaT and 3T3 cells at the tested concentrations, as evidenced by cell viability percentages exceeding 50% across all concentrations (Figure 2 and Figure 3). In all tested concentrations, ascorbic acid demonstrated non-cytotoxic effects on HaCaT cells, as indicated by the lack of notable variance in cell viability between the negative control (untreated cells) and those treated with ascorbic acid. Nonetheless, at a concentration of 100 ppm, ascorbic acid exhibited cytotoxicity towards 3T3 cells. Although, limited studies have explored the cytotoxic effects of ascorbic acid, existing data suggests potential hazards associated with elevated levels of this compound The results confirm the hypothesis that at greater doses, cell viability diminishes, indicating that ascorbic acid appears to exert cytotoxic effects on 3T3 cells at elevated concentrations [55]. Furthermore, the study investigated the cytoprotective properties of the extract against H_2O_2-induced oxidative stress, with cell viability assessed using the MTT assay. Cytoprotective effects

were observed in HaCaT cells at concentrations of 12.5 ppm and 25 ppm and in 3T3 cells at a concentration of 6.25 ppm (Figure 4 and Figure 5). Notably, treatment with 100 ppm ascorbic acid in the cytoprotective experiment led to a significant reduction in cell viability compared to other concentrations. These findings suggest that the protective impact against oxidative stress induced by H_2O_2 may not be sustained at higher concentrations of antioxidants. It is crucial to acknowledge that some outcomes from this study align with prior research, particularly the work of [56] which demonstrated that ascorbic acid can shift from an antioxidant to a pro-oxidant at very high concentrations, potentially causing tissue damage. Additionally, the study by [48] supported these findings, proposing that elevated concentrations of ascorbic acid may act as a pro-drug, leading to the formation of H_2O_2 and cellular harm instead of providing protection against oxidative stress. Moreover, it is important to note that the viability of 3T3 cells decreased significantly at concentrations of 6.25 and 12.5 ppm of ascorbic acid. This phenomenon may be attributed to the inherent instability of ascorbic acid when exposed to light and air, making it susceptible to oxidation in aqueous solutions [57]. The exposure to light causes ascorbic acid to absorb ultraviolet (UV) light, resulting in its degradation. Furthermore, in anaerobic environments, ascorbic acid degradation occurs over time due to ring cleavage and the addition of water molecules, compromising its quality and stability [58]. Therefore, the observed degradation of ascorbic acid could have compromised its ability to protect cells against oxidative damage.

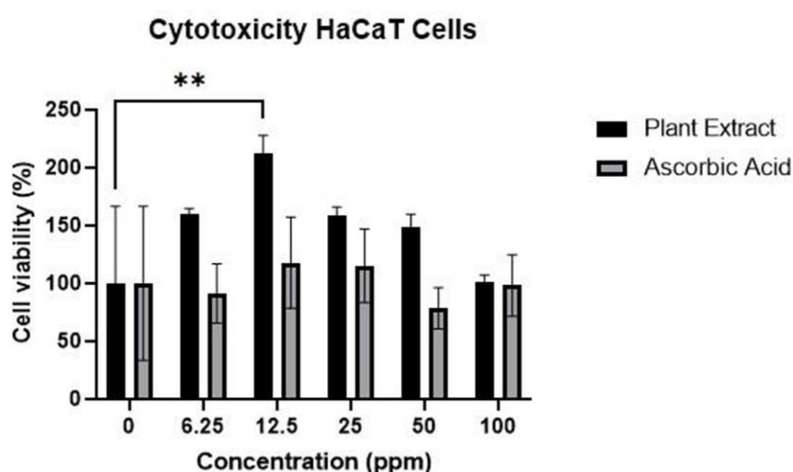

Figure 2.
Cytotoxic effects of treatments on HaCaT cells treated with increasing concentrations of *Litsea oppositifolia* stem extract and ascorbic acid respectively for 24 h
** indicate statistical significant difference against the negative control ($P < 0.01$)

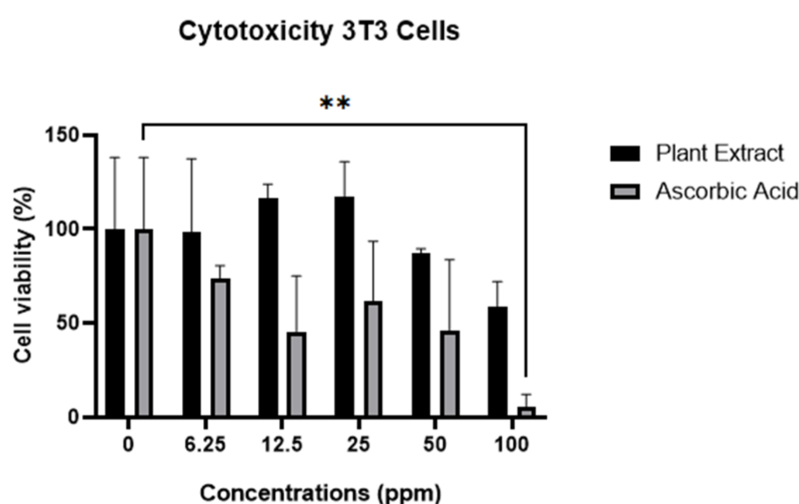

Figure 3.
Cytotoxic effects of treatments on 3T3 cells treated with increasing concentrations of *Litsea oppositifolia* stem extract and ascorbic acid respectively for 24 h
** indicate statistical significant difference against the negative control ($P < 0.01$)

