Components Analysis of Bioactive Essential Oil Combinations (Lavender, Lemon, and Cinnamon) by Gas Chromatography-Mass Spectrometry and their Activities against *In Vitro* Photoaging on Hairless Rat Dorsal Skin

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ABSTRACT

The essential oil of lavender, lemon, and cinnamon (LaLC) combination is rich in antioxidants and potentially be used as an anti-wrinkle and strengthens the collagen tissue. Ultraviolet B (UVB) radiation is a free radical source that accelerates the aging process and reduces collagen production. This study aims to characterize the chemical components of each oil and determine the best combination as an anti-wrinkle substance. The test was conducted on twenty-four Wistar male rats (*Mus musculus*) that were divided into six experimental groups consisting of the normal (N), control (C), vehicle control (V), first treatment (T1), second treatment (T2), and third treatment (T3) groups. Each sample was rubbed upon, and the UVB irradiation was administered frequently to each subject. The embedded skin specimen was analyzed using a digital-capable microscope. Data were analyzed through the Kolmogorov-Smirnov normality test, one-way analysis of variance (ANOVA), and the post-hoc Tukey's Honest Significant Difference test. Lavender, lemon, and cinnamon essential oils contained each most significant component, which was linalool (41.46% peak area), dl limonene (44.74% peak area), and 2-propenal, 3-phenyl- (CAS) (53.89% peak area), respectively according to the Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The T1 showed the best score of all parameters and did not show significant significance compared to the N group. In conclusion, the 1:1:3 combination of LaLC is better in preventing *in vitro* photoaging than other treatment groups.
Keywords : photoaging; UVB irradiation; LaLC essential oils combination; anti-wrinkle; collagen density

INTRODUCTION

Aging is an inevitable process in the living organism and will happen periodically. Loss of skin elasticity becomes the pre-aging indication due to less collagen production. The aging process is followed immediately by phenotypic changes in cutaneous cells, as well as structural and functional changes in extracellular matrix components such as collagen, elastin, and proteoglycans, which are required to provide the skin with tensile strength, elasticity, and hydration (Zhang & Duan, 2018).

Long-term exposure to ultraviolet radiation (UVR), either from the sun or direct irradiation, is the primary cause of extrinsic skin aging, known as photoaging. Skin wrinkling is one of the most visible signs of aging, and it is caused by decreased collagen levels and faster collagen disruption. Collagen is produced from procollagen secreted by dermal fibroblasts. Transforming growth factor β (TGF- β) is responsible for collagen disruption.

Several short-wavelength radiations, such as UV and infrared radiation or even visible light, induce the disintegration of collagen due to the matrix metalloproteinases (MMPs) (Campa & Baron, 2018; Zhang & Duan, 2018).

Two key factors responsible for the cutaneous aging process are intrinsic and extrinsic. The intrinsic factor is affected by the internal aging condition, for instance, excessive free radicals in the body due to the thin, dry skin, fine wrinkles, and gradual dermal atrophy. Furthermore, extrinsic aging is triggered by external environmental factors such as air pollution, smoking, poor nutrition, and sun exposure, resulting in coarse wrinkles, loss of elasticity, laxity, and rough-textured appearance. (Zhang & Duan, 2018).

Wrinkled skin has defined the accumulation of altered elastic fibers and the degradation or degeneration of collagen fibers in the dermis on a histological level. The damaged or dysfunctional mitochondria will be withdrawn regularly via autophagy and replaced by healthy elements

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fusion throughout the mitochondrial network. Autophagy will be activated by UVB p53 stabilization that initiates transcription processes of AMP-activated protein kinase (AMPK), sestrin 2 (SESN2), and UV radiation resistance-associated gene (UVRG). However, when the aging autophagy diminishes, and the mitochondrial fission process is higher than fusion, the total dysfunctional mitochondria will rise. These processes produce numerous reactive oxidative species (ROS) and trigger oxidative damage along with increased membrane permeability. This process leads to the senescence of cells and causes a pro-inflammatory phenotype formation related to the aging process (Gromkowska-Kępka et al., 2021; Rippo et al., 2014; Subedi et al., 2017).

The UVR in minimum dosage causes sun-tan and accelerates photoaging. UVR can be categorized into three main types, UVA (320–400 nm), UVB (280–320 nm), and UVC (100–280 nm) rays. Half of UVB is absorbed by the earth-ozone layer, and the remaining one penetrates the epidermis. The stratum corneum, the epidermis's outermost layer, absorbs nearly all UVB. The chronically UV-irradiated epidermis causes thinning of the epidermis, fine wrinkles, dryness, and a disrupted epidermal barrier function. UVB exposure was found to cause a significant increase in ROS production and a decrease in cell viability in human epidermal keratinocytes (HaCaT) cells. UVB-induced reactive oxidative species (ROS) activates mitogen-activated protein kinase (MAPK) signaling, along with the transcription factors activator protein-1 (AP-1) and nuclear factor-B (NF-κB), allowing inflammation-aging and apoptosis in cells and aging of the skin (Ansary et al., 2021).

