Asian J Agric & Biol. 2023(4).
DOI: 10.35495/ajab.2023.117

AJAB

Original Article

Biological activity of leaves of three *Morinda* species detected by stimulation and suppression of gene expression of collagen, elastin, melanin and other related genes

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Received:
July 20, 2023
Accepted:
August 07, 2023
Published Online:
August 28, 2023

Abstract

Morinda coreia, *M. elliptica* and *M. tomentosa* leaves were investigated. α-EG and pinoresinol were found by GC-MS and GC-FID. MTT and comet assays did not reveal toxicity in normal PBMCs and DHFa cells, but the ethanol and hexane *M. coreia* and *M. tomentosa* extracts were toxic to CHL-1 cancer cells. Biological activities were detected by gene expression through qRT-PCR. Ethanol *M. tomentosa* extract can stimulate collagen type I, II, III and elastin creation through expression of *COL1A*, *COL1A2*, *COL2A1*, *COL-II*, *COLL2*, *COL3A* and *ELN* genes; inhibit enzyme synthesis for collagen and elastin degradation through *ELANE*, *MMP1*, *MMP13*, *TIMP1* genes; does not affect DHFa cells, but does affect CHL-1 cells through the *NRF2* gene; can stimulate DHFa cell proliferation through the *FGF1* and *FGF7* genes; has anti-inflammatory effects on DHFa cells, and has no effects on CHL-1 cells through the *TGF-β* gene. Ethanol *M. tomentosa* extract can suppress melanin production through the *α-MSH* and *TYR* genes. In summary, *M. tomentosa* can be used in human health and skin care products, the research was accomplished with the aim of deducing how more plant species with high bioactivities can be utilized without toxicity, the leaves of *M. tomentosa* can be easier to use correctly than fruits depending on the season and smell.

Keywords: Collagen, Elastin, α-EG, *Morinda* species, Pinoresinol, qRT-PCR

How to cite this:

Sudmoon R, Kaewdaungdee S, Tanee T, Siripiyasing P, Ang A, Lee L, Wong XJ, Lee SY and Chaveerach A. Biological activity of leaves of three *Morinda* species detected by stimulation and suppression of gene expression of collagen, elastin, melanin and other related genes. Asian J. Agric. Biol. 2023(4): 2023117. DOI: https://doi.org/10.35495/ajab.2023.117

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Introduction

Human health and beauty need collagen which is an extracellular matrix protein, the main structural protein found in skin, tendon, and bone, and the most abundant component of extracellular matrix protein in skeletal muscle tissue, (Zhang et al., 2021). However, in order to take advantage of its benefit to the tensile strength of skin, it is not possible to take collagen orally as a nutritional supplement as it is digested into its component amino acids, nor will it penetrate through the skin from outside the body due to large molecular weight. Although collagen hydrolysates, a group of peptides, have been used instead, they function solely as an aid for controlling cell proliferation, water-holding capacity, moisture absorption, retention, and anti-aging in skin (León-López et al., 2019; Zhang et al., 2021; Wang, 2021; Kim et al., 2022). From such mentioned information, the body has to produce collagen and elastin by itself, requiring glycine, proline, hydroxyproline, glycine and proline residues as precursors (Kim et al., 2022; Aziz et al., 2016). There are various structural conformations such as fibrillar collagen which can be found in abundance in human skin, over 90% of the collagen in the human body is collagen type I, contained in almost all connective tissue (Wang, 2021). Collagen type I and III are predominant in skin and can be found in abundance in the dermal layer. Also, a large amount of collagen and elastin are present in the reticular portion of the dermal layer (Aziz et al., 2016). Elastin is another extracellular matrix protein that plays an important role in maintaining cutaneous homeostasis, extensibility and reversible recoil to skin, which allows the skin to withstand repeated mechanical deformation without suffering irreversible plastic damage. It is most abundance in elastic fibers, which contributes to the characteristic properties of elastic recoil (Rauscher and Pomes, 2012; Aziz et al., 2016; Kim et al, 2022). Hyaluronic acid is one more substance effective in the skin. Its ability is to replenish moisture, water holding, resulting in smooth and moisture skin which can be slow down the formation of wrinkles and improve skin texture (Kim et al., 2022). Additionally, it can promote cell regeneration and stimulate the production of collagen. So, it is already used in various products including dermal fillers and cosmetics (Bukhari et al., 2018). One more factor for beauty, melanogenesis is the well-known mechanism for melanin production causing the skin to not be white and bright, but rather dull. There are several compounds that inhibit tyrosinase activity in melanogenesis, thus blocking the synthesis of melanin, such as arbutin (Noikotr et al., 2018; Kaewduangdee et al., 2020), α-EG and pinorasinol (Sudmoon et al., 2022).

Several compounds including synthetics and phytochemicals can stimulate collagen, elastin and hyaluronic acid production in the body, natural substances are likely to be more reliable in these matters such as α-EG increasing normal human dermal fibroblasts (NHDF) proliferation and collagen type I (Bogaki et al., 2017); asiatic ester with diaminobutyl acid stimulates significantly more collagen synthesis in normal human fibroblast than asiatic and betulinic acid (Drąg–Zalesińska et al., 2019); nutraceutical compounds containing lycopene, vitamin C, lutein and manganese have shown in vitro efficacy for stimulating the synthesis of collagen, elastin and hyaluronic acid components that are crucial for providing the dermis' supporting structure, being responsible for the skin's firmness and elasticity (Schalka et al., 2017).

Following the literature review, the authors studied the bioactivity of three *Morinda* species including *M. tomentosa*, *M. elliptica* and *M. coreia* based on phytochemicals related to collagen, elastin, melanin, and anti-inflammatory creation by gene expression detection, for applications without toxicity.

Material and Methods

Plant materials

The leaves of the three *Morinda* species were collected including *M. coreia* Ham, *M. elliptica* (Hook. f.) Ridl at the field of Khon Kaen province, and *M. tomentosa* Heyne ex Roth, from Nakhon Si Thammarat province, Thailand. They were identified by proficient botanist, Prof. Arunrat Chaveerach, Ph.D. Collector numbers are A. Chaveerach 1102-1104, kept at Department of Biology, Faculty of Science, Khon Kaen University. They are not a protected plant in Thailand, nor did we collect from private or protected areas that require permits. They are common species, grow widely in gardens, fields, and forests. The leaves were air-dried at room temperature or dried in oven at 60 ^{o}C, and after that they were kept under low humidity until further use.

Phytochemical extraction

The three studied *Morinda* species' dried leaves were

finely ground. The 25 g leaf powder were soaked separately with ethanol and hexane solvents at a ratio of 1 g: 5 mL. Then, the extracts were incubated for 72 h at room temperature. After that, the extracts were filtered through filter paper (Whatman no 1). Subsequently, the solvents were eliminated by vacuum concentrator and re-dissolved with 10 mL each solvent.

For phytochemical screening and targeted substance analysis, the 2 mL of each extract was fractionated with 800 µL of the mixed solvents between petroleum ether: diethyl ether (2:1 v/v). After precipitation, the clear solution was collected and filtrated through a 0.45 µm syringe filter into vials. For the remaining extract, the solvents were eliminated using a speed vacuum concentrator. After that, the dried extracts were re-dissolved with 10% DMSO and filtered through a 0.45 µm syringe filter. The extracts were 2-fold serially diluted for eight concentrations of working solutions with sterile water and kept at -20 °C for further use in toxicity and gene expression evaluation.

Phytochemical screening by Gas Chromatography-Mass Spectrometry (GC-MS)

The phytochemical components of the studied plant extracts were analyzed by an Agilent Technologies GC 6890 N, MS 5973 inert mass spectrometer fused with a capillary column (30.0 m x 250 µm x 0.25 µm) with a slightly modified protocol from Jahan et al. (2020). Helium gas was used as the carrier at a constant flow rate of 1 mL/min. The injection and mass- transferred line temperature was set at 280°C. The oven temperature was programmed from 70°C to 120°C and 3°C/min, then held isothermally for 2 min and finally raised to 270°C and 5°C/min. The 1 µL of the aliquot was injected in the split mode (1:10). Phytochemical components were identified by comparing the obtained mass spectra with the reference compounds in the Wiley 7N.1 library. The relative percentage of phytochemical constituents was calculated as a percentage by peak area normalization.

α-EG and pinoresinol detection, and quantification by gas chromatography-flame ionization detector (GC-FID)

The extracts were 2-fold serially diluted with the extracted solvent. The targeted compounds α-EG and pinoresinol were examined by GC using an Agilent Technologies 7890B GC system, equipped with flame ionization detector (FID), corresponding to Sudmoon et al. (2022). Helium was used as the carrier gas, at a flow rate of 1 mL/min. The injector and detector temperatures were 260ºC and 320ºC, respectively. The initial temperature for the oven was set at 120ºC and increased by 10ºC/min to a final temperature of 300ºC, which was held for 10 min. Then, 1 µL of each sample was injected into a HP-5 capillary column (30.0 m x 0.32 mm i.d. x 0.25 µm film thickness) with a split mode (1:10).

The 1 mg of 98% α-EG (Combi-Blocks, USA) and 95% pinoresinol (Sigma, USA) standards were prepared and dissolved in 1 mL ethanol. The standard solution was 2-fold serially diluted for 5 levels (0.0625-1 mg/mL). The retention time of the standard was used for α-EG and pinoresinol identification in the extracts. Additionally, the areas of the standards were plotted with its concentration to generate linear calibration equation curves of $y=mx+c$ and a correlation coefficient (R^2). For α-EG and pinoresinol quantification, the peak areas of the extracts were substituted in y to calculate x as a concentration and content of the targeted substance.

