HASIL CEK_Components Analysis of Bioactive Essential Oil Combinations

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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. One of the essential oils becomes the primary active compound, while the others are the secondary active compounds. Ultraviolet B (UVB) radiation is a free radical source that accelerates the aging process and reduces collagen production. This study aims to identify the combination of essential oils in inhibiting collagen reduction and determine the best combination as an anti-wrinkle substance. The test was conducted in 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 let absorb into the rat skin for twenty minutes. Subsequently, the UVB irradiation was administered frequently to each subject for ten minutes with certain intensity of exposure dose. The excision biopsy was made by making 3x3 cm of skin specimen after killing each subject and 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 largest component which were 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 C group was significantly different from the treatment (T) group ($P<0.05$) in both wrinkle and

collagen density parameters. The T1 shows the highest score of all parameters and it does not show significantly significance compared to the N group. In conclusion, the combination of LaLC essential oils can avert collagen density reduction process so it increases the anti-wrinkle activity.

Keywords: photoaging, UVB irradiation, LaLC essential oils combination, anti-wrinkle, collagen density

INTRODUCTION

Aging is an inevitable process in living organism and will happen periodically. Loss of skin elasticity becomes the pre-aging indication due to the 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 collagens, 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, which is 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 the 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 that are responsible for the cutaneous aging process include the intrinsic and extrinsic. The intrinsic factor is affected by internal aging condition, for instance, excessive free radical in the body due to the thin, dry skin, fine wrinkles, and gradual dermal atrophy. Furthermore, extrinsic aging is triggered by external environment factors such as air pollution, smoking, poor nutrition, and sun exposure, resulting in rough 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 it will be replaced by healthy elements 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, a total of 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 that is 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 the 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 into 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 as well as 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).

Increasing incidence in collagen impairment that is related to the wrinkly skin has become an encouragement for 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 has been widely used for protecting skin for UVA and UVB irradiation. However, these metal-based substances have become a major concern due their safety and potential toxicity, therefore, the natural-based products containing antioxidants have been investigated to overcome the problems (Dunaway et al., 2018; Geoffrey et al., 2019).

Antioxidant have the potential to form a new and increasing a 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 have widely known as the natural skin aging treatment and some fractions of them have been studied by scientific research (Campa & Baron, 2018; Miguel, 2010).

One of the extracted plant products is 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 which 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 that were 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 major roles in pharmacological and biological activities. The minor constituents are also contained in the species plant which depend 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 in vitamin C compound, so this substance has been used as the main component of skin aging cosmetics. LEO can reduce the damages caused by excessive oxidative process (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 in concentration of less than 100 μg/mL. Combination of several essential oils can enhance their performance within the skin and cause a significant skin aging 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 onto the skin, it may cause a direct physical or chemical irritation to the outer protective layer which is also known as irritant contact dermatitis. This reaction will take place faster than the systematic allergy process after direct application. Therefore, a pharmaceutical vehicle is needed to be a medium for the administration of essential oils combination. Olive oil can increase the stratum corneum integrity and skin barrier function. The transepidermal water loss (TEWL) was 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 future study.

METHODOLOGY

Materials

Twenty-four male Wistar rats (200-300 grams/rat daily, six to nine weeks old) from UD. Wistar Stockbreeding were 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% of formalin (Brataco®), and Mallory's trichrome tissue stain.

Table 1 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**

Methods

1. **Design of Experiment**

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

2. Subject and sample preparations

This research has approved by the Ethical Committee of Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approvals: 012104020 and -21 for anti-wrinkle activity and collagen density observations, respectively) (Supplementary Figure 1 (a-b)). All of the male Wistar rats were prepared as the model organism and were 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 31ºC 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 aqua bidest was given daily to the rats.

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

Each combination was diluted in 60% of 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.

3. 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 observed 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 Integrated Laboratory of Universitas Ahmad Dahlan.

3.1 Color observation (SNI 06-3734-2006)

Each sample was dropped onto the watch glass covered with a white paper on its back to ease the observation. Each sample's color was directly observed 30 cm of distance looking.

3.2 Smell observation (SNI 06-3734-2006)

Each sample was dropped on the 2x4 cm of test paper and was characterized its smell.

4. Parametric analysis

4.1 Anti-wrinkle activity

This analysis was approved by the Ethical Committee of Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approval: 012104020)

4.1.1 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 for 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, 7mW/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 for gaining the objective results.

4.1.2 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 the description of each are presented in Table 2.