The increasing incidence of collagen impairment related to wrinkly skin has encouraged the research community to investigate and formulate the best skin photo-protection alternatives. Several inorganic sunscreen substances such as titanium dioxide, kaolin, zinc oxide, talc, and calamine have been widely used for protecting skin from UVA and UVB irradiation. However, these metal-based substances have become a significant concern due to their safety and potential toxicity. Therefore, natural-based products containing antioxidants have been investigated to overcome the problems (Dunaway et al., 2018; Geoffrey et al., 2019).

Antioxidants have the potential to form a new and increasing steady radical via intramolecular hydrogen bonding and oxidation process. The antioxidants in the skin are affected by UV radiation. All layers of UVB-exposed skin are reduced in ascorbate, glutathione (GSH), superoxide dismutase (SOD), catalase, and ubiquinol (Petruk et al., 2018). The exogenous antioxidant should be applied to elevate the cellular antioxidant activity. Any kind of exogenous antioxidants can be found in natural plant products. Plant products are widely known as the natural skin aging treatment, and some fractions have been studied by scientific research (Campa & Baron, 2018; Miguel, 2010).

One of the extracted plant products is an essential oil that can be defined as the organic complex mixture from medicinal and aromatic plants extracted by non-heating distillation techniques such as hydro-, steam, or dry distillation (Miguel, 2010). Lavender, lemon, and cinnamon (LaLC) are several aromatic plants that are often extracted as commercialized products. Lavender essential oil (LaEO) possesses numerous therapeutic properties and biological activities. According to Hajhashemi et al. and Prashar et al. studies summarized by Cardia et al. (2018), *Lavandula angustifolia* is rich in aromatic compounds and mainly contains 1,8-cineole, camphor, and endo-borneol. These compounds play significant roles in pharmacological and biological activities. The minor constituents are also contained in the species plant, which depends on the geographical origin and environmental conditions. Lemon essential oil or LEO (*Citrus lemon* L.) is one of the most effective substances against skin aging due to its abundant vitamin C compound. This substance has been used as the main component of skin aging cosmetics. LEO can reduce the damages caused by excessive oxidative processes (Guzmán & Lucia, 2021; Happy et al., 2021). In the study of Banglao et al. (2020), cinnamon essential oil had an antioxidant activity, which its capacity depended on the concentration, according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) assays. Also, cinnamon oil had abilities to inhibit tyrosinase, collagenase, and elastase synthesizes. Additionally, this essential oil had no *in vitro* cytotoxicity effect on normal human fibroblast cells at a concentration of less than 100 µg/mL. A combination of several essential oils can enhance their performance within the skin and cause significant skin aging to relieve (Guzmán & Lucia, 2021).

However, it is noteworthy that essential oils are rich in plant products and chemical compounds which can trigger allergic reactions. Once the pure essential oils are applied to the skin, it may cause a direct physical or chemical irritation to the outer protective layer, also known as irritant contact dermatitis. This reaction will occur faster than the

Table I. Combinations of samples

Essential oils	Samples for treatment (mL)		
	T1	**T2**	**T3**
Lavender	1	1	3
Lemon	1	3	1
Cinnamon	3	1	1
Olive	7.5	7.5	7.5
Total volume	**12.5**	**12.5**	**12.5**

systematic allergy process after direct application. Therefore, a pharmaceutical vehicle is needed to be a medium for administering essential oils. Olive oil can increase the stratum corneum integrity and skin barrier function. The trans-epidermal water loss (TEWL) declined after the topical application of olive oil to the forearms skin of several adult volunteers who gained atopic dermatitis or not (Lin et al., 2018).

According to the explanation above, combined essential oils become importantly studied for treating photoaging observed from the collagen density and wrinkle level. The combined LaLC essential oils in different ratios were diluted in the olive oil to letting off the irritation and increase the product value. The best combination can be used for product assembling in the future study.

METHODOLOGY
Materials
Twenty-four male Wistar rats (200-300 grams/rat daily, six to nine weeks old) from UD. Wistar Stockbreeding was used as the test subject. Lavender (*Lavandula angustifolia*), lemon (*Citrus limon* L.), and cinnamon essential oils (*Cinnamomum burmannii* B.) were purchased from Eloxa®. LaLC essential oils-used ratios are listed in Table 1. All other materials were olive oil vehicle substance, 37% formalin (Brataco®), and Mallory's trichrome tissue stain.

Methods
Design of Experiment
This research was a type of experimental research designed as a posttest-only control group design.

Subject and sample preparations
The Ethical Committee has approved this research of the Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approvals: 012103020 and -04021 for collagen density and anti-wrinkle activity observations, respectively) (Supplementary Figure. 1 (a-b)). All male Wistar rats were prepared as the model organism and pre-

treated for seven days. 3x4 cm of each dorsal rat was shaved and once more acclimated for twenty-four hours. The room temperature was 31^0C and 40-70% of humidity. The pre-treatment of subject lighting was set in accord with the standard (twelve hours for on- and off- lighting, respectively). Daily feeding was done to each rat by giving the AD1 feed. Additionally, a bottle of double-distilled water was given daily to the rats.

A total of the included subject groups were counted by Frederer's formula that was re-cited by Ihwah et al. (2018). Six groups of subjects, four rats per group, were resulted for the subsequent analysis.