Cell preparation: Normal Human Peripheral Blood Mononuclear Cells (PBMCs) Isolation

PBMCs were isolated from sodium heparin anticoagulated venous blood from a blood bank of Srinagarind Hospital using Ficoll-Paque™ Plus (Cytiva, Sweden) as recommended. The method is slightly modified from Freshney (2010). The PBMCs were isolated with viability of at least 98% to use for the toxicity testing. The cells were suspended in modified RPMI-1640 medium (Cytiva, USA) with L-glutamine, supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin (Gibco Life Technologies, USA). The cells were calculated as a concentration of 10^6 cells/mL for toxicity testing.

Cell line preparation: Dermal Human Fibroblast (DHFa) and Human Melanoma Cells (CHL-1) Culture

The frozen DHFa and CHL-1 cells were rapidly thawed at 37°C and transferred into 15 mL tube, then centrifuged at 2,200 rpm for 10 mins. The supernatant was discarded and the cells were gently suspended with fresh DMEM with low glucose, supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin as a culture media. The suspended cells were transferred into 60 mL cultured flask and incubated at 37°C for cell activation. For cell harvesting, the adherent cells were trypsinizied using

0.25% trypsin-EDTA (1X) and centrifuged at 2,200 rpm for 10 mins. The supernatant was removed and resuspended with fresh media. The cells were utilized for toxicity and biological activity testing at a concentration of 10^4-10^5 cell/mL.

Cytotoxicity testing by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction (MTT) assay

The prepared PBMCs (1x10^6 cell/well), DHFa, and CHL-1 (2x10^4 cell/well) were seeded into 96-well plates, 125 µL per well. 12.5 µL of the eight concentration levels of *Morinda* leaf extract working solutions were added to the corresponding wells. The plates were incubated for 24 h in a humidified CO_2 incubator under 37°C and 5% CO_2 condition, 10% DMSO were performed as vehicle controls. For MTT assay, the plates were centrifuged at 1,500 rpm for 10 min and the supernatant was discarded. The MTT solution was added to a final concentration of 0.5 mg/mL in volume of 10 µL per well. After that, the plates were incubated for 4 h at 37°C. Next, the formazan crystals were solubilized by adding 100 µL DMSO to each well and measured at 570 nm with a fluorescence microplate reader (SpectraMax M5 series, Molecular Devices). Each treatment was performed in triplicate. Cellular reduction of MTT formed a violet crystal formazan through mitochondrial succinate dehydrogenase activity of the viable cells, and the violet crystal formazan was quantified following Freshney (2010) method. The percentage of cell viability was calculated using the equation,

$$Cell\ viability\ (\%) = \frac{\text{Average viable of treated cells}}{\text{Average viable of untreated cells (negative control)}} x\ 100$$

To reveal the cytotoxicity of the plant extracts, doses inducing 50% inhibition of cell viability (IC_{50}) was calculated using nonlinear regression, between the extract concentrations and cell viability by GraphPad Prism version 8.0.2 (GraphPad Software, Boston, USA). The IC_{50} value was further used for the LD_{50} calculation (Walum, 1998) to release hazardous levels, according to the World Health Organization (WHO, 2009).

Genotoxicity evaluation by comet assay

Comet assay was performed according to the method described by Singh et al. (1988) with a few modifications. The concentration at IC_{50} or a maximum working concentration, in case no IC_{50} value was detected, were applied as a treatment. The 100 µL of treated cells was combined with 100 µL of 0.5% low melting point agar (LMA) and dropped onto the 1% normal melting point agarose pre-coated slides. Then, the slides were kept at 4°C. After 10 min, all slides were soaked with lysis buffer solution (pH 10) at least 1 h and transferred to electrophoresis buffer solution for 40 min. The electrophoresis was performed for 25 min at 26 V, 300 mA at 4°C. After electrophoresis, the slides were immediately neutralized with tris buffer solution (pH 7.5) for 5 min and repeated for three times. The slides were stained with 1 µg/mL ethidium bromide (50 µL per slide). To verify the result, all slides were visualized under a fluorescent microscope (Nikon, Minato-ku, Japan) at 200 magnifications equipped with a 560 nm excitation filter, 590 nm barrier filter, and a CCD video camera PCO (Kelheim, Germany). At least 150 cells per experiment were captured using ImageJ. The CASP software version 1.2.3 (CASPlab, Wroclaw, Poland) was used to analyze the olive tail moment (OTM), which is the relative amount of DNA in the tail of the comet multiplied by the median migration distance. The untreated cells were employed as a negative control, and the positive control was treated with UVC light.

Cell treatment, RNA extraction and cDNA synthesis

To evaluate gene expression involving collagen, elastin, melanin, and anti-inflammatory genes, the DHFa and CHL-1 cells (2x10^5 cell/well) were seeded into 6-well plates. The substances for the experiments were the three *Morinda* leaf extracts at the highest working concentration, the α-EG and pinoresinol standards at 1 mg/mL in 10% DMSO employed to authenticate the potential of inducing targeted gene expression, and the commercial products hyaluronic and whitening serums (from a beauty store in Khon Kaen province, Thailand), used to compare with the extracts to study gene expression efficiency. The two kind of cells were treated with all mentioned substances for 24 hr. The untreated cell was used as a negative control. All experiments were performed in triplicate. After cell harvesting, the RNA extraction was performed according to manufacturer's protocol using PureLink™ RNA mini kit. The concentration of total RNA was quantified with nanodrop spectrophotometer (DeNovix, Wilmington, Delaware, USA). Subsequently, cDNA synthesis was performed using SuperScript®VILO™ MasterMix according to manufacturer's protocol. The cDNA was kept at -20°C until use.

Gene expression analysis by quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR assay was performed using Roche equipped with LightCycler®480 software (Mannheim, Germany). Amplifications were carried out with the final reaction solution as 20 µL containing 10 µL, 2x SensiFAST SYBR® No-ROX Mix, 6 µL of first-stranded cDNA (diluted 1:10), 0.8 µL of each specific primer (10 µM) and 2.4 µL nuclease-free water. PCR conditions slightly modified from Freitas et al. (2019), were programed following pre-denaturation at 95°C for 3 min followed by 55 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s, for denaturation, annealing and extension, respectively. The melting curve analysis were set to verify specificity of each pair of primers, following 95°C for 30 s, 60°C for 2 min and a continuous raise in temperature to 95°C at 0.11°C/s ramp rate. The cycle of threshold (C*t*) in each treatment was applied to calculate ΔC*t* for relative gene expression calculation. The expression of target genes was normalized by the GAPDH expression value. The sequences of the primers are listed in Table 1S.

All values were expressed as the mean ± S.D. The relative gene expression value was derived using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Yuan et al., 2006), the percentage of relative gene expression was calculated from determined equation as;

$$Relative\ gene\ expression\ (\%) = \frac{\text{Average of relative gene expression (treatment)}}{\text{Average of relative gene expression (control)}} x100$$

Statistical analysis

All experiments were performed in triplicates. The values were expressed as the mean ± standard deviations (S.D.) using Microsoft Office Excel 2019. Additionally, the unpaired *t*-test was performed for comparison between groups using GraphPad Prism version 8.0.2, and $p<0.05$ was considered as the statistically significant value.

Results

Phytochemical Profile Screening and Analysis

The hexane and ethanol *M. coreia*, *M. elliptica* and *M. tomentosa* leaf extracts were analyzed to elucidate their phytochemical constituents (Table 1). The GC-MS chromatograms are shown in Fig. 1. There are 48 phytochemical substances found at different percentages via different retention times (R*t*). The major compound is ethyl alpha-d-glucopyranoside (α-EG) detected in the ethanolic extracts of *M. coreia*, *M. elliptica*, and *M. tomentosa* as 52.33%, 43.65% and 42.72%, respectively. Phytol is the next highest content compound, found in the hexane extracts of the three studied species at 33.26%, 24.49% and 14.46%, respectively (Table 1 and Figure 1).

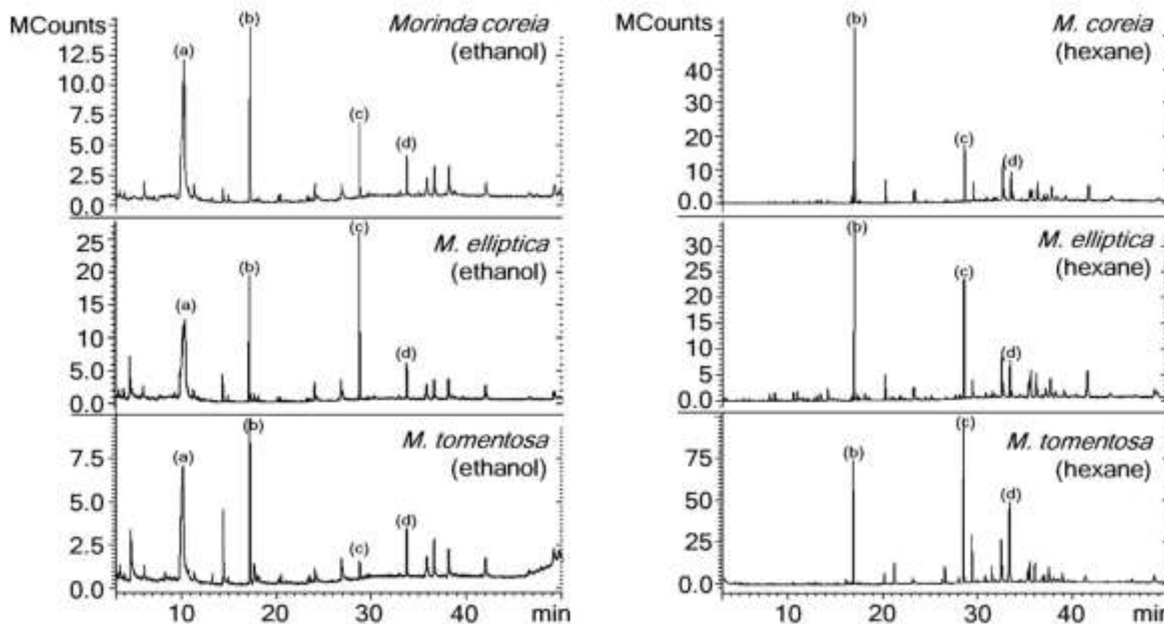


Figure-1. The GC-MS chromatograms of the ethanol and hexane leaf extracts of the three *Morinda* species containing α-EG (a), phytol (b), squalene (c) and α-tocopherol (d)

Table-1. The phytochemical profiling from GC-MS analysis of the three *Morinda* leaf extracts with ethanol and hexane solvents.