Table 2 Grading wrinkle scales and each characteristic (Bissett et al., 1987)

Scores*	Wrinkle characteristics
0	No wrinkles (Numerous fine striations covering back and flanks of body. Fine striations run length of body (head-to-tail direction) and appear and disappear with motion).
1	A few shallow rough wrinkles (All fine striations on back along spine gone. A few shallow coarse wrinkles across back (run perpendicular to head-to-tail direction) which appear and disappear with motion.
2	Several permanent rough wrinkles (All fine striations gone. Some coarse wrinkles across back (run perpendicular to head-to-tail direction) which are permanent).
3	Several permanent deep wrinkles (All fine striations gone. Several deep coarse wrinkles across back (run perpendicular to head-to-tail direction) which 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 that hardly returned to the steady state indicating that the skin was lost its elasticity.

4.2 Observation of histological collagen density

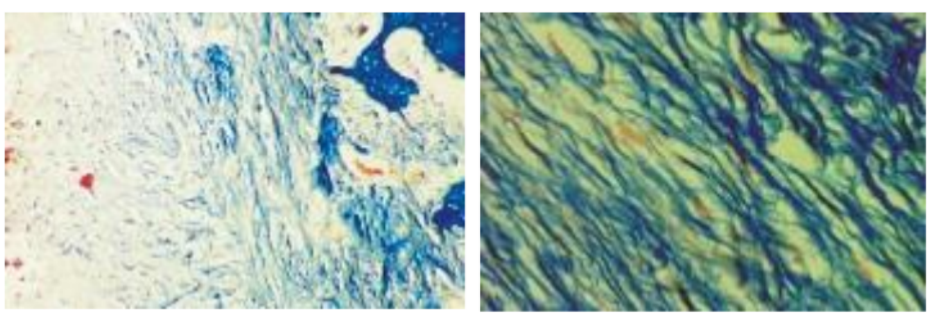
This analysis was approved by the Ethical Committee of Faculty of Pharmacy, Universitas Ahmad Dahlan (No. Ethical Approval: 012104021). All subjects were arrest for forty-eight hours from the UVB irradiation to prevent the acute injury. The subjects were sacrificed for the observation of histological collagen density via cervical spinal dislocation.

4.2.1 Histological specimen preparation

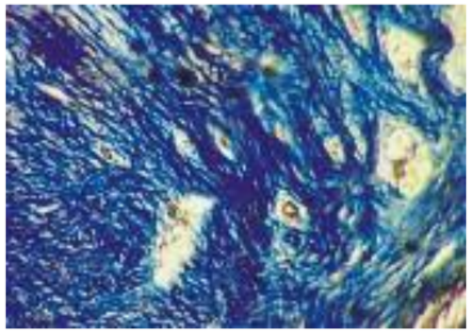
The skin-irradiated region was taken by 3x3 cm of excision biopsy and the tissue was fixed with 10% of buffer formalin. The histological specimen of each subject was assembled at Laboratory of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada and the Mallory's trichrome staining was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

4.2.2 Collagen density observation

Each specimen was analyzed on three of microscope field of views (FOV). The grading scale method was done according to Tandelilin et al. (2006) which is shown in Figure 1.



a. b.



c.

Figure 1 Reference for collagen densities observation. (a.) The tissues show a low-, (b.) medium-, (c.) and high collagen density (All the figures were taken from (Tandelilin et al., 2006))

5. Statistical analysis

Data were analyzed using SPSS 23.0 software and all of them are presented in mean value ± standard deviation (SD). The normality test was conducted by One-sample Kolmogorov-Smirnov 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 were significantly difference to one another according to the preliminary testing. The RStudio software was performed to annotate the significance between treatments.

RESULT AND DISCUSSION

1. Identification of samples

The samples were identified for ensuring the type of essential oils containing in the product. Each essential oil should have characteristics of fresh and did not contaminate based on the color identification. The organoleptic observations of sample's smell and color are all listed in Table 3.

Lavender possesses numerous volatile compounds in certain composition and proportion which are good 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 as well as lemon rinds (Kiecolt-Glasera et al., 2008), while the cinnamon essential oil gives a specific spicy aroma due to the abundant cinnamaldehyde (Siripatrawan, 2016).

Table 3 Organoleptic test results of each essential oil

	Colors			Smells		
Type of essential oils	**Theory-based characteristics[1]**	**CoAs[2]**	**Obtained results[3]**	**Theory-based characteristics**	**CoAs**	**Obtained results**
LaEO	Pale yellow	Colourless 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 cinnamo odor

Lavender essential oil possesses a pale yellow in color, clear mobile liquid in 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 mobile, clear liquid which may become clear in lower temperature, pale yellow to dark green in color, and possesses a characteristic of fresh lemon In another reference by Boughendjioua & Djeddi (2017), LEO is an 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. Cinnamon essential oil should possess a clear and mobile liquid, light to dark amber in color, and gives a spice-like odor reminiscing the aroma of eugenol (ISO 3524:2003a). In this study, all the samples conformed the reference characteristics (Table 3).

