Journal Pre-proof

Anti-skin aging activities of green tea (*Camellia sinensis* (L) Kuntze) in B16F10 melanoma cells and human skin fibroblasts

Puxvadee Chaikul (Conceptualization) (Methodology) (Formal analysis) (Investigation) (Project administration), Tawanun Sripisut (Methodology) (Investigation), Setinee Chanpirom (Methodology) (Investigation), Naphatsorn Ditthawutthikul (Methodology) (Investigation)

Please cite this article as: Chaikul P, Sripisut T, Chanpirom S, Ditthawutthikul N, Anti-skin aging activities of green tea (*Camellia sinensis* (L) Kuntze) in B16F10 melanoma cells and human skin fibroblasts, *European Journal of Integrative Medicine* (2020), doi: https://doi.org/10.1016/j.eujim.2020.101212

Research paper

Anti-skin aging activities of green tea (*Camellia sinensis* (L) Kuntze) in B16F10 melanoma cells and human skin fibroblasts

Puxvadee Chaikul[1,2*], Tawanun Sripisut[1,2], Setinee Chanpirom[1,2], Naphatsorn Ditthawutthikul[1,2]

[1]*School of Cosmetic Science, Mae Fah Luang University, Chiang Rai 57100, Thailand*

[2]*Phytocosmetics and Cosmeceuticals Research Group, Mae Fah Luang University, Chiang Rai 57100, Thailand*

*Corresponding Author: Phone: +66 5391 6832   Fax: +66 5391 6831

E-mail address: puxvadee@mfu.ac.th

ABSTRACT

Introduction: Skin aging is a multifactorial process caused by chronological changes and environmental factors. Green tea, a processed tea plant (*Camellia sinensis* (L) Kuntze), possesses health benefits and has been used in traditional medicines and natural products. However, the anti-skin aging effect of green tea in clinical trials has been a controversial issue. To clarify this without the interference of systemic involvement, the objectives of study were to investigate the activities of green tea against skin aging in B16F10 melanoma cells and human skin fibroblasts. *Methods*: Green tea leaves were extracted to standardise the phenolic profile. The cytotoxicity and the anti-skin aging activities, including melanogenesis assay, antioxidant activity, collagen content analysis, and matrix metalloproteinase-2 inhibitory assay, were evaluated in cell culture. *Results*: Green tea in this study composed mainly of epigallocatechin gallate ($1,903.77 \pm 33.59$ μg/mL), epigallocatechin (930.24 ± 27.04 μg/mL), and epicatechin gallate (607.84 ± 22.65 μg/mL). The cell viabilities of 0.5% (0.0875 mg/mL) green tea, the noncytotoxic concentration, were 96.04 ± 6.76 and $93.44 \pm 0.95\%$ in B16F10 melanoma cells and human skin fibroblasts, respectively. Green tea exhibited the activities against skin aging, including the significant suppression of melanin production via inhibition of tyrosinase and tyrosinase-related protein-2 activities, the potent antioxidant, and the significant matrix metalloproteinase-2 inhibition ($p<0.001$).
Conclusions: The study results have shown that green tea is a functional processed plant for utilisation as an anti-skin aging agent in the natural remedies, including food, health, and cosmetic products.

Key words: Anti-skin aging; antioxidant; green tea; matrix metalloproteinase-2; melanogenesis assay; phenolics

1. Introduction

Skin aging is a multifactorial biological process caused by combination of chronological changes (intrinsic factors) and environmental (extrinsic) factors, including ultraviolet (UV) and infrared irradiation, cigarette smoke, and air pollution [1]. The changes in aging skin include uneven pigmentation, decreased skin thickness, impaired skin elasticity and strength, wrinkles, and rough-textured characteristics [2,3]. Matrix metalloproteinases (MMPs), the zinc-containing enzymes, play the important role in degradation of extracellular matrix proteins, including collagen, gelatin, and elastin. The increased activity of MMPs is associated with age, free radicals, and UV irradiation [4,5]. Presently, the anti-aging research have extensively studied the molecular mechanisms as well as strategies for treatment of aging process [3,6]. Among a variety of strategies, the application of natural products, including nutrition and remedies, have shown to be the preferred choices for reversal of aging characteristics, due to the health considerations, environmental awareness, and safety of synthetic chemicals [7].