Each combination was diluted with 60% olive oil (Astuti & Fitri, 2020), and the final volumes were obtained (Table 1). Untreated (N), UVB-induced (C), and vehicle substance (V) rats were prepared for comparison.

Organoleptic and chemical sample identifications
Each sample was tested via organoleptic and chemical analysis for proofing the Certificate of Analysis (CoA) data given by Eloxa® (Supplementary Figure. 2 (a-c)). The organoleptic tests were carried out at the Pharmacology Laboratory of Universitas Ahmad Dahlan, and the experimental parameters were color and smell. The chemical compounds of each sample were assessed using GC integrated with MS (GC-MS-QP2010 SE Shimadzu) by injecting 1 µL of each essential oil into the instrument. The analysis was carried out at the Integrated Laboratory of Universitas Ahmad Dahlan.

Color observation (SNI 06-3734-2006)
Each sample was dropped onto the watch glass covered with white paper on its back to ease the observation. Each sample's color was directly observed 30 cm distance looking.

Smell observation (SNI 06-3734-2006)
Each sample was dropped on 2x4 cm of test paper and was characterized by its smell.

Parametric analysis
Anti-wrinkle activity

This analysis was approved by the Ethical Committee of the Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approval: 01210 4021)

Subject treatments

Two drops of each sample and vehicle control were rubbed upon the dorsal skin (T1, T2, T3, V), and the time for sample absorption was set to twenty minutes. The UVB (Kernel KN-4003, Phillip®) was placed 4 cm on top of the subjects and was irradiated for ten minutes by applying 311 nm of wavelength, $7 mW/cm^2$ of ray intensity, and $4.20 J/cm^2$ of irradiation dose. The UVB irradiation was administered for five days weekly, and the wrinkle scoring was assessed after two weeks by three anonymous panelists to gain objective results.

Wrinkle scoring assessment

The UVB-irradiated skins were measured by the Bissett method of wrinkle scoring (Bissett et al., 1987). The wrinkle grading scales and each description are presented in Table II in quotation form.

Observation of histological collagen density

This analysis was approved by the Ethical Committee of the Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approval: 012103020). All subjects were arrested for forty-eight hours after the UVB irradiation to prevent acute injury. The subjects were sacrificed to observe histological collagen density via cervical spinal dislocation.

Histological specimen preparation

The skin-irradiated region was taken by 3x3 cm of excision biopsy, and the tissue was fixed with 10% of buffer formalin. The histological specimen of each subject was assembled at the Laboratory of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, and the Mallory's trichrome staining was used for the microscopic analysis. The specimen was analyzed for collagen density by a digital-capable microscope (Olympus CX23) at the Pharmacology Laboratory of Universitas Ahmad Dahlan.

Collagen density observation

Each specimen was analyzed on three microscope fields of view (FOV). The grading scale method was done according to Tandelilin et al. (2006).

Statistical analysis

Data were analyzed using SPSS 23.0 software, all presented in mean value ± standard deviation (SD). The One-sample Kolmogorov-Smirnov test conducted the normality test. Subsequently, the preliminary test was chosen by considering the resulted distribution of mean values: one-way Analysis of Variance (one-way ANOVA) for the normal-distributed data and Kruskal-Wallis analysis otherwise. The post-hoc Tukey's Honest Significance Difference was conducted when the data differed significantly from one another according to the preliminary testing. The RStudio software was performed to annotate the significance between treatments.

RESULT AND DISCUSSION
Identification of samples

The samples were identified to ensure the type of essential oils contained in the product. Each essential oil should have characteristics of freshness and did not contaminate based on the color identification. The organoleptic observations of the sample's smell and color are all listed in Table III.

Lavender possesses numerous volatile compounds in specific compositions and proportions, which are suitable for health. In the study of Guo & Wang (2020), who analyzed the descriptive sensory data from several panelists, LaEO smelled spicy, camphor-like, herbal, woody, pine-like, clove-like, hay, and medicine-like, with a strong floral note. The LEO emits strong and stimulant odors and lemon rinds (Kiecolt-Glasera et al., 2008), while the cinnamon essential oil gives a specific spicy aroma due to the abundant cinnamaldehyde (Siripatrawan, 2016).

The lavender essential oil has a pale yellow clear mobile liquid appearance and predominantly gives sweet, fresh floral, woody, camphor, fruity, and herb notes (ISO 3515:2002; Xiao et al., 2017). According to ISO 855:2003, LEO is a mobile, transparent liquid that may become clear at a lower temperature, pale yellow to dark green, and possesses a characteristic of fresh lemon. In another reference by Boughendjioua & Djeddi (2017), LEO is either transparent, pale yellow, or greenish-yellow in color, has a strong aromatic odor and bitter taste, and is soluble in fat and also other non-polar solvents. The cinnamon essential oil should possess a clear and mobile liquid, light to dark amber, and gives a spice-like odor reminiscing the aroma of eugenol (ISO 3524:2003a). In this study, all the samples conformed to the reference characteristics (Table III).

Table II. Grading scale of mouse skin wrinkling (Bissett et al., 1987)

Grades*	Description of skin
0	Numerous fine striations cover the back and flanks of the body. Fine striations run the length of the body (head-to-tail direction) and appear and disappear with motion.
1	All fine striations on the back along the spine are gone. A few shallow coarse wrinkles across the back (run perpendicular to head-to-tail direction) appear and disappear with motion.
2	All fine striations are gone. Some coarse wrinkles across the back (run perpendicular to head-to-tail direction) are permanent.
3	All fine striations are gone. Several deep coarse wrinkles across the back (run perpendicular to head-to-tail direction) are permanent.