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

Each essential oil was analyzed using GC-MS since it has generally used in separation and analysis of volatile compounds without breaking the chemical structure. GC-MS is a commonly used technique for the profiling of 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 key parameters in determining the type of compounds present in the sample. This technique allows 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 2, 3, and 4.

1.1 Lavender essential oil (LaEO)

Twenty-five peaks presented on the chromatogram and five of them were the highest ones including peak 10^{th}, 14^{th}, 6^{th}, 11^{th}, and 22^{nd}. Based on Figure 2, 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. It is noteworthy that peak area corelates to the concentration of a component compound in the sample (Guiochon & Guillemin, 1988). Linalool was the major compound (peak area: 41.46%) and was followed by linalyl acetate as the second largest 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 are 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.

==== Shimadzu GCMS Report ====
LABORATORIUM PENELITIAN TERPADU
FAKULTAS FARMASI - UNIVERSITAS AHMAD DAHLAN

Data File : E:\Hasil Analisa LPT-UAD\2021\06-Juni\Minyak Atsiri\Lavender.qgd
Method File : C:\GCMSsolution\Data\Metode Analisa\Metode Minyak Atsiri 3.qgm
Analyzed by : Admin
Analyzed : 17/06/2021 12:24:47
Sample Type : Unknown
Level # : 1
Sample Name : S21II004
Sample ID : 006

Method

[Comment]

==== Analytical Line 1 ====

[AOC-20i]
# of Rinses with Presolvent :2
# of Rinses with Solvent(post) :2
# of Rinses with Sample :1
Plunger Speed(Suction) :High
Viscosity Comp. Time :0.2 sec
Plunger Speed(Injection) :High
Syringe Insertion Speed :High
Injection Mode :Normal
Pumping Times :5
Inj. Port Dwell Time :0.3 sec
Terminal Air Gap :No
Plunger Washing Speed :High
Washing Volume :8uL
Syringe Suction Position :0.0 mm
Syringe Injection Position :0.0 mm
Use 3 Solvent Vial :1 vial

[GC-2010]
Column Oven Temp. :75.0 °C
Injection Temp. :175.00 °C
Injection Mode :Split
Flow Control Mode :Pressure
Pressure :45.9 kPa
Total Flow :83.8 mL/min
Column Flow :0.80 mL/min
Linear Velocity :32.9 cm/sec
Purge Flow :3.0 mL/min
Split Ratio :100.0
High Pressure Injection :OFF
Carrier Gas Saver :OFF
Splitter Hold :OFF
Oven Temp. Program

Rate	Temperature(°C)	Hold Time(min)
-	75.0	5.00
10.00	250.0	10.00
20.00	300.0	2.00

Equilibrium Time :3.0 min

[GC Program]

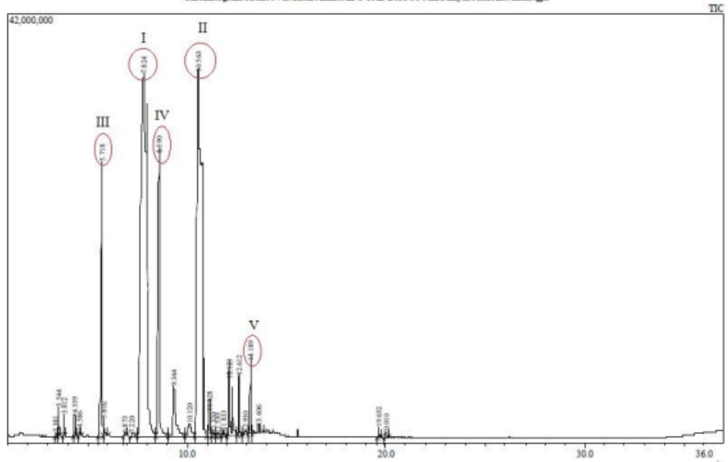
[GCMS-QP2010 SE]
IonSourceTemp :200.00 °C
Interface Temp. :225.00 °C
Solvent Cut Time :1.00 min
Detector Gain Mode :Relative
Detector Gain :1.04 kV +0.00 kV
Threshold :0

[MS Table]
--Group 1 - Event 1--
Start Time :1.00min
End Time :37.00min
ACQ Mode :Scan
Event Time :0.30sec
Scan Speed :2000
Start m/z :10.00
End m/z :500.00