Green tea, the processed tea plant (*Camellia sinensis* (L) Kuntze), is prepared by steaming tea leaves for deactivation of color changing enzyme and drying [8]. It has been a popular beverage for consumers in Japan, China, Korea, and Morocco, due to several health benefits [9]. The usage of green tea in traditional medicines are to prolong life, detoxify, and treat various conditions, including headache, body ache, and digestive problems. Additionally, green tea has been used for prevention and treatment of cardiovascular diseases and cancer, photoprotection, and body weight control [9,10]. However, the clinical efficacies of green tea against skin aging have been a subject of controversy [11,12]. To clarify this without the interference of systemic involvement, the study was aimed to evaluate the anti-skin aging activities of green tea in cell culture by a number of assays, including melanogenesis assay, antioxidant activity, collagen content analysis,

and MMP-2 inhibitory assay. The standardisation of phenolic profile in green tea and the

cytotoxicity assay were also performed.

2. Materials and methods

2.1 Chemicals

Green tea leaves were purchased from a farm in Kyoto, Japan. The plant voucher specimen

(CS_L MFU15) was deposited at our laboratory herbarium, Mae Fah Luang University, Chiang

Rai, Thailand, for using as a reference. Dulbecco's-modified Eagle medium (DMEM), fetal

bovine serum, and penicillin/streptomycin solution were obtained from Gibco (Maryland, USA).

The lysis buffer and protease inhibitor cocktail were purchased from Thermo Fisher Scientific

(USA), and Roche® (Germany), respectively. The other reagents were of analytical grade.

2.2 Preparation of green tea extract

Green tea was extracted as a previous method with some modification [13]. Briefly, green tea

leaves were ground and then extracted by distilled water at 75-80°C for 30 min. The proportion

of tea leaves and water was 1:10 (w/v). The extract was filtered through filter paper, and the

filtrate was concentrated by using a spray drier. The dried extract was dissolved in a mixture of

water and butylene glycol at a ratio of 1:1 for further analysis. The final concentration of extract

was 17.5 mg/mL.

2.3 Standardisation of phenolic profile

The analysis was performed by high-performance liquid chromatography (HPLC) [14]. The

standards, including gallic acid (GA), gallocatechin (GC), epigallocatechin (EGC), catechin (C),

epicatechin (EC), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epicatechin

gallate (ECG), and caffeine, were mixed to prepare the standard calibration curve. The

4

standardisation of phenolic profile in the extract was determined using a Waters 966 HPLC photodiode array detector equipped with a pump system (Waters, USA). The analysis was conducted on a Platinum EPS C18 reversed-phase column (3 μm, 53×7 mm i.d.) using the isocratic elution system, which was consisted of 0.05% trifluoroacetic acid mixed with water and acetonitrile (83:17), at 30°C. The flow rate, injection volume, and detection wavelength were set at 2 mL/min, 10 μL, and 210 nm, respectively. The peak identification was compared to the retention time of each standard and the content was calculated from the standard calibration curve. The analysis was performed in triplicate.

2.4 Cytotoxicity assay

2.4.1 Cell culture

B16F10 melanoma cells (ATCC® CRL-6475™) and human skin fibroblasts (ATCC® CRL-2097™ at the 11th-18th passages) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution in a humidified incubator at 37°C with 5% carbon dioxide. Cells were harvested to seed into the culture plates. All cell culture experiments were performed in triplicate.

2.4.2 Cytotoxicity assay

A sulforhodamine B (SRB) assay was used for cytotoxicity assay [15]. Briefly, cells were seeded and incubated for cell adhesion. The B16F10 melanoma cells were treated with 0.5-2% green tea, 0.0001-1 mg/mL theophylline and kojic acid, and solvent, whereas the human skin fibroblasts were treated with noncytotoxic concentration of green tea, 0.0001-1 mg/mL vitamin C, and solvent. After 72 h incubation, cells were fixed and then dyed with SRB. The excess dye was washed with diluted acetic acid and the bound dye was solubilized in tris buffer. The sample

5

absorbance was measured at 540 nm by a microplate reader. The percent of cell viability was calculated in compared to solvent control.