* Half-grade increments can be used with this scale.
Each skin was hand-pinched to observe the lowering of elasticity. The pinched skin hardly returned to the steady state, indicating that the skin was lost its elasticity.

Table III. Organoleptic test results of each essential oil

Type of essential oils	Colors			Smells		
	Theory-based characteristics[1]	CoAs[1]	Obtained results[1]	Theory-based characteristics	CoAs	Obtained results
LaEO	Pale yellow	Colorless to pale yellow	Trans-parent	Gives sweet, fresh floral, woody, camphor, fruity, and herb notes	Charaste-ristic	Characteristic lavender odor
LEO	Transparent, pale yellow to greenish-yellow	Yellowish to slightly greenish	Yellow	Strong aromatic odor	Fresh lemon peel	Characteristic lemon odor
CEO	Light to dark amber	Clear yellow to brownish-yellow liquid	Clear yellow	Spice-like odor	Characte-ristic cinnamon odor	Characteristic cinnamon odor

[1] Theory-based characteristic is the characteristic of each essential oil according to the international standard and previous organoleptic studies.
[2] CoA is a certificate delivered by Eloxa® which explains the characteristics of each essential oil product.
[3] The obtained result is the resulted organoleptic analysis of an under-investigated essential oil.

Each essential oil was analyzed using GC-MS since it is generally used in separating and analyzing volatile compounds without breaking the chemical structure. GC-MS is a commonly used technique for profiling aromatic compounds by separating, detecting, and assessing each base compound in a complex one. An obtained peak in chromatogram represents a compound that is successfully separated and also for observing the condition of the running GC-MS. The retention time and width of a peak were the critical parameters in determining the type of compounds present in the sample. This technique allowed the identification of an injected sample by analyzing the resulted mass spectra and was compared to the National Institute of Standards and Technology (NIST) standard, while the obtained retention indices (RIs) are compared to the related reference studies (Forgács & Cserháti, 2003; Xie et al., 2013; Yang et al., 2018). The NIST and Wiley Mass Spectral Libraries were used for this study, and the chromatograms are presented in Figure. 1, 2, and 3.

Lavender essential oil (LaEO)
Twenty-five peaks were presented on the chromatogram; five were the highest, including peaks 10th, 14th, 6th, 11th, and 22nd. Based on Figure. 1, all the highest five peaks could be identified as linalool (I); linalyl acetate (II); 1,8-cineole (III); camphor (CAS) (IV); and trans-caryophyllene (V), respectively. Notably, the peak area correlates to the concentration of a component compound in the sample (Guiochon & Guillemin, 1988). Linalool was the major compound (peak area: 41.46%), followed by linalyl acetate as the second-largest
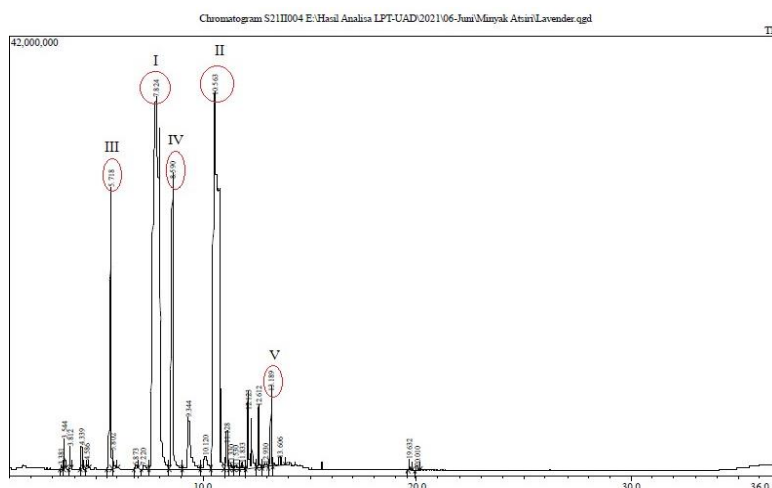
Figure 1. GC-MS data of LaEO. The red circle means the five chosen components since they give a wider %peak area than others. The I is linalool (peak 10) that becomes a dominant component, the II is linalyl acetate (peak 14), the III is 1,8-cineole (peak 6), and the IV is camphor (peak 11), and the V is trans-caryophyllene (peak 22).
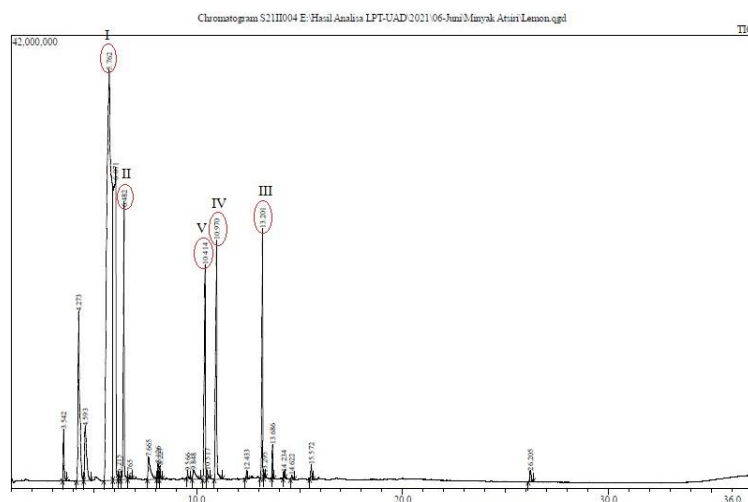