Sample Inlet Unit :GC

[MS Program]
Use MS Program :OFF

Chromatogram S21II004 E:\Hasil Analisa LPT-UAD\2021\06-Juni\Minyak Atsiri\Lavender.qgd



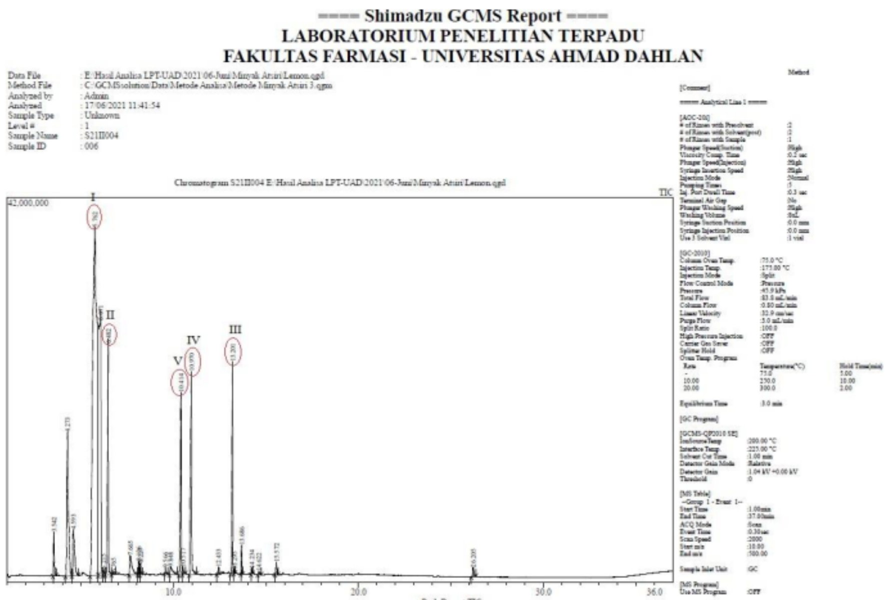
Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	3.381	3.335	3.465	841796	0.05	335914	0.20	2.51	Tricyclene
2	3.544	3.485	3.635	6211603	0.34	2877804	1.73	2.16	.ALPHA.-PINENE, (-)-
3	3.812	3.750	3.895	5041254	0.28	2113157	1.27	2.39	Camphene (CAS)
4	4.339	4.285	4.430	5860239	0.32	2172758	1.31	2.70	BICYCLO[3.1.1]HEPTANE, 6,6-DIMETHYL-2-METHYLENE-, (1S)-
5	4.586	4.530	4.695	2571747	0.14	811927	0.49	3.17	.beta.-Myrcene
6	5.718	5.515	5.770	124440682	6.86	26556732	16.00	4.69	1,8-Cineole
7	5.802	5.770	6.030	5988408	0.33	1674455	1.01	3.58	.ALPHA.-PINENE, (-)-
8	6.873	6.780	6.990	1799130	0.10	313084	0.19	5.75	trans-Linalool oxide
9	7.220	7.130	7.480	2564412	0.14	312199	0.19	8.21	trans-Linalool oxide
10	7.824	7.480	8.430	752186752	41.46	35511831	21.40	21.18	Linalool
11	8.590	8.430	9.050	151077373	8.33	26317251	15.86	5.74	Camphor (CAS)
12	9.344	9.050	9.890	49446613	2.73	4867327	2.93	10.16	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- (CAS)
13	10.120	9.890	10.390	15496504	0.85	1197003	0.72	12.95	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)- (CAS) p-Menth-1-en-8-ol, (S)-(-)-
14	10.563	10.390	10.990	590330523	32.53	35485844	21.39	16.64	LINALYL ACETATE
15	11.128	10.990	11.250	12015485	0.66	3225505	1.94	3.73	NERYL ACETATE
16	11.330	11.250	11.470	2189752	0.12	417221	0.25	5.25	1,5-Heptadien-4-ol, 3,3,6-trimethyl- (CAS)
17	11.530	11.470	11.690	1342999	0.07	231296	0.14	5.81	BICYCLO[3.3.1]HEPT-3-EN, 2-FORMYLMETHYL-4,6,6-TRIMETHYL-
18	11.833	11.690	11.990	2375608	0.13	533142	0.32	4.46	.BETA.-TERPINYL ACETATE
19	12.123	12.010	12.530	36293736	2.00	5989379	3.61	6.06	.ALPHA.-TERPINENYL ACETATE
20	12.612	12.530	12.750	13837882	0.76	5493718	3.31	2.52	Geranyl acetate
21	12.930	12.750	13.070	2986646	0.16	275699	0.17	10.83	5-Isopropenyl-2-methyl-7-oxabicyclo[4.1.0]heptan-2-ol
22	13.189	13.070	13.270	18806259	1.04	7114485	4.29	2.64	trans-Caryophyllene
23	13.606	13.270	13.850	6990624	0.39	756853	0.46	9.24	.beta.-Farnesene
24	19.632	19.570	19.790	2741954	0.15	1016746	0.61	2.70	(6E,8E,10E)-2,6,11,15-Tetramethyl-2,6,8,10,14-hexadecapentaene
25	20.010	19.910	20.150	1012416	0.06	334474	0.20	3.03	.beta. bisabolene
				1814450397	100.00	165935804	100.00		