2.5 Analysis of anti-skin aging activities

2.5.1 Melanogenesis assay

Melanogenesis assay was performed as previously described [7]. Briefly, B16F10 melanoma cells were plated and treated with noncytotoxic concentration of green tea, theophylline, kojic acid, and solvent for 72 h. The cell pellets were then harvested to determine melanin content and activities of tyrosinase and tyrosinase-related protein-2 (TRP-2). Total protein content was also analyzed by the Bradford protein assay for calculation of the actual melanin content and enzyme activities. The percent of relative ratio of melanin content and enzyme activities was calculated in compared to solvent control.

2.5.2 Antioxidant activity assay

Antioxidant activity was evaluated as previous method [15]. Briefly, human skin fibroblasts were seeded and treated with noncytotoxic concentration of green tea, vitamin C, and solvent. After 24 h incubation, all samples were replaced with fresh culture medium containing 150 µM hydrogen peroxide. After a 4-h incubation, cells were fixed, stained with SRB dye, and rinsed off the unbound dye. The bound dye was solubilized and measured the absorbance at 540 nm. The percent of viable cells was calculated in compared to solvent control.

2.5.3 Collagen content analysis

Collagen content analysis was performed as a previous study with some modifications [16].

Human skin fibroblasts were seeded and treated with the greatest noncytotoxic concentration of green tea, vitamin C, and solvent for 48 h. Cells were lysed with the mixture of lysis buffer and protease inhibitor cocktail and then centrifuged to collect the cell pellets. Sirius red dye reagent

6

was added and shaken for 30 min. The mixture was centrifuged to collect the red precipitate. The

unbound dye was washed, and the precipitate was dissolved in sodium hydroxide. The sample

absorbance was measured at 550 nm. The percent of collagen content was calculated in compared

to solvent control.

2.5.4 MMP-2 inhibitory assay

The activity of MMP-2 enzyme was performed as previously described [5]. Human skin

fibroblasts were seeded and incubated overnight. The culture medium was replaced with fresh

medium without serum supplement. The greatest noncytotoxic concentration of green tea,

vitamin C, and solvent, was added and incubated for 72 h. The culture medium was collected for

MMP-2 quantification by SDS-PAGE zymography with gelatin as the substrate. The

zymographic bands were scanned and determined with the Bio-Rad Gel Doc Imaging System.

The percent of MMP-2 inhibitory activity was calculated in compared to solvent control.

2.6 Statistical analysis

Data were presented as the means ± standard error of mean of three independent experiments.

The one-way analysis of variance (ANOVA) and the least significant difference (LSD) test were

used to analyse treatment differences with a significance level of p-value<0.05.

3. Results

3.1 Standardisation of phenolic profile

Fig. 1 presents the HPLC chromatograms of standard mixture and green tea. The standard

calibration curve consisted of 100 μg/mL caffeine and 8 phenolic standards, including GA, GC,

EGC, C, EC, EGCG, GCG, and ECG. As shown in Table 1, green tea consisted of several

7

phenolic compounds and caffeine in different contents. EGCG was the major phenolic acid in extract, followed by EGC and ECG, respectively.

3.2 Cytotoxicity assay

The cytotoxicity assay was performed to obtain the noncytotoxic concentrations of samples for anti-skin aging assays. Fig. 2 demonstrates the cytotoxicity of green tea, theophylline, kojic acid, and vitamin C in B16F10 melanoma cells and human skin fibroblasts. In B16F10 melanoma cells, the cell viabilities of 0.5% green tea (0.0875 mg/mL), 0.0001 - 0.1 mg/mL theophylline and kojic acid treatment were greater than 80%, indicating the noncytotoxic concentration. The increased concentration of green tea, theophylline, and kojic acid was shown the decreased cell viabilities to less than 80%, which exhibited the cytotoxicity. In human skin fibroblasts, the cell viability of 0.5% green tea (noncytotoxic concentration) treatment was $93.44 \pm 0.95\%$, whereas those of 0.0001 - 0.1 mg/mL vitamin C treatment were greater than 90%. However, the decreased cell viability ($75.23 \pm 1.87\%$) was observed when treated with 1 mg/mL vitamin C, which was shown the cytotoxic concentration. The noncytotoxic concentration of green tea along with theophylline, kojic acid, and vitamin C, were further investigated the anti-skin aging activities.