Figure 2. GC-MS data of LEO. The red circle means the five chosen components since they give a wider peak area than others. The I is dl-limonene (peak 4) that becomes a dominant component, the II is gamma-terpinene (peak 14), the III is trans-caryophyllene (peak 18), the IV is E-citral (peak 16), and the V is Z-citral (peak 14).

compound (peak area: 32.53%). The other three largest compounds were 1,8-cineole (peak area: 6.86%), camphor (peak area: 8.33%), and trans-caryophyllene (peak area: 1.04%). These results were in accord with the previous studies. Białoń et al. (Białoń et al. 2019) reported that the ETJA lavender oil contained linalool (41.8%) and linalyl acetate (32.7%), while the Crimean lavender oil contained 34.71% or 52.7% of linalool and 23.3% or 36.6% of linalyl acetate later was separated by two different matrix columns. The International Organization for Standardization (ISO) 3515 (ISO 3515:2002) stated that the linalool of lavender oils

is in the range of 28-38% and linalyl acetate are of 25-45%. However, the obtained data are in contrast to the CoA of LaEO (Supplementary Figure. 2a). The linalool was just 3.92%, and there was no geraniol presented in the observed LaEO. This present data could re-confirm the number and type of all constituents in the LaEO.

Lemon essential oil (LEO)

Twenty-four peaks are present on the chromatogram; five are the highest, including peaks 4th, 7th, 18th, 16th, and 14th. Based on Figure. 2, all the highest five peaks can be identified as dl-
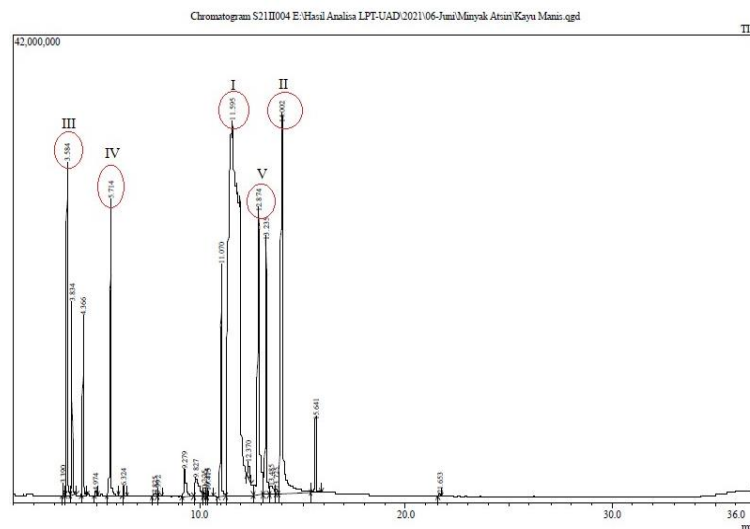
Figure 3. GC-MS data of CEO. The red circle means the five chosen components since they give a wider peak area than others. The I is 2-propenal, 3-phenyl- (CAS) (peak 16) that becomes a dominant component, the II is 2-propen-1-ol, 3-phenyl-, acetate (CAS) (peak 22), the III is tricyclene (peak 2), the IV is 1,8-cineole (peak 6), and the V is 3-allyl-6-methoxyphenol (peak 18).

limonene (peak area: 44.74%), gamma-terpinene (peak area: 6.04%), trans-caryophyllene (peak area: 4.92%), E-citral (peak area: 6.72%), and Z-citral (peak area: 5.46%), respectively. Limonene is substantial and has an impact on the LEO aroma. Limonene can reach about 60-95% in the form of oil, decreasing up to 48% in *Citrus limon*. Also, the gamma-terpinene is contained in the *Citrus* sp. for about 23% (González-Mas et al., 2019). Another previous study by Gök et al. (2015) stated that limonene in Kibris lemon (*Citrus limon* (L.) Burm. f.) oil extract was 72.48%, and 8.91% of gamma-terpinene in which they were extracted through the hydro distillation and cold pressing methods, respectively.

According to the CoA of LEO by Eloxa®, the limonene is 83.62% contained in the sample. The obtained data prove that all of them, especially limonene and gamma-terpinene, are present in the LEO and are related to the previous studies. However, the limonene concentration stated in CoA (Supplementary Figure. 2b) do not appropriate to the obtained data. This obtained data was slightly different from the CoA. This might be due to the storage condition or other indicative factors.