Figure 2 GC-MS data of LaEO. The red circle means the five chosen components since they give 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), the IV is camphor (peak 11), and the V is trans-caryophyllene (peak 22).

1.2 Lemon essential oil (LEO)

Twenty-four peaks are present on the chromatogram and five of them are the highest ones including peaks 4th, 7th, 18th, 16th, and 14th. Based on Figure 3, all the highest five peaks can be identified as dl-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, and it will decrease 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.



Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	3.542	3.495	3.675	13342310	0.95	4764257	2.42	2.80	.ALPHA.-PINENE, (-)-
2	4.273	4.175	4.515	89397733	6.39	15692555	7.98	5.70	.beta.-Phellandrene
3	4.593	4.515	4.855	32640902	2.33	5068999	2.58	6.44	.beta.-Myrcene
4	5.762	5.495	5.935	626041032	44.74	37946999	19.30	16.50	dl-Limonene
5	6.071	5.935	6.185	251145875	17.95	27837274	14.16	9.02	dl-Limonene
6	6.215	6.185	6.315	3078341	0.22	669509	0.34	4.60	1,3,6-Octatriene, 3,7-dimethyl-, (Z)- (CAS)
7	6.482	6.315	6.655	84575269	6.04	25520933	12.98	3.31	.gamma.-Terpinene
8	6.765	6.655	6.875	1063481	0.08	201529	0.10	5.28	CYCLOHEXENE, 1-ISOPROPENYL-4-METHYL-
9	7.665	7.605	8.065	18051563	1.29	2110710	1.07	8.55	Linalool
10	8.126	8.065	8.185	3441959	0.25	1447590	0.74	2.38	Limonene oxide
11	8.227	8.185	8.335	2912931	0.21	1196671	0.61	2.43	Limonene oxide
12	9.566	9.495	9.705	1638581	0.12	689640	0.35	2.38	Decanal (CAS) n-Decanal
13	9.848	9.775	10.185	8216317	0.59	842259	0.43	9.76	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)- (CAS)
14	10.414	10.295	10.485	76389075	5.46	19555335	9.95	3.91	Z-Citral
15	10.517	10.485	10.645	2635518	0.19	813801	0.41	3.24	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)- (CAS)
16	10.970	10.855	11.265	94060406	6.72	21759625	11.07	4.32	E-Citral
17	12.433	12.345	12.485	2060983	0.15	819530	0.42	2.51	.alpha.-Copaene
18	13.201	13.085	13.255	68912133	4.92	22694150	11.54	3.04	trans-Caryophyllene
19	13.295	13.255	13.345	1097337	0.08	454720	0.23	2.41	GERMACRENE-D
20	13.686	13.635	13.755	6703122	0.48	3097774	1.58	2.16	.alpha.-Humulene
21	14.234	14.195	14.295	1326659	0.09	613430	0.31	2.16	Valencene
22	14.622	14.555	14.755	1230857	0.09	397869	0.20	3.09	.delta.-Cadinene
23	15.572	15.495	15.665	4453395	0.32	1359262	0.69	3.28	(-)-Caryophyllene oxide
24	26.205	26.095	26.345	4828032	0.35	1047931	0.53	4.61	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS) Bis(2-ethylhexyl) phthalate
				1399243811	100.00	196602352	100.00		