3.3 Analysis of anti-skin aging activities

3.3.1 Melanogenesis assay

Melanogenesis assay of green tea was performed in compared to the greatest noncytotoxic concentration of theophylline and kojic acid, which were used as the positive and negative controls, respectively. As shown in Fig. 3, the melanin content, tyrosinase, and TRP-2 activities of 0.5% green tea treatment were 85.05 ± 3.45, 89.70 ± 7.83, and $90.87 \pm 6.87\%$, respectively. The melanin content and enzyme activities were increased when treated with 0.1 mg/mL theophylline, whereas those were decreased when 0.1 mg/mL kojic acid was tested. Similar

8

decrease in melanin content of green tea and kojic acid treatment was observed with a *p*-value of

0.26.

3.3.2 Antioxidant activity assay

After treatment with noncytotoxic concentration of samples, hydrogen peroxide was added to

induce the oxidative stress. The cell viability was determined to observe the cellular protective

effect of antioxidant and the damaged cells caused by oxidative stress. Generally, the cell

viability of antioxidant treatment would be greater than that of hydrogen peroxide treatment

(oxidative control). Fig. 4 presents the antioxidant activity assay in human skin fibroblasts treated

with green tea and vitamin C in compared to solvent control and hydrogen peroxide treatment.

The viability of cells treated with hydrogen peroxide was decreased to $75.51 \pm 0.14\%$, indicating

the cytotoxicity. The cell viabilities of 0.5% green tea and 0.1 mg/mL vitamin C treatment were

94.36 ± 1.94 and $97.12 \pm 3.00\%$, respectively, which were comparable to solvent control.

3.3.3 Collagen content analysis

The collagen content assay was performed by colorimetric method using Sirius Red dye reagent.

The effect of green tea and vitamin C on collagen content in human skin fibroblasts demonstrates

in Fig. 5. The collagen contents of green tea and vitamin C treatment were 103.37 ± 6.09 and

$131.49 \pm 4.59\%$, respectively. Similar collagen content of green tea and solvent control was

observed.

3.3.4 MMP-2 inhibitory assay

MMP-2 is one of the important enzymes involved in the degradation of extracellular matrices

during skin aging. Green tea and vitamin C were tested the MMP-2 inhibitory effect in human

skin fibroblasts in compared to solvent control. Fig. 6 presents the zymogram and the inhibitory

activity. The enzyme inhibition of green tea was $44.18 \pm 3.80\%$, whereas that of vitamin C was

9

$25.50 \pm 2.36\%$. Green tea significantly exhibited the enzyme inhibitory effect greater than vitamin C (p-value=0.009).

4. Discussion

Phenolic acids in plant play the main role in the biological activities, so the analysis of these compounds is reasonable to determine as the markers of plant extract [17]. According to the literature, the variation in botanical and herbal preparations influences pharmacological activities which means that the analysis for the chemical standardisation of marker compounds or active ingredients in preparations is required [18]. The standardisation of phenolic acids was shown that the green tea in this study composed of a variety of phenolic compounds. The major phenolic constituent in extract, EGCG, was in agreement with previous studies [19,20]. However, different contents in EGCG and other phenolic acids in the extract were observed. This may be due to the variation of tea plant, including difference in genetic, agronomic conditions, and harvest time, as well as the different extraction conditions, such as extraction solvents, temperatures, and extraction times [19,21].