R*t* (min)	Phytochemical Names	Molecular Formula	Relative content (%)					
			Morinda coreia **(ethanol)**	***M. coreia*** **(hexane)**	***M. elliptica*** **(ethanol)**	***M. elliptica*** **(hexane)**	***M. tomentosa*** **(ethanol)**	***M. tomentosa*** **(hexane)**
4.06	2,3-Dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one	$C_6H_8O_4$	0.5	-	0.79	-	-	-
4.51	Methyl aspirin	$C_{10}H_{10}O_4$	-	-	-	-	-	0.3
4.70	1,2-Benzenediol	$C_6H_6O_2$	-	-	4.25	-	6.21	-
5.09	1,2-di-tert-Butylbenzene	$C_{14}H_{22}$	-	-	-	-	-	0.24
6.11	2-Methoxy-5-Vinylphenol	$C_9H_{10}O_2$	1.56	-	0.58	-	0.99	-
10.33	Ethyl alpha-d-glucopyranoside	$C_8H_{16}O_6$	52.33	-	43.65	-	42.72	-
11.37	Myristic acid	$C_{14}H_{28}O_2$	-	-	-	-	0.78	-
12.11	Phytol acetate	$C_{22}H_{42}O_2$	-	-	-	-	-	0.18
12.21	2-Pentadecanone,6,10,14,-trimetyl-	$C_{15}H_{30}O$	-	-	-	-	-	0.2
13.44	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	-	-	-	-	-	0.19
14.37	Palmitic acid	$C_{16}H_{32}O_2$	1.33	-	2.23	1.82	5.65	-
14.96	Ethyl palmitate	$C_{18}H_{36}O_2$	0.43	-	0.41	-	0.24	-
16.16	13-Hexyloxacyclotridec-10-en-2-one	$C_{18}H_{32}O_2$	-	-	-	-	-	0.72
16.81	Linolenic acid, methyl ester	$C_{19}H_{32}O_2$	-	1.31	-	0.71	-	-
17.02	Phytol	$C_{20}H_{40}O$	13.19	33.26	10.33	24.49	13.28	14.46
17.47	2-Cyclohexylpiperidine	$C_{11}H_{21}N$	-	-	-	-	-	0.36
17.70	Oleic Acid	$C_{18}H_{34}O_2$	-	-	0.62	-	1.55	-
18.22	Linolenic acid, ethyl ester	$C_{20}H_{34}O_2$	0.39	-	0.41	-	-	-
20.24	Chloropyrilene	$C_{14}H_{18}C_lN_3S$	0.43	-	0.23	-	-	-
20.28	1,3-Dipalmitoylglycerol	$C_{35}H_{68}O_5$	0.52	3.66	0.32	3.19	0.56	1.25
23.21	9,12-Octadecadienal	$C_{18}H_{32}O$	-	1.88	-	1.49	-	0.31
23.32	9-Octadecenoic acid	$C_{18}H_{34}O_2$	-	1.73	-	1.37	-	-
23.35	9,12,15-Octadecatrienal	$C_{18}H_{30}O$	-	1.98	-	1.46	-	0.42
24.07	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	1.43	-	1.58	-	1.18	-
26.62	Heptacosane	$C_{27}H_{56}$	-	0.46	-	-	-	1.98
26.86	Glyceryl monooleate	$C_{21}H_{40}O_4$	1.92	-	3.75	-	2.23	-
28.01	Octacosane	$C_{28}H_{58}$	-	-	-	-	-	0.6
28.58	Squalene	$C_{30}H_{50}$	4.97	8.88	13.37	15.28	1.18	19.74
29.50	Nonacosane	$C_{29}H_{60}$	-	3.35	-	2.27	-	6.21
30.79	Triacotane	$C_{31}H_{64}$	-	-	-	-	-	0.97
32.57	Hentriacontane	$C_{31}H_{64}$	-	8.81	-	7.09	-	7.37
32.59	1-Hexacosanol	$C_{26}H_{54}O$	-	-	-	-	-	1.46
32.74	1-Pentacontanol	$C_{50}H_{102}O$	-	2.44	-	-	-	-
33.44	dl-alpha-Tocopherol	$C_{29}H_{50}O_2$	4.3	6.61	3.74	7.28	5.09	15.48
33.57	alpha-Tocopherolquinone	$C_{29}H_{50}O_3$	-	1.91	-	1.18	-	0.96
35.61	Hexadecanal	$C_{16}H_{32}O$	-	2.81	-	6.61	-	7.67
35.82	Campesterol	$C_{28}H_{48}O$	2.9	4.02	2.49	4.94	2.73	2.41
36.60	Stigmasterol	$C_{29}H_{48}O$	4.21	5.97	2.93	6.27	5.25	3.83
36.72	Dotriacontane	$C_{32}H_{66}$	-	-	-	-	-	1.07
36.99	1-Dotriacontanol	$C_{32}H_{66}O$	-	-	-	-	-	1.48
37.97	Columbin	$C_{20}H_{22}O_6$	-	-	-	-	-	1.43
38.12	gamma-Sitosterol	$C_{29}H_{50}O$	4.22	4.67	3.52	5.39	3.94	3.17
38.92	Cedryl acetate	$C_{17}H_{28}O_2$	-	-	-	-	-	1.82
41.61	Octadecanal	$C_{18}H_{36}O$	-	6.24	-	9.21	-	-
42.03	Vitamin E	$C_{29}H_{50}O_2$	1.8	-	2.36	-	3.25	-
46.61	Methyl arjunolate	$C_{31}H_{50}O_5$	0.86	-	0.59	-	-	-
48.59	Urs-12-en-28-al	$C_{30}H_{48}O$	-	-	-	-	-	3.74
49.20	Methyl oleanonate	$C_{31}H_{48}O_3$	2.72	-	1.85	-	3.18	-

α-EG and pinoresinol standards were prepared at five concentration levels, ranging between 0.062-1 mg/mL.

The α-EG standard was detected at R*t* 6.853 min. Each concentration was applied for peak area calculation shown at 18.41, 26.37, 44.13, 63.51 and 157.22, respectively. The calibration equation and correlation coefficient (R^2) were derived from the plotted graphs using concentrations and peak areas of α-EG standard, y=145.28x+5.6469 and 0.9774 for R^2 value.

Pinoresinol standard was detected at R*t* 16.834 min, and its peak areas were 11.19, 36.60, 67.62, 169.64 and 404.92, respectively. The calibration equation of the pinoresinol standard was y=420.62x-24.952, with an 0.9935 R^2 value.

The three studied species hexane and ethanol leaf extracts were investigated for α-EG and pinoresinol. The α-EG substance was identified at R*t* as 6.881 min, 0.043 mg/g in ethanol *M. tomentosa*, was not found in the hexane extracts, and was found in both hexane and ethanol extracts of the other two studied species. The pinoresinol compound was detected at R*t* 16.992, 17.014 and 16.978 min in the ethanol solvent only for *M. coreia*, *M. elliptica* and *M. tomentosa* at 0.018, 0.015 and 0.013 mg/g sample, respectively, but was not found in any of the hexane extracts (Table 2 and Figure 2).

Table-2. The peak area, concentration and amount of α-EG and pinoresinol from the three *Morinda* species leaf extracts with hexane and ethanol solvents; α-EG was found in the ethanol *M. tomentosa* extract only, pinoresinol was found in all the three studied species extracted with ethanol solvent.