Cinnamon essential oil (CEO)
Twenty-four peaks are present on the chromatogram, and five are the highest, including peaks 16th, 22nd, 2nd, 6th, and 18th. Based on Figure. 3, all the highest five peaks can be identified as 2-propenal, 3-phenyl- (CAS) (peak area: 53.89%) that becomes a dominant component, the II is 2-propen-1-ol, 3-phenyl-, acetate (CAS) (peak area: 12.49%), the III is tricyclene (peak area: 4.35%), the IV is 1,8-cineole (peak area: 5.13%), and the V is 3-allyl-6-methoxyphenol (peak area: 7.51%); respectively.

The 2-propenal, 3-phenyl- (CAS) compound is also known as trans-cinnamaldehyde (Ashakirin et al., 2017), and the phenyl group is attached to the type of aldehyde structure. The cinnamaldehyde contained in cinnamon bark essential oil is about 90%, giving a specific spicy aroma, flavor, and biological activity. Another study by Trinh et al. (Trinh et al., 2015) found that the Vietnamese *Cinnamomum cassia* essential oil contained 90.08% of trans-cinnamaldehyde with a retention time of 16.17. Based on this present study, the trans-cinnamaldehyde (2-propenal, 3-phenyl- (CAS)) gives a %area of 53.89%. According to the CoA by Eloxa® (Supplementary Figure. 2c), the cinnamic aldehyde content of the cinnamon bark essential oil product is 71.03%. This obtained data was slightly different either from the CoA or previous studies. This might be due to the storage condition or other indicative factors.

Sample combinations against photoaging on rat's dorsal skin
The photoaging observation is based on two parameters: anti-wrinkle and collagen density.

Table IV. Results of wrinkle grading

Experimental groups	Wrinkle grading scales of each subject group				Mean ± standard deviation*
	1	2	3	4	
N	0	0	0	0	0[c]
C	3	2.3	2.6	2.3	2.55 ± 0.28[a]
V	2	1	1.6	1.3	1.47 ± 0.36[ab]
T1	0	1.3	1.3	2	1.15 ± 0.72[bc]
T2	2	1.6	0.6	1.6	1.45 ± 0.51[ab]
T3	1	1.6	2	1	1.40 ± 0.42[ab]

*different annotation indicates the presence of statistical significance between two groups.

Anti-wrinkle parameter

This research is referred to as the modification of Citrawan et al. (2019) and Rahmi et al. (2013). The anti-wrinkle test aims to observe the effect of three essential oil combinations mixed with olive oil on rat-induced UVB irradiation. The dorsal skin was characterized by determining the wrinkle grade scale (Table II). After the irradiation, each rat felt very dehydrated, and several fine wrinkles were formed on the dorsal skin. The wrinkle grading scales of each group were presented in Table IV.

Table IV, the average score of the N group (untreated group) was 0. It was in contrast to the C group (mean wrinkle score: 2.55±0.28), which the permanent shallow coarse wrinkles found on the skin. The V group showed slight protection against UVB irradiation and was significantly different from the N group (1.47±0.36), which the shallow coarse wrinkles found on dorsal skin. The shallow coarse wrinkles were formed in the T1 group (mean wrinkle score: 1.45±0.72), while the T2 group was much better in protecting than other treatments and the V group (1.15±0.72). The T3 group defended the skin from UVB better than V and T1 groups (1.40±0.42).

According to the post-hoc analysis (Table IV), the C, V, T2, and T3 groups show statistical significance compared to the N group. The V group did not show a statistical significance compared to the C group. Therefore, the individual olive oil could not be claimed as effective against wrinkles caused by UVB. The T2 group did not show statistical significance compared to the C and T3 groups. These were contrary to the T1 group, significantly different from the C group. However, it was not significant to the N group. It revealed that the T1 group could reduce the wrinkle formation genuinely as well as the N group. The visual observation of all experiments is presented in Figure. 4.

Essential oils are dominated by terpene compounds such as monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Tongnuanchan & Benjakul, 2014). Mimica-Dukić et al. (2016) stated that essential oils h ave a solid potential to scavenge free radicals and inhibit lipid peroxidation. In the study of Mimica-Dukić et al. (2016), volatile compounds in essential oils can act as antioxidants individually and in a mixture. Therefore, the capacity of antioxidants may be increased in combined essential oils.