The noncytotoxic concentration of green tea in B16F10 melanoma cells and human skin fibroblasts was 0.5% (0.0875 mg/mL). The cytotoxicity of green tea was associated with the phenolic constituents. Previous studies have shown that EGCG and gallic acid demonstrate the inhibitory effect on cell viability and cell proliferation, particularly on cancerous cells [20,22]. Theophylline inhibits the cell proliferation by mitotic inhibition, cellular morphology alteration, and cytotoxicity [23], whereas the cytotoxic effect of kojic acid and vitamin C at high concentration is associated with the acidified culture environment [24]. Additionally, ascorbyl stearate, the vitamin C derivative, exhibits the inhibitory effect on cancer cell proliferation and clonogenicity in a dose-dependent manner [25].

Skin pigment is produced within the epidermal melanocytes located at basal layer of skin epidermis [26]. The production of pigment is influenced by environment and several factors secreted by neighboring keratinocytes and fibroblasts [27]. Tyrosinase is a rate-limiting enzyme that controls the pigment production [24]. The uneven pigmentation and hyperpigmentation, that are correlated with the increased activity of tyrosinase, are the conditions affecting psychosocial aspects of skin disorders. Moreover, photoaging, a major type of extrinsic aging caused by exposure to UV irradiation, is markedly associated with the hyperpigmentation in Caucasian and Asian skin [28]. Many studies have investigated the pigment reducing agents with the additional benefit of anti-skin aging [5,29]. The decreased melanin production of green tea was mediated via inhibitory effect of two melanogenic enzyme activities, tyrosinase and TRP-2, in melanin biosynthesis pathway. The phenolic constituents in green tea, including EGCG and gallic acid, have shown to inhibit the pigment synthesis and tyrosinase expression [30,31]. Theophylline stimulates the pigment formation through the activation of cyclic adenosine monophosphate (cAMP) signaling pathway and the increased number of dopa- and gamma-glutamyl transpeptidase-reactive cells [32], whereas kojic acid suppresses the pigment via acting as an inhibitor of tyrosinase in a nonclassical manner [33].

Free radicals, including reactive oxygen and nitrogen species, are associated with several disorders and skin problems, such as inflammation, hyperpigmentation, and aging. Antioxidants can prevent the radical formation and/or scavenge the radicals, resulting in the attenuated oxidative damage. The cytotoxicity induced by hydrogen peroxide is mediated by leakage of cell membrane and DNA damage [34]. The observed antioxidant activity of green tea and vitamin C was in agreement with previous reports [7,9]. Phenolics in extract, including EGCG, ECG, EGC, EC, and gallic acid, may synergistically act on the antioxidant activity via chelating redox-active transition metal ions and scavenging reactive oxygen and nitrogen species [31,35].

11

Collagen, an abundant structural protein, gives tensile strength and supports human skin.

Markedly decreased collagen content is associated with the decreased skin integrity in aging skin.

In Addition, various factors, including free radicals and upregulation of MMPs, have been shown

to elevate the collagen metabolism, resulting in the wrinkle formation and the decreased skin

thickness [5]. Sirius red dye can interact with [Gly-x-y] triple-helix structure in collagen fiber and

lead to the colorimetric analysis of collagen content [36]. Previous study has shown that EGCG

can suppress the fibroblast proliferation and collagen production [37]. In addition, ECG, EGC,

and EGCG have demonstrated the reduced expression of transforming growth factor-β (TGF-β),

a collagen stimulating protein [38,39]. The significantly increased collagen production of

vitamin C is associated with the increased mRNA expression of procollagen and elastin in

fibroblasts [40].

As known, the activation of MMPs is associated with free radicals by oxidizing enzyme catalytic

center [41]. The potent MMP-2 inhibition of green tea may be attributed to the presence of

phenolic acids. EGCG has demonstrated to form complex with zinc ion, an essential site for

enzyme function, leading to the inhibition of MMP-2 and MMP-9 enzymes [42]. Additionally,

EGCG can decrease MMP expression and production through regulation of nuclear factor kappa

B (NF-κB), activator protein 1 (AP-1), and mitogen-activated protein kinases (MAPKs) signaling

pathway [43]. Gallic acid in green tea also exhibits the MMP-2 inhibitory activity in fibroblast

culture [31]. Vitamin C is mediated the enzyme suppression by antioxidant activity [44] along

with the increased expression of tissue inhibitor of matrix metalloproteinases [45].