Plant samples	α-EG			Pinoresinol		
	Peak area	Concentration (mg/mL)	Amount (mg/g sample)	Peak area	Concentration (mg/mL)	Amount (mg/g sample)
Morinda coreia (ethanol)	-	-	-	52.08	0.18	0.018
M. coreia (hexane)	-	-	-	-	-	-
M. elliptica (ethanol)	-	-	-	36	0.15	0.015
M. elliptica (hexane)	-	-	-	-	-	-
M. tomentosa (ethanol)	68.44	0.43	0.043	27.84	0.13	0.013
M. tomentosa (hexane)	-	-	-	-	-	-

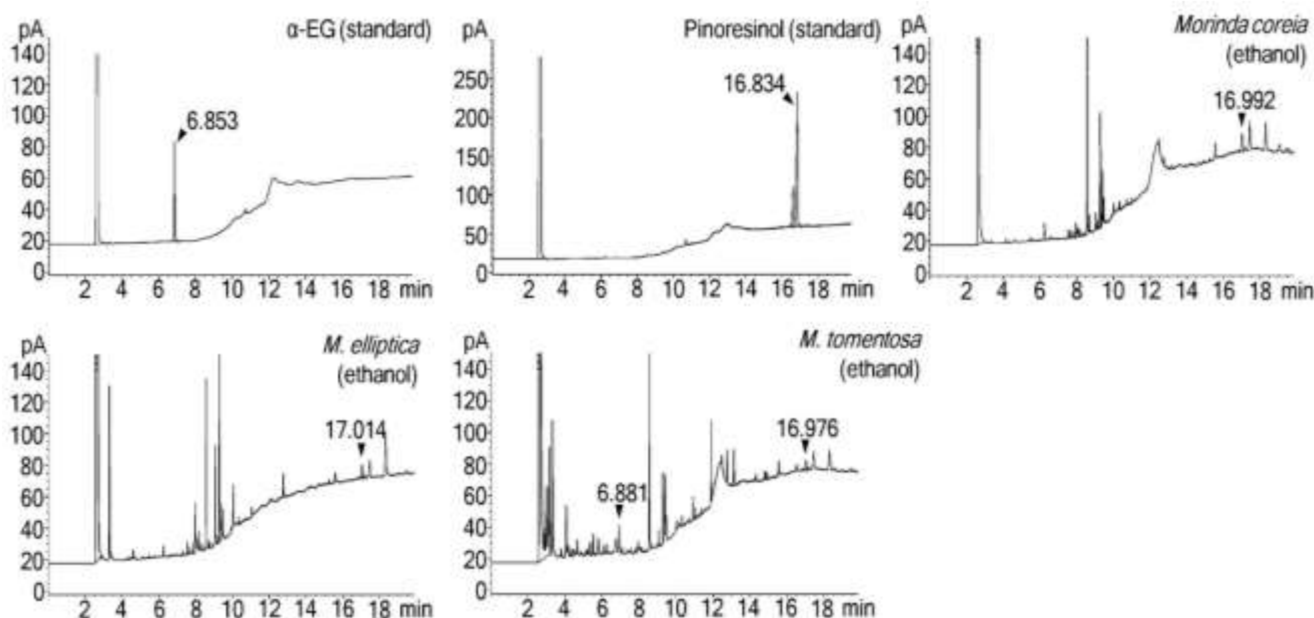


Figure-2. The GC-FID chromatograms of α-EG and pinoresinol detection in the three ethanol *Morinda* leaf extracts; α-EG was found in *M. tomentosa*; pinoresinol was found in all the three studied species extracted with ethanol solvent

Toxicity evaluation of the three *Morinda* leaf extracts through MTT and Comet Assay

The ethanol and hexane extracts of the three *Morinda* leaves were prepared for eight concentration levels from the first concentration extract in 100% DMSO (Table 3). The two normal cells, PBMCs and DHFa, and a cancer cell, CHL-1, were employed for cell and DNA toxicity. For cytotoxicity testing, the results revealed that all extracts of the studied samples have no toxic effects on PBMCs, DHFa, and CHL-1 cells indicated by lacking any IC_{50} values and having the highest cell viability percentage, except for the ethanol and hexane *M. coreia* and *M. tomentosa* extracts which expressed toxic activity on CHL-1 cells with IC_{50} values at 3.63, 5.98, 2.61 and 4.09 mg/mL, respectively. The LD_{50} values was calculated using IC_{50} values and categorized as class II toxicity (moderately hazardous class) (Table 3 and Figure 3A). The concentration at IC_{50} values at 3.63, 5.98, 2.61 and 4.09 mg/mL of ethanol and hexane *M. coreia* and *M. tomentosa* extracts were used for the comet assay, but in the cases with no IC_{50} values, the highest working concentrations were used instead, at 18.56 and 17.60 mg/mL of ethanol and hexane *M. elliptica* extracts. The results showed that the highest working concentration of the extracts did not significantly ($p>0.05$) induce DNA damage on PBMCs and DHFa as indicated by DNA condensing in cells. However, the concentration at IC_{50} values of ethanol and hexane *M. coreia* and *M. tomentosa* extracts significantly ($p<0.0001$) induced DNA damage on CHL-1 cells detected by DNA fragments throughout the cell membrane. All treatments were compared to each negative control as indicated by the OTM values (Table 4 and Figure 3B).

Table-3. The percentages of cell viability, IC_{50}, and LD_{50} values of PBMCs, DHFa and CHL-1 cells from the leaf extracts of the three *Morinda* species treatment; the result revealed that all the studied extracts have no toxicity on PBMCs, DHFa, and CHL-1, except for the ethanol and hexane *M. coreia* and *M. tomentosa* leaf extracts which had IC_{50} values on CHL-1 cancer cells.

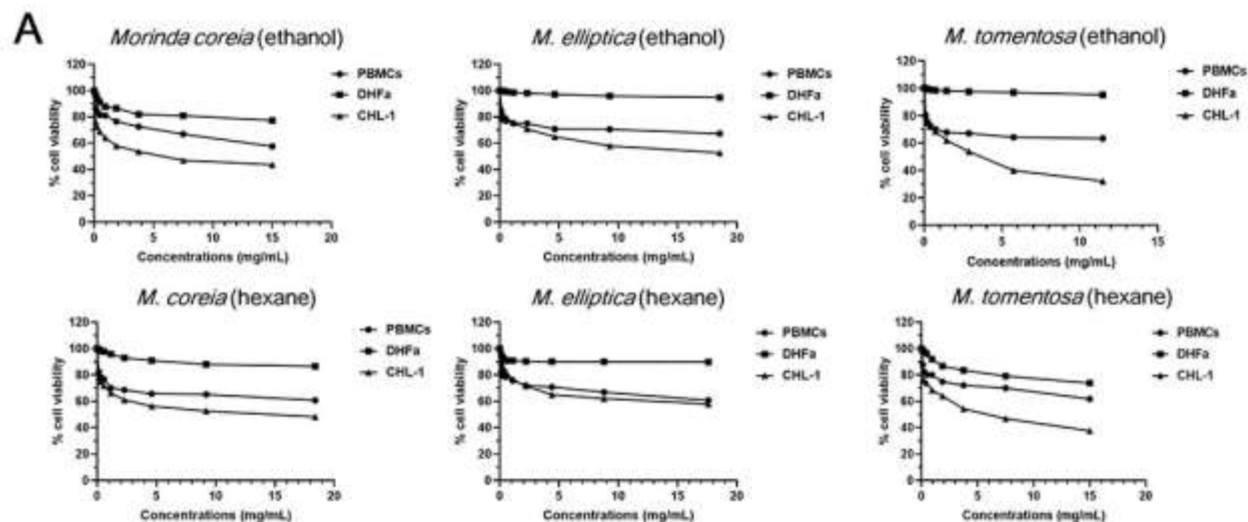
Plant samples (highest concentration)	PBMCs			DHFa			CHL-1		
	%cell viability	IC_{50} (mg/mL)	LD_{50} (mg/Kg rat)	%cell viability	IC_{50} (mg/mL)	LD_{50} (mg/Kg rat)	%cell viability	IC_{50} (mg/mL)	LD_{50} (mg/Kg rat)
Negative control	100±0.00	-	-	100±0.00	-	-	100±0.00	-	-
10% DMSO	97.79±2.84	-	-	98.36±1.03	-	-	98.08±1.74	-	-
Morinda coreia (ethanol) (15.02 mg/mL)	57.62±0.06	-	-	77.31±0.06	-	-	43.72±0.05	3.63	2,229.90
M. coreia (hexane) (18.40 mg/mL)	60.78±0.02	-	-	86.57±0.01	-	-	48.26±0.05	5.98	2,684.93
M. elliptica (ethanol) (18.56 mg/mL)	67.34±0.17	-	-	94.78±0.04	-	-	52.71±0.06	-	-
M. elliptica (hexane) (17.60 mg/mL)	60.77±0.04	-	-	89.83±0.05	-	-	57.96±0.08	-	-
M. tomentosa (ethanol) (11.47 mg/mL)	63.47±0.05	-	-	95.22±0.02	-	-	32.28±0.20	2.61	1,972.38
M. tomentosa hexane (15.00 mg/mL)	61.80±0.08	-	-	73.88±0.02	-	-	37.66±0.05	4.09	2,331.10

Table-4. Olive Tail Moment (OTM) and *p* values of each sample to indicate DNA damage level on PBMCs, DHFa, and CHL-1 cells; the highest working concentration of the extracts did not significantly ($p>0.05$) induce DNA damage on PBMCs and DHFa compared to negative control, the concentration at IC_{50} values of ethanol and hexane *M. coreia* and *M. tomentosa* leaf extracts significantly ($p<0.0001$) induced DNA damage compared to negative control on CHL-1 cells.