Essential oils are liquid substances containing many volatile compounds extracted from various aromatic plants and have been well-known by people as a safer product than chemical cosmetics. Antioxidant plays a role in secondary prevention, i.e., minimizing impaired condition. Endogenous antioxidant enzyme levels can be affected by UV exposure. The higher the intensity of UV irradiation, the lower the endogenous antioxidant will result. Therefore, administering exogenous antioxidants is essential for increasing skin protection against free radicals caused by UV irradiation. Antioxidants from secondary metabolites of plants can work by three different mechanisms, 1) absorbing the UV radiations, 2) impeding all of the free radical reactions caused by UV radiations, and 3) regulating the endogenous antioxidant and inflammatory systems of post-irradiations. In general, after the skin cell is irradiated by UVB, the first line of antioxidant mechanism will be occurred by a defense process. Subsequently, the antioxidants in the second line prevent free radical chain initiation and disrupt chain propagation processes. The cell can stimulate the transcription and translation of de novo enzymes which are essential in fixing the response to oxidative stress. If a cell can overcome the stress damage, it will adapt and recover normal antioxidant levels. When the cells are exposed to extreme stress, the programmed cell death will be automatically done (Petruk et al., 2018).
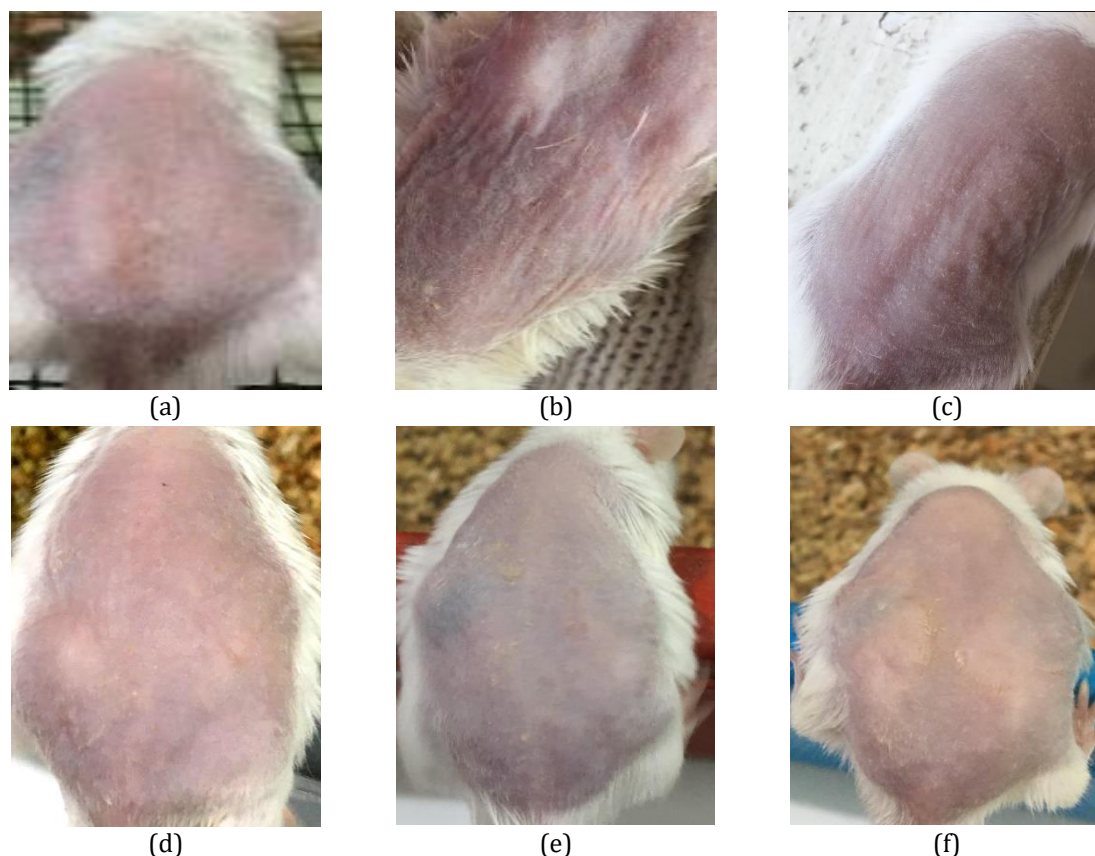
Figure 4. Wrinkles visualization. (a.) N, (b.) C, (c.) V, (d.) T1, (e.) T2, (f.) T3 group. The light burn is also shown on each rat-induced UVB.

Collagen density parameter

Collagen is the most important structural protein that makes up the human body. It is widely presented in tissues such as tendons, skin, and teeth and is seen as a fiber appearance. Collagen fibers in tissues are typically white, opaque, and easily identifiable. It is a viscoelastic material with enhanced tensile strength but low inextensibility. It creates support nets throughout the cellular structures to ensure the strength and durability of the human components. However, collagen will impair over time due to either internal or external aging and causes wrinkled skin (Avila Rodríguez et al., 2018). It can be implied that the collagen density observation is essential in analyzing the wrinkle caused by photoaging.

The collagen density can be observed microscopically and is determined on each scale by comparing it to the standard. The histological observation of each subject's collagen density using a digital-capable light microscope is presented in Figure. 5.

The microscopic observation was based on the three fields of view and observers. The density scaling was conducted on each subject, referring to Tandelilin et al. (2006). Based on histological observations (Figure. 5), there were several differences in collagen density among groups. The collagen fibers emerged with a high-dense appearance in the T1, T2, and T3. Based on the observations of each color contrast, the C group was reduced dramatically along with the V group. The result revealed that applying serial combinations of LaEO, LEO, and CEO to the photoaged rat could maintain the collagen density.