To clarify the efficacy of green tea, the study results had suggested that green tea in this study

composed of a variety of phenolic acids and exhibited biological activities against skin aging,

including the melanogenesis inhibition through suppression of tyrosinase and TRP-2 activities,

the potent antioxidant, and the MMP-2 inhibition. The controversy of green tea in clinical trials

may be associated with several factors, including the design of study protocol, the contents of

phenolic constituents, as well as the skin penetration. The design of study protocol, including the

differences in treatment regimens and characteristics of the recruited volunteers, may influence

the clinical effects of tested product. Moreover, the contents of phenolics in green tea, mainly

EGCG, should be taken into consideration. EGCG, the major constituent in green tea, has

exhibited several activities against skin aging, including the potent antioxidant activity, the

inhibition of melanin formation, and the suppression of MMPs [46-48]. Nevertheless, the

degradation of EGCG during product manufacturing and storage is associated with the reduction

of green tea activities [46]. Antioxidant, such as vitamin C and butylated hydroxytoluene (BHT),

is recommended to incorporate into the preparations containing green tea for prolonged phenolic

components in extract and the health advantages [49]. For application of green tea in topical

formulations, the percutaneous absorption is an important factor influencing the efficacy.

Previous study has demonstrated that EGCG is more likely to accumulate in the stratum corneum

than in viable epidermis and dermis after topical application [50], thereby resulting in the topical

effect.

4. Conclusions

The green tea used in this study consisted of a variety of phenolic acids. EGCG, EGC, and ECG,

were shown to be the three major phenolics in this extract. The noncytotoxic concentration of

green tea, 0.5% or 0.0875 mg/mL, exhibited the anti-skin aging activities, including the

significant suppression of melanin production by inhibition of tyrosinase and TRP-2 activities,

the potent antioxidant, and the significant MMP-2 inhibitory activity. These results have revealed

that green tea is a functional processed plant for utilisation as an anti-skin aging agent in the natural healthcare products, including food, health, and cosmetic products.

Authors contribution

Puxvadee Chaikul: Conceptualization, Methodology, Formal analysis, Investigation, Writing manuscript and editing, and Project administration

Tawanun Sripisut: Methodology and Investigation

Setinee Chanpirom: Methodology and Investigation

Naphatsorn Ditthawuthikul: Methodology and Investigation

Declaration of Competing Interests

The authors declare that there are no conflicts of interest.

Data availability

The plant voucher specimen and the research data used to support the finding of this study are

available from the corresponding author on reasonable request.