Plant samples (concentration)	PBMCs		DHFa		CHL-1	
	OTM	*p*-value	OTM	*p*-value	OTM	*p*-value
Negative control	0.51±0.25[a] 0.01±0.05[b]	-	0.12±0.22	-	0.08±0.11	-
10% DMSO	0.49±0.5[a]	0.7094	0.11±0.20	0.7480	0.09±0.17	0.7607
Morinda coreia (ethanol) (15.02 and 3.63* mg/mL)	0.46±0.42[a]	0.2468	0.10±0.20	0.4108	0.008±0.03	<0.0001
M. coreia (hexane)	0.47±0.42[a]	0.4489	0.11±0.23	0.6452	0.01±0.02	<0.0001

(18.40 and 5.98* mg/mL)						
M. elliptica (ethanol) (18.56 mg/mL)	0.51±0.47[a]	0.8851	0.15±0.22	0.2163	0.07±0.10	0.3276
M. elliptica (hexane) (17.60 mg/mL)	0.52±0.39[a]	0.8498	0.11±0.18	0.7434	0.07±0.14	0.4904
M. tomentosa (ethanol) (11.47 and 2.61* mg/mL)	0.01±0.03[b]	0.7067	0.11±0.20	0.7534	0.001±0.01	<0.0001
M. tomentosa (hexane) (15.00 and 4.09* mg/mL)	0.01±0.05[b]	0.9784	0.10±0.18	0.4343	0.008±0.02	<0.0001

* = the concentration at IC_{50} was used for CHL-1 treatment merely, a = the data was compared with 0.51 value as a negative control, b = the data was compared with 0.01 value as a negative control



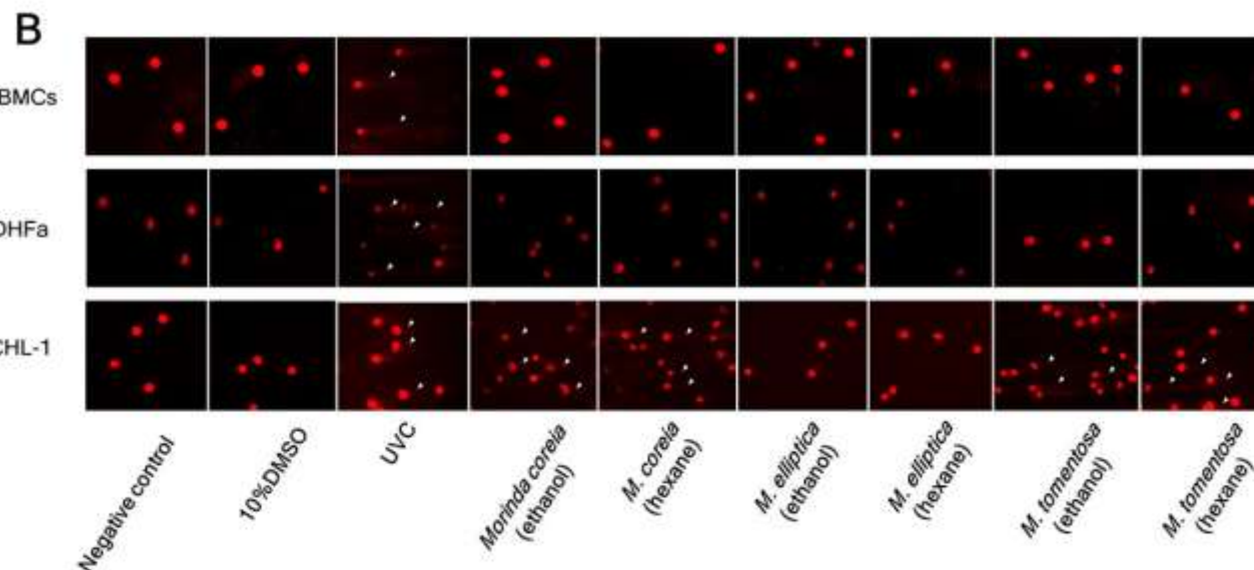


Figure-3. The toxicity evaluation of the three ethanol and hexane *Morinda* leaf extracts on PBMCs, DHFa and CHL-1 cells; (A) Cytotoxicity testing with the MTT assay showing all the studied extracts have no toxicity on PBMCs, DHFa, and CHL-1, except for the extracts that expressed IC_{50} values on CHL-1 cancer cells; (B) Genotoxicity evaluation through comet assay images (200x), compared to negative control and UVC as a positive control, the DNA damage fragments were indicated by white arrow.

Gene expressions and quantifications on collagen type I, II, III, elastin, and related genes in DHFa cells treated with the three ethanol *Morinda* leaf extracts

The ethanol extracts of the three studied *Morinda* species were used to study gene expression for collagen and elastin stimulation, as well as related genes involved in connective tissue formation and degradation in DHFa cells compared to the α-EG and pinoresinol standards and hyaluronic serum. The relative gene expressions were calculated with cycle of threshold (C*t*) values of the *GAPDH* reference gene. The results showed that the standard solution of α-EG, pinoresinol, and hyaluronic serum can significantly ($p<0.0001$) activate overexpression on collagen type I (*COL1A*, *COL1A2*), II (*COL2A1*, *COL-II*, *COLL2*), III (*COL3A*) and elastin (*ELN*) genes with high relative gene expression levels, aside from the *COL3A* gene which when treated with hyaluronic serum showed lower relative gene expression levels than the standards (Table 5 and Figure 4).

For the experiments, the two extracts of ethanol *M. coreia* and *M. elliptica* that contained pinoresinol in 0.018 and 0.015 mg/g sample can significantly ($p<0.0001$) stimulate the DHFa cells to overexpress high levels of *COL1A*, *COL1A2* (collagen type I); *COL2A1*, *COL-II*, *COLL2* (collagen type II), and *ELN* (elastin) genes (Fig. 4) with the same efficacy as hyaluronic serum, but the percentages of *COL3A* (collagen type III) gene expression levels were lower as 605% and 411%, than the collagen type I and II (Table 5). Remarkably, for the ethanol *M. tomentosa* extract that contained both α-EG and pinoresinol in amount of 0.043 and 0.013 mg/g sample, it stimulated the DHFa cells to significantly ($p<0.0001$) induce higher gene expression level on all collagen type I, II, III, and elastin formation, with higher efficacy than commercial hyaluronic serum (Table 5 and Figure 4).

Interestingly, the ethanol *M. tomentosa* extract can significantly ($p<0.05$) suppress the *ELANE*, *MMP1,* and *MMP13* genes, which is the gene involving elastase and matrix metalloproteinases produced to degrade elastin and collagen. Their percentages of relative gene expression were 28%, 35% and 31%, respectively and lower than other samples, also, ethanol *M. coreia* and *M. elliptica* extracts can stimulate the suppression, but was not significant, while as ethanol *M. elliptica* extract can significantly ($p<0.05$) suppress the *ELANE* gene (Table 5).

Table-5. The relative gene expression on collagen type I (*COL1A*, *COL1A2*), II (*COL2A1*, *COL-II*, *COLL2*), III (*COL3A*) and elastin (*ELN*), and related genes as *ELANE, FGF1*, *FGF7*, *MMP1*, *MMP13*, *NRF2*, *TGF-β* and *TIMP1* in DHFa cells showing the efficacy of expression in the role of stimulation of collagen type I, II, and III by all studied plant extracts, and the highest suppression by ethanol *M. tomentosa* extract in *ELANE*, *MMP1*, and *MMP13* for delaying elastin and collagen degradation. Also listed are supporting genes such as *FGF1* and *FGF7*, *TGF-β* and *NRF2*.

Genes	Negative control		10% DMSO		α-EG		Pinoresinol		Hyaluron serum®		*Morinda coreia* ethanol		*M. elliptica* ethanol		*M. tomentosa* ethanol	
	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	%Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression
COL1A1	1.00 ±0.17	100	0.99 ±0.02	99	16.51 ±0.04	1,636	18.32 ±0.04	1,815	10.72 ±0.36	1,063	12.16 ±0.04	1,205	13.22 ±0.28	1,310	18.10 ±0.01	1,794
COL1A2	1.00 ±0.16	100	1.00 ±0.34	99	17.45 ±0.71	1,731	18.63 ±1.12	1,847	10.45 ±1.75	1,037	10.13 ±0.60	1,004	13.70 ±1.41	1,359	17.87 ±2.98	1,772
COL2A1	1.01 ±0.17	100	0.99 ±0.36	98	15.00 ±0.96	1,485	19.64 ±1.85	1,944	11.69 ±0.28	1,157	11.10 ±0.29	1,098	12.03 ±0.48	1,191	14.91 ±1.45	1,475
COL-II	1.00 ±0.12	100	1.03 ±0.39	102	14.84 ±0.71	1,477	17.91 ±0.71	1,783	12.36 ±0.37	1,230	11.50 ±0.22	1,145	10.84 ±0.85	1,079	13.99 ±1.28	1,394
COLL2	1.03 ±0.31	100	1.00 ±0.38	97	14.23 ±0.96	1,378	13.73 ±1.22	1,330	11.40 ±0.37	1,104	11.46 ±0.73	1,110	10.13 ±0.77	982	12.30 ±0.83	1,191
COL3A1	1.01 ±0.17	100	0.99 ±0.32	98	11.07 ±0.60	1,095	11.36 ±1.04	1,124	7.13 ±0.47	705	6.11 ±0.65	605	4.15 ±0.01	411	10.14 ±0.73	1,003
ELN	1.01 ±0.18	100	1.03 ±0.26	102	12.33 ±0.05	1,219	13.77 ±0.22	1,361	16.80 ±0.22	1,661	11.90 ±0.21	1,177	13.64 ±1.86	1,348	14.33 ±1.26	1,416