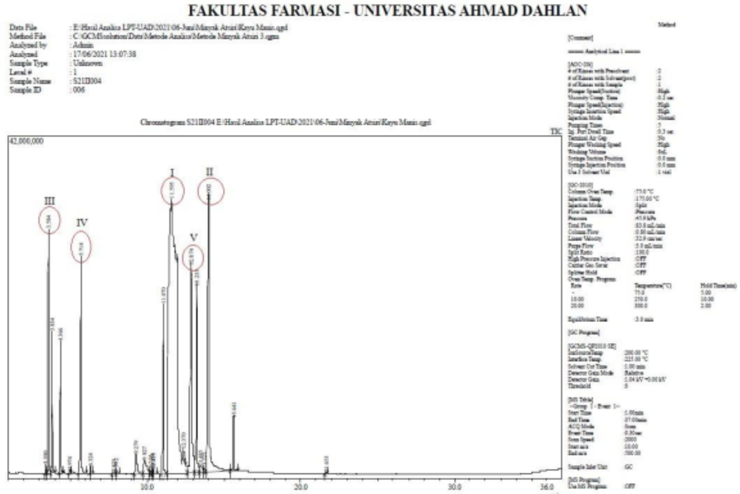
Figure 3 GC-MS data of LEO. The red circle means the five chosen components since they give wider peak area than others. The I is dl-limonene (peak 4) that becomes a dominant component, the II is gamma-terpinene (peak 14), the III is trans-caryophyllene (peak 18), the IV is E-citral (peak 16), and the V is Z-citral (peak 14).

According to the CoA of LEO by Eloxa®, the limonene is 83.62% containing in the sample. The obtained data proves all of them, especially for limonene and gamma-terpinene, 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 to the CoA. This might be due to the storage condition or other indicative factors.

1.3 Cinnamon essential oil (CEO)

Twenty-four peaks are present on the chromatogram and five of them are the highest ones including peaks 16th, 22nd, 2nd, 6th, and 18th. Based on Figure 4, 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.

==== Shimadzu GCMS Report ====
LABORATORIUM PENELITIAN TERPADU
FAKULTAS FARMASI - UNIVERSITAS AHMAD DAHLAN



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	3.390	3.330	3.490	3533882	0.16	1110914	0.47	3.18	Tricyclene
2	3.584	3.490	3.745	96206600	4.35	29990546	12.60	3.21	.ALPHA.-PINENE, (-)-
3	3.834	3.745	4.050	45490545	2.06	17367832	7.30	2.62	Camphene (CAS)
4	4.366	4.280	4.550	46933008	2.12	16114730	6.77	2.91	BICYCLO[3.1.1]HEPTANE, 6,6-DIMETHYL-2-METHYLENE-, (1S)-
5	4.974	4.905	5.065	1181004	0.05	336957	0.14	3.50	l-Phellandrene
6	5.714	5.500	6.075	113556771	5.13	26442538	11.11	4.29	1,8-Cineole
7	6.324	6.275	6.505	2537986	0.11	833822	0.35	3.04	.gamma.-Terpinene
8	7.825	7.705	7.955	2012713	0.09	194808	0.08	10.33	Linalool
9	7.992	7.955	8.155	1022724	0.05	211587	0.09	4.83	.ALPHA.-CAMPHOLENE ALDEHYDE
10	9.279	9.155	9.715	20538147	0.93	2415185	1.02	8.50	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)- (CAS)
11	9.827	9.715	10.195	23874455	1.08	1657942	0.70	14.40	3-Cyclohexene-1-methanol, .alpha.,.alpha.,4-trimethyl-, (S)- (CAS) p-Menth-1-en-8-ol, (S)-(-)-
12	10.225	10.195	10.305	1621051	0.07	302621	0.13	5.36	CIS-P-2-MENTHEN-1-OL
13	10.354	10.305	10.385	1650452	0.07	525189	0.22	3.14	ALPHA-CYCLOGERANIOLACETAT
14	10.415	10.385	10.675	2025879	0.09	502960	0.21	4.02	1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate
15	11.070	10.885	11.265	84604365	3.82	19846659	8.34	4.26	ACETIC ACID 1,7,7-TRIMETHYL-BICYCLO[2.2.1]HEPT-2-YL ESTER
16	11.595	11.265	12.605	1192236801	53.89	33682653	14.16	35.40	2-Propenal, 3-phenyl- (CAS)
17	12.370	12.305	12.525	6970071	0.32	1352273	0.57	5.15	2-Propenal, 3-phenyl- (CAS)
18	12.874	12.605	13.085	166044318	7.51	24482827	10.29	6.78	3-Allyl-6-methoxyphenol
19	13.235	13.085	13.385	89480582	4.04	19749656	8.30	4.53	trans-Caryophyllene
20	13.485	13.385	13.685	9904051	0.45	711618	0.30	13.92	Phenol, 2-methoxy-3-(2-propenyl)- (CAS) Phenol, 3-allyl-2-methoxy- (CAS) 3-Allylguaiacol
21	13.725	13.685	13.845	3570214	0.16	482624	0.20	7.40	.alpha.-Humulene
22	14.002	13.845	15.385	276261501	12.49	33124575	13.92	8.34	2-Propen-1-ol, 3-phenyl-, acetate (CAS)
23	15.641	15.385	15.905	19959640	0.90	6280039	2.64	3.18	(-)-Caryophyllene oxide
24	21.653	21.545	21.725	940548	0.04	210151	0.09	4.48	CYCLOPROPANE, 2-BENZYL-1,1-DIMETHYL-
				2212155308	100.00	237930706	100.00		