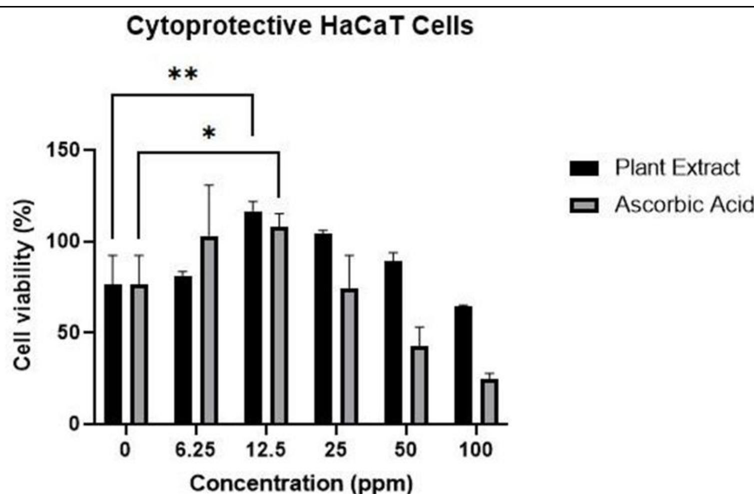

Figure 4.

The cytoprotective impacts of treatments on HaCaT cells subjected to 50 µM H_2O_2 along with escalating doses of *Litsea oppositifolia* stem extract and ascorbic acid for 24 hours were investigated

* denotes a statistically significant difference compared to the negative control ($P < 0.05$), while

** indicates a statistically significant difference against the negative control ($P < 0.01$)

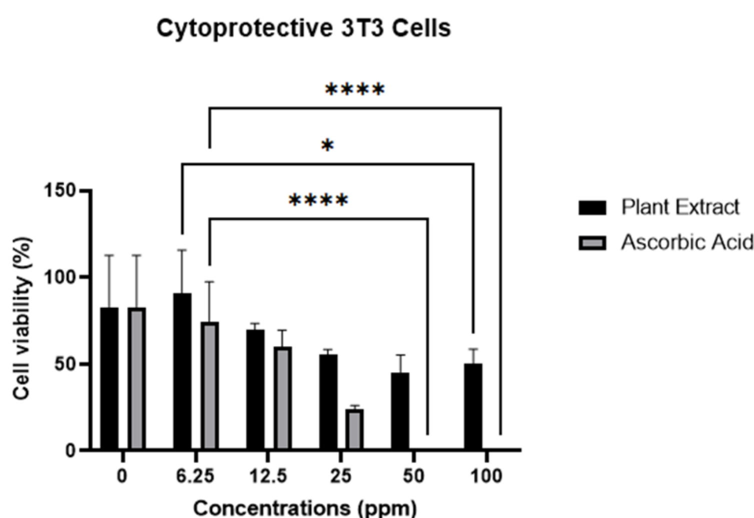

Figure 5.

The cytoprotective impacts of treatments on 3T3 cells exposed to 50 µM H_2O_2 alongside rising levels of *Litsea oppositifolia* stem extract and ascorbic acid for 24 hours were examined

Cell viability was assessed through the MTT assay

*** denotes a statistically significant difference compared to the negative control ($P < 0.001$), while

**** indicates a statistically significant difference against the negative control ($P < 0.0001$)

Conclusions

Plants are one of the most essential natural ingredients in cosmetics, particularly skincare. Considerable evidence suggests that plants possess anti-ageing qualities by functioning as antioxidants, which is one of the most prominent highlights of their skincare benefits. Our phytochemical profiling study of *L. oppositifolia* stem extract revealed phytoconstituent profiles associated with antioxidant properties. Moreover, our findings demonstrated significant antioxidant activity through *in vitro* assessments employing DPPH and FRAP techniques and *ex vivo* evaluations conducted on skin cell lines (HaCaT and 3T3 cells). According to our findings, *L. oppositifolia* stem extract emerges as a promising candidate for use as a skincare product. However, more research is needed to establish its comprehensive phytoconstituent profiles, substantiate its efficacy and explore its full potential, including conducting *in vivo* investigations. These necessary steps are essential before considering its integration into skincare products.

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Conflict of interest

The authors declare no conflict of interest.

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