ELANE	1.01 ±0.18	100	0.97 ±0.37	96	0.69 ±0.03	69	0.46 ±0.02	45	1.91 ±0.01	188	1.07 ±0.04	106	0.71 ±0.02	70	0.28 ±0.02	28
FGF1	1.00 ±0.16	100	0.98 ±0.38	97	2.44 ±0.07	241	2.73 ±0.14	271	0.71 ±0.05	71	1.52 ±0.06	151	1.77 ±0.07	175	1.61 ±0.13	160
FGF7	1.01 ±0.17	100	0.99 ±0.39	99	2.75 ±0.07	273	2.09 ±0.07	207	1.28 ±0.01	127	0.72 ±0.01	71	1.14 ±0.04	113	1.50 ±0.13	149
MMP1	1.00 ±0.16	100	0.99 ±0.32	98	0.76 ±0.05	75	0.63 ±0.06	62	0.77 ±0.03	77	0.77 ±0.02	76	0.75 ±0.06	74	0.36 ±0.14	36
MMP13	1.01 ±0.17	100	0.97 ±0.32	96	0.63 ±0.03	63	0.83 ±0.04	82	0.77 ±0.03	77	0.54 ±0.04	53	0.73 ±0.03	73	0.31 ±0.02	30
NRF2	1.01 ±0.18	100	0.99 ±0.35	98	0.97 ±0.05	96	1.02 ±0.02	101	1.03 ±0.03	102	1.01 ±0.02	99	0.99 ±0.02	97	0.99 ±0.05	98
TGF-β	1.01 ±0.17	100	0.99 ±0.31	98	6.45 ±0.25	638	5.47 ±0.07	541	5.89 ±0.03	582	5.15 ±0.38	509	6.98 ±0.06	690	7.29 ±0.44	721
TIMP1	1.01 ±0.16	100	1.06 ±0.36	105	2.39 ±0.08	237	2.37 ±0.03	235	1.30 ±0.02	129	2.42 ±0.06	240	2.14 ±0.06	212	2.74 ±0.10	271

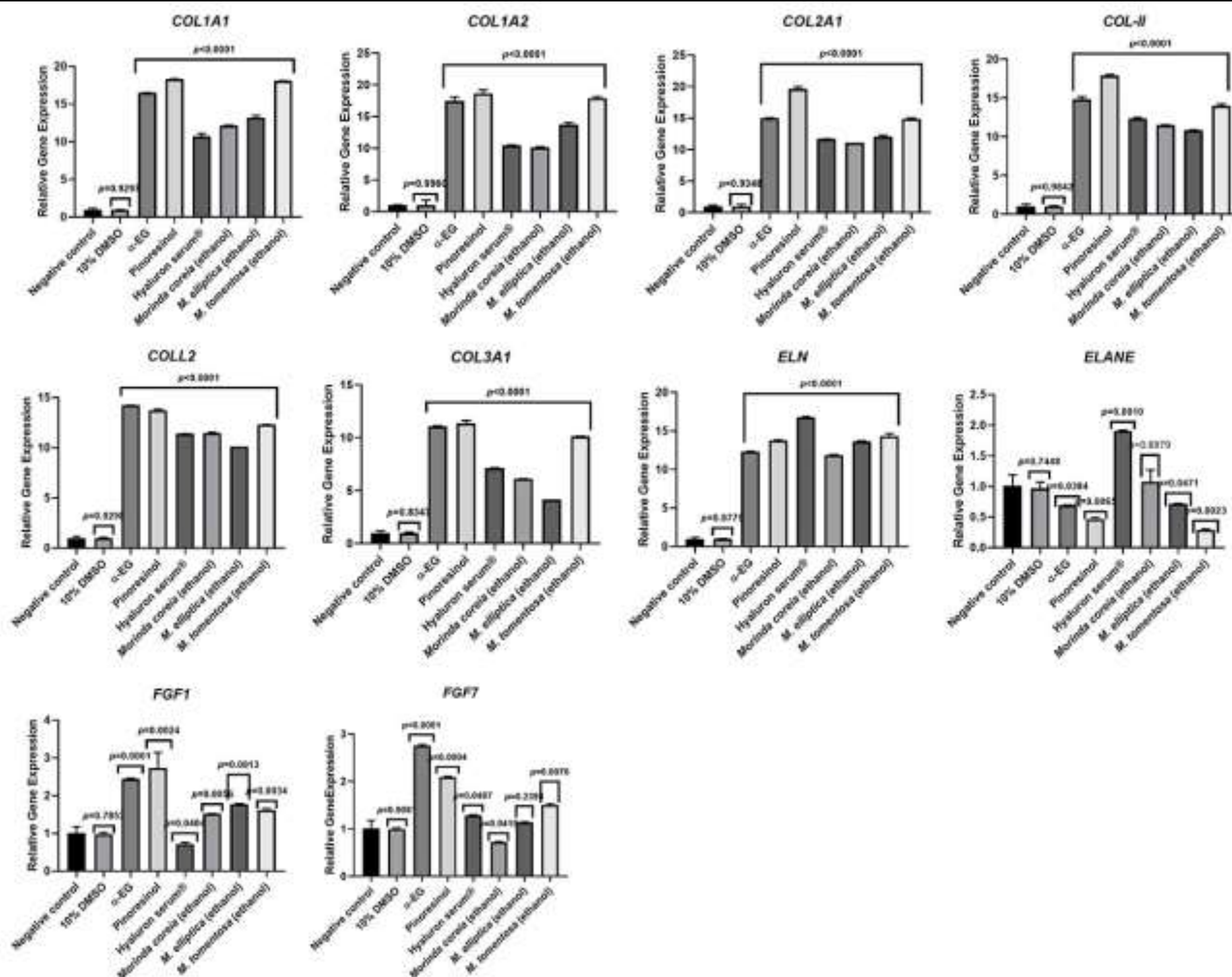


Figure-4. The comparison of relative gene expression levels in stimulation and suppression roles between α-EG and pinoresinol standards, commercial hyaluron serum, and the three *Morinda* leaf ethanol extracts The *COL1A*, *COL1A2*, *COL2A1*, *COL-II*, *COLL2*, and *COL3A* genes are responsible for collagen type I, II and III production; the *ELN* gene with an elastin formation function, and the *ELANE* gene which functions to produce elastase to break down elastin; and supporting genes *FGF1* and *FGF7* which function to produce fibroblast growth factor. All samples were compared to negative control. The column charts were located under the same bar indicating equal *p*-value.

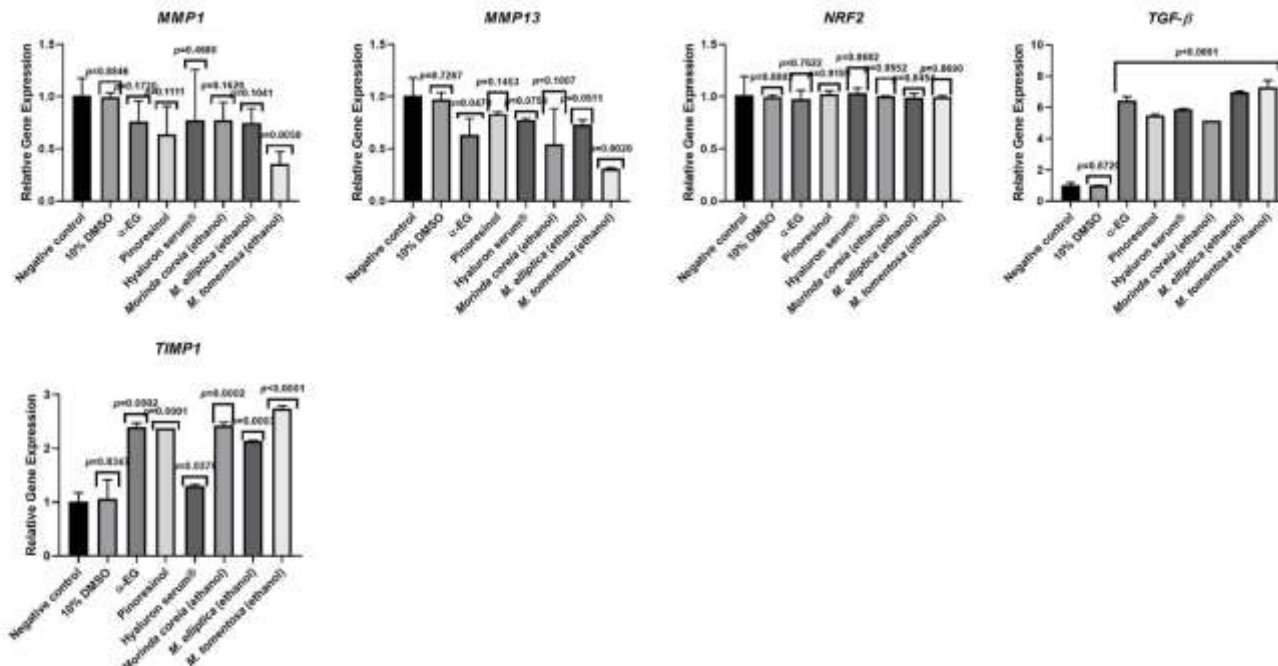


Figure-5. The comparison of relative gene expression levels with stimulation and suppression roles between α-EG and pinoresinol standards, commercial hyaluron serum, and the three *Morinda* leaf ethanol extracts; *MMP1* and *MMP13* genes are function of metalloproteinases production for collagen degradation; *NRF2* gene functions of toxicity and oxidative stress defense in cells; *TGF-β* gene functions as an anti-inflammatory response; *TIMP-1* function of metalloproteinases production inhibition to delay collagen degradation; all samples were compared to negative control. The column charts were located under the same bar indicating equal *p*-value.