Figure 4 GC-MS data of CEO. The red circle means the five chosen components since they give 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).

The 2-propenal, 3-phenyl- (CAS) compound is also known as trans-cinnamaldehyde (Ashakirin et al., 2017) which the phenyl group is attached to the type of aldehyde structure. The cinnamaldehyde contained in cinnamon bark essential oil is as much as about 90% and it gives a specific spicy aroma, flavor, and its biological activity. Another study by Trinh et al. (Trinh et al., 2015) the Vietnamese *Cinnamomum cassia* essential oil contained 90.08% of trans-cinnamaldehyde with the retention time of 16.17. Based on this present study, the trans-cinnamaldehyde (2-propenal, 3-phenyl- (CAS)) gives the %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 to the CoA or previous studies. This might be due to the storage condition or other indicative factors.

2. Sample combinations against photoaging on rat's dorsal skin

The photoaging observation is based on two parameters including anti-wrinkle and collagen density.

2.1 Anti-wrinkle parameter

This research is referred to the Citrawan et al. (2019) and Rahmi et al. (2013) with modification. 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 2). Each rat felt very dehydrated after the irradiation and several fine-wrinkles were formed on the dorsal skin. The wrinkle grading scales of each group were presented on Table 4.

Table 4 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}

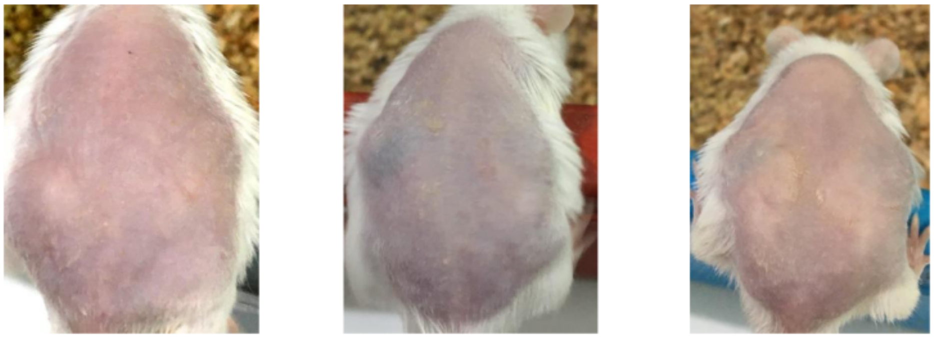
T3	1	1.6	2	1	1.40 ± 0.42[ab]

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

Since the data had a normal distribution (P>0.005) according to the Kolmogorov-Smirnov test, the ANOVA was conducted as the preliminary test (data not shown). Based on Table 4, 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 4), the C, V, T2, and T3 groups show statistical significances 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 wrinkle caused by UVB. T2 group did not show a statistical significance compared to the C group as well as T3 group. These were contrary to the T1 group that was significantly different compared to the C group, however, it was not significant to the N group. It revealed that T1 group could reduce the wrinkle formation genuinely as well as the N group. The visual observation of all experiments is presented in Figure 5.

a. b. c.



d. e. f.

Figure 5 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.

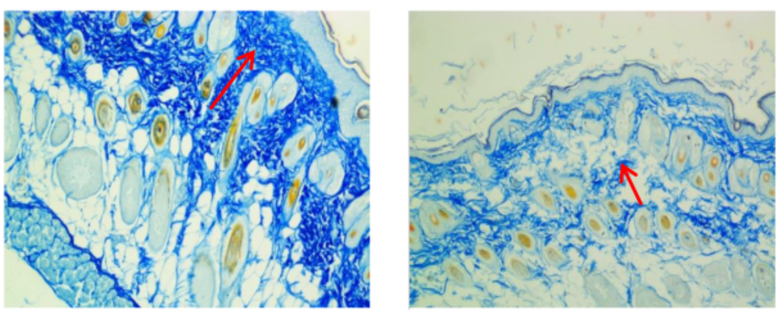
Essential oils are dominated by terpene compounds such as monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Tongnuanchan & Benjakul, 2014). Mimica-Dukić et al. (2016) stated that essential oils have a strong potential to scavenge free radicals and inhibit lipid peroxidation. In the study of Mimica-Dukić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