The data were normally distributed (P>0.05) according to the Kolmogorov-Smirnov test. Then, the data were conducted by preliminary tested of one-way ANOVA. The results revealed that the C group showed statistical significance over the other groups. It can define that the collagen density decreased significantly due to UVB irradiation. The data were supported by Figure. 5b, which showed a decrement in collagen color after the staining process. The T1 showed the highest protection against UVB irradiation. Meanwhile, all treatment groups did not show statistical significance.
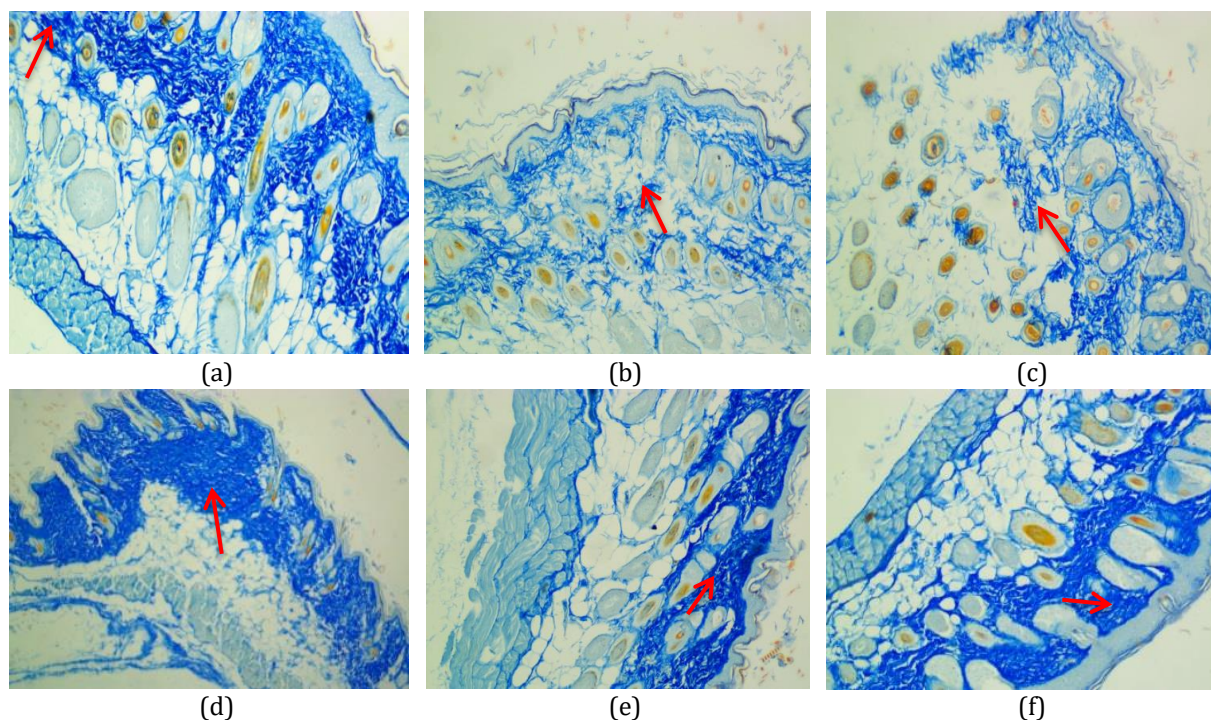
Figure 5. Histological observation of collagens on mice dorsal (*Mus musculus*) using Mallory's trichrome under the CX23 microscope of 40x magnification. The red arrow indicates the collagen histology of (a.) N, (b.) C, (c.) V, (d.) T1, (e.) T2, (f.) T3 group. The thickness of collagen can be seen clearly in this Figure.

Table V. Results of collagen density grading scores

Experimental groups	Collagen density grading scales of each subject group				Mean ± standard deviation*
	1	2	3	4	
N	2.77	2.77	2.33	2.88	2.68 ± 0.24[a]
C	1.44	1.55	1.44	1.55	1.49 ± 0.06[c]
V	2.44	2.11	1.55	2	2.02 ± 0.36[b]
T1	2.22	2.66	2.44	2.55	2.46 ± 0.18[ab]
T2	2.22	2.22	2.44	2.44	2.33 ± 0.12[ab]
T3	2.11	2.77	2.33	2.55	2.44 ± 0.28[ab]

*different annotation indicates the presence of statistical significance between two groups.

T1 has more lemon content, according to the results of the identification conducted by researchers on lemon essential oil using the GC-MS method, which states that it contains 44.74% of dl-Limonene, 6.04% of Gamma terpinene, which is a monoterpene compound, and E-Citral of 6.72%. In addition, essential oils contain vitamin C, which can promote collagen synthesis and increase dermal fibroblasts' proliferation and migration process. This vitamin C is also involved in the activation and stability of the hypoxia-inducible factor (HIF)-1, which is essential in controlling collagenase-related gene expression. (Pullar et al., 2017). The data were also supported by Anshori et al. (2017), which stated that oral administration of lemon peel extract could reduce MMP-1 expression and increase the amount of collagen in the skin tissue of male Wistar rats exposed to UV-B light.

CONCLUSION

Lavender, lemon, and cinnamon essential oils contained each most significant component, which was linalool (41.46% peak area), dl limonene (44.74% peak area), and 2-propenal, 3-phenyl- (CAS) (53.89% peak area), respectively according to the GC-MS analysis. The LaLC essential oils combination can prevent wrinkles and maintain collagen density. The T1 (LaLC 1:3:1) showed the best score for both anti-wrinkle activity (1.15 ± 0.72) and collagen density (2.46 ± 0.18) parameters, and it did not show statistical significance compared to the N group (0 and 2.68 ±

0.24 for anti-wrinkle and collagen density parameters, respectively). This LaLC combination can potentially be commercialized and used for preventing photoaging in a naturally effective way due to its bioactive compounds.

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