Consequently, the ethanol *M. coreia*, *M. elliptica* and *M. tomentosa* extracts can significantly ($p<0.05$) induce *TIMP1* gene expression, which is a gene involved in metalloproteinases inhibition production for the delay of collagen degradation, and shows higher efficacy than the commercial product. The *FGF1* and *FGF7* genes as fibroblast growth factor production to support cell proliferation, can be stimulated by all treatments, excepted for commercial hyaluron serum and ethanol *M. coreia*. Meanwhile, all treatments can significantly ($p<0.05$) stimulate *TGF-β* gene expression, as an anti-inflammatory cytokine response. Lastly, the *NRF2* which is the gene related to cellular defense against toxic and oxidative stress response for detoxification, alternatively called a transcription factor for stress response, showed similar expression levels in all treatments compared to untreated cells as a negative control, meaning the extracts are non-toxic reagents for DHFa cells (Table 5 and Figure 4-5).

Gene expressions and quantifications of tyrosinase and melanin creation in CHL-1 cells treated with the three ethanol *Morinda* leaf extracts

The highest concentration of the *Morinda* leaf ethanol extracts were applied to evaluate the gene expression suppression on tyrosinase and melanin creation compared to the commercial whitening serum on CHL-1 cells, including *TYR* and *α-MSH* genes. The relative gene expressions were calculated with C*t* values of the *GAPDH* reference gene. The α-EG and pinoresinol standards and ethanol *M. tomentosa* extract significantly ($p<0.0001$) showed gene suppression by low percentage expression of the *TYR* gene at 14%, 16% and 16%, and the *α-MSH* gene at 5%, 3.75% and 7.43%, respectively, while the ethanol *M. coreia* and *M. elliptica* extracts showed less significant gene suppression than the ethanol *M. tomentosa* extract at 47% and 43%, and 38% and 49%, respectively, without the efficacy of *TYR* and *α-MSH* genes suppression of the commercial whitening product.

Moreover, the ethanol *M. coreia* and *M. tomentosa* extracts can significantly ($p<0.0001$) induce the *NRF2* gene expression, which regulates the cellular defense against toxic and oxidative stress, at high levels of 183% and 251% in the CHL-1 cell. So, the extracts acted as toxic reagents on the CHL-1 cancer

cell line. The pinoresinol standard, ethanol *M. elliptca* and *M. tomentosa* extracts can less significantly ($p<0.0001$) induce low expression of the *TGF-β* gene, an anti-inflammatory cytokine response, at 23%, 29% and 18%, respectively. This means the extracts did not support anti-inflammatory activity on CHL-1 cells. The mentioned results have been shown in Table 6 and Figure 6.

Table-6. The relative gene expression on tyrosinase and melanin (*α-MSH* gene is α-melanocyte-stimulating hormone gene, stimulating melanogenesis and *TYR* is an essential enzyme in melanin synthesis) in the suppression role; ethanol *M. tomentosa* extract has the higher efficacy than ethanol *M. coreia* and *M. elliptica* extracts; related genes, *NRF2* showed high value of toxic activity on CHL-1 cells, and *TGF-β* had low values of anti-inflammatory cytokine activity in CHL-1 cells.

Genes	Negative control		10% DMSO		α-EG		Pinoresinol		Whitening serum®		*Morinda coreia* ethanol		*M. elliptica* ethanol		*M. tomentosa* ethanol	
	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	%Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression	Relative gene expression	% Relative gene expression
α-MSH	1.00 ±0.01	100	0.99 ±0.07	97	0.049 ±0.001	5	0.037 ±0.001	3.75	0.84 ±0.01	84	0.38 ±0.01	38	0.49 ±0.01	49	0.074 ±0.002	7.43
TYR	1.00 ±0.02	100	0.92 ±0.06	92	0.14 ±0.01	14	0.16 ±0.01	16	0.72 ±0.001	72	0.47 ±0.03	47	0.43 ±0.005	43	0.16 ±0.01	16
NRF2	1.00 ±0.03	100	0.98 ±0.06	98	1.62 ±0.02	162	1.75 ±0.05	175	1.35 ±0.01	135	1.83 ±0.07	183	1.69 ±0.09	169	2.49 ±0.08	249
TGF-β	1.00 ±0.07	100	0.98 ±0.11	98	0.39 ±0.03	39	0.23 ±0.01	23	0.59 ±0.01	60	0.47 ±0.01	47	0.29 ±0.01	29	0.18 ±0.01	18

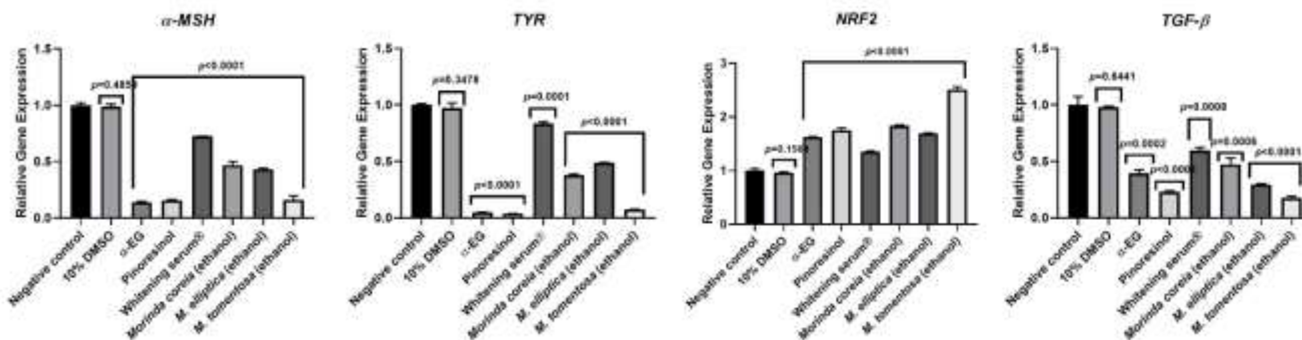


Figure-6. The comparison of relative gene expression levels between α-EG, pinoresinol, commercial hyaluron serum, and the three *Morinda* leaf ethanol extracts; the *α-MSH* gene's function in melanin production; the *TYR* gene's function in tyrosinase enzyme production, the *NRF2* gene's function in toxicity and oxidative stress defense of cells, the *TGF-β* gene's function of anti-inflammatory activity; ethanol *M. tomentosa* has a higher efficacy than ethanol *M. coreia* and *M. elliptica* extracts, and related genes, *NRF2* showed high toxic activity on CHL-1 cells, and *TGF-β* showed low anti-inflammatory cytokine activity in CHL-1 cells. All samples were compared to negative control. The column charts were located under the same bar indicating equal *p*-value.

Discussion

Phytochemical components in a plant group provides many clues for methods in plant systematics, showing the evolutionary lines. This convinced the authors to investigate a group of *Morinda* species in addition to the well-known *Morinda citrifolia* which has been used throughout the world for many benefits derived from its phytochemical contents and activities revealed by many publications, such as anti-inflammatory, anti-cancer, antioxidant properties, inhibition of elastase and tyrosinase (Masuda et al., 2009; Sanni et al., 2017; Sousa et al., 2017a) without any significant disturbances in liver or kidney outside of immune modulation (Sousa et al., 2017b; Soni, 2018), and that noni wine could effectively prevent high-fat diet-

induced oxidative stress and obesity (Zhang et al., 2020). Additionally, Matsuda et al., 2013 reported that noni seeds contain pinoresinol and americanin, which inhibit tyrosinase activity and α-melanocyte stimulated melanogenesis leading to a skin whitening effect, wrinkle inhibition, inhibition of poor blood fluidity and platelet aggregation, and fibrinolytic activity. Finally, Sudmoon et al., 2022 reported that *M. citrifolia* parts contain α-EG and pinoresinol, leading to collagen creation, tyrosinase and α-*MSH* inhibition with the effect of wrinkle inhibition and whitening stimulation. The foregoing, advantages of the species seem to correspond with abundant chemicals that produce its properties. So, in order to use the various phytochemicals in more varied plant species in the genus *Morinda*, the leaves of *M. coreia*, *M. elliptica* and *M. tomentosa* were collected and several aspects were investigated, including phytochemicals, toxicity, and biological activities by gene expression detection through the qRT-PCR method that will be discussed as follows.

For *M. citrifolia,* which contains α-EG and pinoresinol (Matsuda et al., 2013; Sudmoon et al, 2022), the important phytochemicals found in the three *Morinda* species which are in the same genus, were α-EG and pinoresinol extracted with an ethanol solvent, but were absolutely not found in the hexane extract. The dominant α-EG and pinoresinol substances were found in the ethanol *M. tomentosa* extract, while the other two species extracts showed only the pinoresinol component, by GC-MS and GC-FID methods (Tables 1-2 and Figure 1-2).

The α-EG substance acts as a collagen creation stimulant, an extracellular matrix protein which is the major component of the dermis. Therefore, changes in dermal collagen content greatly affect dermal homeostasis (Nishiyama et al., 1988). Normally, collagen is synthesized mainly by fibroblasts in the body which is the main structural protein of the skin, tendon, and bone. So, collagen has been presently mentioned as a good component to apply in cosmetic medicine, food, and pharmaceutical factors, but is broken down to amino acids when consumed (Drąg–Zalesińska et al., 2019; Wang, 2021). Pinoresinol has many functions, which has been found in ethanol *M. tomentosa* extract. Pinoresinol has several activities, such as a tyrosinase inhibitory activity in melanogenesis, inhibitory effect on *MMP*1 secretion (*MMP1* is a major collagenolytic enzyme responsible for collagen damage, *MMP*1 has expression promoted by UV irradiation), inhibitory effect on wrinkle formation by *HLE* creation which degrades collagen I and elastic fiber in human skin (Matsuda et al., 2013). The importance of the two compounds therefore requires further study in these three *Morinda* species.