Essential oils are liquid substances containing many volatile compounds extracted from various aromatic plants and have well-known by people as safer product than chemical cosmetics. Antioxidant plays a role in secondary prevention, i.e. it minimizes the impaired condition. Endogenous antioxidant enzyme levels can be affected by UV exposure. The higher the intensity of UV irradiation is, the lower the endogenous antioxidant will be resulted. Therefore, the administer of exogenous antioxidant is essential for increasing the skin protection against free radicals caused by UV irradiation. Antioxidant 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, 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 occured by a defense process and subsequently the antioxidants in the second line prevent free radical chain initiation and/or disrupt chain propagation processes. The cell can stimulate the transcription and translation of de novo enzymes which are important 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 the extreme stress, the programmed cell death will be automatically done (Petruk et al., 2018).

2.2 Collagen density parameter

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

The collagen density can be observed through the microscopic way and is determined each scale by comparing to the standard. The histological observation of each subject’s collagen density using digital-capable light microscope is presented in Figure 6.



a. b.

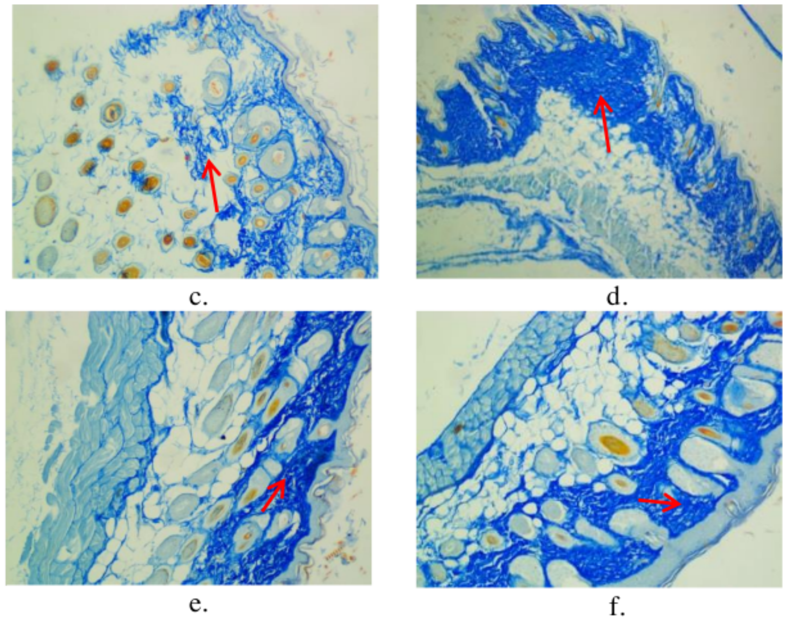


Figure 6 Histological observation of collagens on mice dorsal (*Mus musculus*) using Mallory's trichrome under the CX23 microscope of 40x magnification. Red arrow indicates the collagen histology of (a.) N, (b.) C, (c.) V, (d.) T1, (e.) T2, (f.) T3 group. The thickness of collagen can be seen clearly in this figure.

The microscopic observation was according to the three fields of view and three observers. The density scaling was conducted in each subject referring to the Tandelilin et al. (2006) (Figure 1). Based on histological observations (Figure 6), there were several differences of collagen density among groups. The collagen fibers emerged a high-dense appearance as well as 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 the application of serial combinations of LaEO, LEO, and CEO to the photoaged rat could maintain the collagen density.

Table 5 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 present of statistical significance between two groups.

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 6b which showed a decrement in collagen's color after the staining process. The T1 showed the highest protection against UVB irradiation. Meanwhile, all treatment groups did not show statistical significance.

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 the collagen synthesis and increasing the proliferation and migration process of dermal fibroblasts. This vitamin C is also involved in the activation and stability of the hypoxia-inducible factor (HIF)-1, which is important in the control of collagenase-related gene expression. (Pullar et al., 2017). The research data were also supported by research conducted by Anshori et al. (2017) which stated that oral administration of lemon peel extract can reduce MMP-1 expression and increase the amount of collagen in the skin tissue of male Wistar rats exposed to UV-B light.

CONCLUSION

The LaLC essential oils combination can prevent the wrinkle process and also maintain the collagen density. The T1 shows the highest score of all parameters and it does not show significantly significance compared to the N group.

ACKNOWLEDGMENT

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