MTT and comet assays used for toxicity tests in the studied extracts showed that all studied extracts were not toxic to normal cells, including PBMCs and DHFa, do not have IC_{50} values, and showed high cell viability percentages (Table 3 and Figure 3A), but the ethanol and hexane *M. coreia* and *M. tomentosa* extracts were toxic to CHL-1 cancer cells, according to in-depth toxicity in DNA exposed to the extracts IC_{50} values significantly ($p<0.05$) induced DNA damage in CHL-1 (Table 4 and Figure 3B). When researching up to this stage, it can be seen that the extracts or leaves of the three studied plants used in this research should be able to be used for human benefit. It showed non-toxicity in both cellular and DNA levels of normal human cells, such as PBMCs and DHFa, and had selective properties, that is cytotoxic to cancer cells, but not to normal cells. When these three studied species are used, biological activity has to be verified through gene expression detection by qRT-PCR. The results revealed that all studied ethanol *M. coreia*, *M. elliptica* and *M. tomentosa* extracts can stimulate collagen type I, II, III and elastin (*COL1A*, *COL1A2*; *COL2A1*, *COL-II*, *COLL2*; *COL3A* and *ELN*) gene expression in DHFa cells which is responsible for collagen type I, II, II creation (Yuan et al., 2016; Wang et al., 2022; Lubbers et al., 2020; Ezure et al., 2019), at the same efficacy as the α-EG and pinoresinol standards, better efficacy than hyaluronic serum, and *M. tomentosa* extract is the best extract according to the effects. Ethanol *M. tomentosa* extract has the additional activity of suppressing *ELANE* gene function of elastase production to break down elastin (Ezure et al., 2019), with low expression percentage of 28%, a better efficacy than the two standards. On the other hand, commercial hyarulonic serum cannot suppress *ELANE* gene. All studied extracts can stimulate *FGF1* and *FGF7* which are genes involved in fibroblast growth factor production (Sudmoon et al., 2022), and the best extract showing the highest activity is ethanol *M. tomentosa* extract (Table 5 and Figure 4). So, the DHFa cells indicated highly effective cell proliferation, and finally collagen and elastin creation. *MMP1* and *MMP13* genes produce metalloproteinases for collagen degradation (Ezure et al., 2019; Sun et al., 2019) and was suppressed by ethanol *M. tomentosa* extract, since they expressed as low percentages of 35% and 31%, better efficacy than the two standards

and hyaluronic serum, meaning that the ethanol *M. tomentosa* extract can effectively inhibit collagen degradation. All studied extracts stimulated *NRF2* expression the same as the control (untreated cells) and the standards, the gene related to detoxifying cells against toxic and oxidative stresses (Sudmoon et al., 2022), meaning that the extracts did not effect on DHFa cells (Table 5 and Figure 5). However, they did effect CHL-1 cells by showing higher expression than the control and the standards, meaning that the extracts were toxic to CHL-1 cells. The *TGF-β* gene functions as anti-inflammatory response (Hillege et al., 2020), and all studied extracts induced higher expression than the control, the same as the two standards, and the ethanol *M. tomentosa* extract showed the highest stimulation up to 721%, meaning that the extracts has higher anti-inflammatory effects on DHFa cells compared to without the extracts. On other hand, ethanol *M. tomentosa* extract has low anti-inflammatory effects on CHL-1 cells at 18%, lower than the control and standards. *TIMP1* inhibits metalloproteinases production to delay collagen degradation (Wang et al., 2020). The extracts can stimulate higher expression than the two standards and control, and ethanol *M. tomentosa* extract showed the most stimulation. The expression values and function of this gene is inverse to the expression of the *MMP1* and *MMP13* genes, when the *TIMP1* gene is expressed in a large number, the *MMP1* and *MMP13* genes must be expressed in a small amount, that means the extract can slow down the breakdown of collagen effectively.

For the suppression activities shown in Table 6 and Figure 6, the genes to study are *α-MSH* and *TRY*, an essential enzyme in melanin synthesis. The α-MSH gene is α-melanocyte-stimulating hormone gene, stimulating melanogenesis or melanin creation (Sudmoon et al., 2022) in CHL-1 cells. These factors cause dull skin and aging. The ethanol *M. tomentosa* extract has the highest suppression (expression at low percentages, 7.43% in α-*MSH* and 16% in *TYR*), similar to the standards' activity (expression at low percentages 5%, 3.75% in *α-MSH*; 14%, 16% in *TYR*), followed by the effect of the ethanol *M. coreia* and *M. elliptica* extracts on *α-MSH* and *TRY*, which means ethanol *M. tomentosa* extract and with slightly less effect, the ethanol *M. coreia* and *M. elliptica* extracts, can inhibit melanin production in the cells. Additionally, *M. tomentosa* extract can inhibit *TGF-β* expression showing a lower value at 18% vs. ethanol *M. coreia* (47%) and *M. elliptica* (29%) extracts, and the standards, implying that ethanol *M. tomentosa* extract cannot induce anti-inflammatory effect in CHL-1 cells.

Following from of all these research results, it can be assumed that the three studied species in the form tested (ethanol *M. coreia*, *M. elliptica* and *M. tomentosa* extracts) can be used for the benefit of humans, showing non-toxicity on both the cellular and DNA levels of normal human cells, which in this study are PBMCs and DHFa, and more activities with selective properties, including being cytotoxic to cancer cells. Implementation will be possible or it will not be. It must be considered with the studied plant bioactivity tested by investigating the expression of genes directly responsible for target functions and associated genes. The genes *COL1A*, *COL1A2*, *COL2A1*, *COL-II*, *COLL2*, *COL3A*, *ELN*, *ELANE*, *MMP1*, *MMP13*, *TIMP1* were tested on DHFa given that collagen synthesis takes place in fibroblast cells (Rodriguez et al., 2018; Wang, 2021). Fibroblasts produce substances that constitute the basic structure of skin and a decrease in their proliferation and activity causes the deterioration of the skin structure (Varani et al., 2006). *α-MSH* and *TYR* gene expression was tested in CHL-1 melanoma cancer cells as the process of melanin synthesis and distribution (melanogenesis) based on melanocytes present among the basal cells of the epidermis. Melanin formed in melanocyte are then stored in the basal layer of epidermal cells (Maranduca et al., 2019). *NRF2* and *TGF-β* gene expression was tested both in normal DHFa and CHL-1 cancer cells to show stimulation or suppression activity on normal or cancer cells.

This research has revealed the plant efficacy for applications in humans. The leaf of *M. tomentosa* which contain both α-EG and pinoresinol has empirical stimulation properties through this genes expression as it can stimulate collagen type I, II, III and elastin creation (*COL1A*, *COL1A2*, *COL2A1*, *COL-II*, *COLL2*, *COL3A* and *ELN*); inhibit enzyme synthesis for collagen degradation (*ELANE*, *MMP1*, *MMP13*, *TIMP1*); had no effect on DFHa cells, but affected CHL-1 cells (*NRF2*); can stimulate DHFa cell proliferation; stimulate collagen and elastin creation (*FGF1* and *FGF7*); has anti-inflammatory effects on DHFa cells; and has no anti-inflammatory effects on CHL-1 cells (*TGF-β*). Additionally, the leaf of *M. tomentosa* has suppression properties on melanin production through α-*MSH* and *TYR* gene expression.

It is well known that various structural conformations and types of collagens can be found in abundance in human skin with over 90% of the collagen in the

human body being collagen type I contained in almost all connective tissue (Wang, 2021), collagen type I and III predominate in skin and can be found in abundance in the dermal layer, and a high content of collagen and elastin are mostly present in the reticular portion of the dermal layer (Aziz et al., 2016). Though collagen cannot be taken orally as it is digested into amino acids, or penetrate through the skin from outside into body due to its large molecular weight, which contributes to it increasing the tensile strength of skin (Wang, 2021; Kim et al., 2022). Given that it has essential factors for the human body, in older people with higher collagen and elastin degradation than creation, stimulation to create more collagen should be better than letting the body create collagen without a stimulant. There are plenty of natural substances functioning as stimulants such as pinoresinol from noni seeds (Matsuda et al., 2013), α-EG found in *M. citrifolia* parts, madecassoside, madecassic and asiatic acids in *Centella asiatica*, ascorbic acid as a co-factor of proline hydroxylase and botulinic acid (Drąg–Zalesińska et al., 2019), and have property for melanin suppression. Here, the research has promoted one more species, *M. tomentosa* which can be further used for identical activity, but more efficacy than the above-mentioned species. These advantages are: the species can stimulate creation of elastin, was not toxic and has anti-inflammatory effects on normal cells.

Conclusion

The *M. tomentosa* species was found to be the best for use in various areas of human health and beauty production in various products such as face foam, liquid soap, supplements, medicine, etc. The research was accomplished with the aim that more plant species be utilized that contain effective phytochemicals, without toxicity on the cellular and DNA levels, and a high efficacy of bioactivity. Moreover, the leaves of *M. tomentosa* can be collected more easily and used instead of fruits with a bad smell when ripe as in *M. citrifolia*.

Acknowledgment

This research was financially supported by Research and Graduate Studies, Khon Kaen University, Thailand.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

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