REFERENCES

1. D.J. Tobin, Introduction to skin aging, J. Tissue Viability. 26 (2017) 37-46. https://doi.org/10.1016/j.jtv.2016.03.002.
2. R. Ganceviciene, A.I. Liakou, A. Theodoridis, E. Makrantonaki, C.C. Zouboulis, Skin anti-aging strategies, Dermatoendocrinol. 4 (2012) 308-319. https://doi.org/10.4161/derm.22804.
3. S. Zhang, E. Duan, Fighting against skin aging: the way from bench to bedside, Cell. Transplant. 27 (2018) 729-738. https://doi.org/10.1177/0963689717725755.
4. T. Quan, Z. Qin, W. Xia, Y. Shao, J.J. Voorhees, G.J. Fisher, Matrix-degrading metalloproteinases in photoaging, J. Investig. Dermatol. Symp. Proc. 14 (2009) 20-24. https://doi.org/10.1038/jidsymp.2009.8.
5. N. Lourith, M. Kanlayavattanakul, P. Chaikul, C. Chansriniyom, P. Bunwatcharaphansakun, *In vitro* and cellular activities of the selected fruits residues for skin aging treatment, An. Acad. Bras. de Ciênc. 89 (2017) 577-589. https://doi.org/10.1590/0001-3765201720160849.
6. X. Li, J. Zhang, C. Sun, Y. Zhang, R. Cai, S. Fu, J. Zheng, D. Huang, Application of biological age assessment of Chinese population in potential anti-ageing technology, Immun Ageing. 15 (2018) 33. https://doi.org/https://doi.org/10.1186/s12979-018-0140-9.
7. P. Chaikul, N. Lourith, M. Kanlayavattanakul, Antimelanogenesis and cellular antioxidant activities of rubber (*Hevea brasiliensis*) seed oil for cosmetics, Ind. Crop. Prod. 108 (2017) 56-62. https://doi.org/10.1016/j.indcrop.2017.06.009.
8. D.F. Holderbaum, T. Kon, T. Kudo, M.P. Guerra, Enzymatic browning, polyphenol oxidase activity, and polyphenols in four apple cultivars: dynamics during fruit development, HortScience. 45 (2010) 1150-1154. https://doi.org/10.21273/HORTSCI.45.8.1150.
9. C. Cabrera, R. Artacho, R. Gimenez, Beneficial effects of green tea—a review, J. Am. Coll. Nutr. 25 (2006) 79-99. https://doi.org/10.1080/07315724.2006.10719518.
10. T. Aburjai, F.M. Natsheh, Plants used in cosmetics, Phytother. Res. 17 (2003) 987-1000. https://doi.org/10.1002/ptr.1363.
11. A.E. Chiu, J.L. Chan, D.G. Kern, S. Kohler, W.E. Rehmus, A.B. Kimball, Double-blinded, placebo-controlled trial of green tea extracts in the clinical and histologic appearance of photoaging skin, Dermatol. Surg. 31 (2005) 855-860. https://doi.org/10.1111/j.1524-4725.2005.31731.
12. M.D. Gianeti, D.G. Mercurio, P.M. Campos, The use of green tea extract in cosmetic formulations: not only an antioxidant active ingredient, Dermatol. Ther. 26 (2013) 267-271. https://doi.org/10.1111/j.1529-8019.2013.01552.x.
13. Y. Yoshida, M. Kiso, T. Goto, Efficiency of the extraction of catechins from green tea, Food Chem. 67 (1999) 429-433. https://doi.org/10.1016/S0308-8146(99)00148-X.
14. T. Theppakorn, A. Luthfivyyah, K. Ploysri, Simultaneous determination of caffeine and 8 catechins in Oolong teas produced in Thailand, Int. Food Res. J. 21 (2014) 2055-2061.
15. P. Chaikul, T. Sripisut, S. Chanpirom, K. Sathirachawan, N. Ditthawutthikul, Melanogenesis inhibitory and antioxidant effects of *Camellia oleifera* seed oil, Adv. Pharm. Bull. 7 (2017) 473-477. https://doi.org/10.15171/apb.2017.057.

16. H. Tullberg-Reinert, G. Jundt, *In situ* measurement of collagen synthesis by human bone cells with a Sirius Red-based colorimetric microassay: effects of transforming growth factor β2 and ascorbic acid 2-phosphate, Histochem. Cell Biol. 112 (1999) 271-276.

17. G. Miliauskas, P.R. Venskutonis, T.A. van Beek, Screening of radical scavenging activity of some medicinal and aromatic plant extracts, Food Chem. 85 (2004) 231-237. https://doi.org/10.1016/j.foodchem.2003.05.007.

18. E.S. Ong, Extraction methods and chemical standardization of botanicals and herbal preparations, J Chromatogr B. 812 (2004) 23-33. https://doi.org/https://doi.org/10.1016/j.jchromb.2004.07.041.

19. G. Rusak, D. Komes, S. Likić, D. Horžić, M. Kovač, Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used, Food Chem. 110 (2008) 852-858. https://doi.org/10.1016/j.foodchem.2008.02.072.

20. L. Schröder, P. Marahrens, J.G. Koch, H. Heidegger, T. Vilsmeier, T. Phan-Brehm, S. Hofmann, S. Mahner, U. Jeschke, D.U. Richter, Effects of green tea, matcha tea and their components epigallocatechin gallate and quercetin on MCF‑7 and MDA-MB-231 breast carcinoma cells, Oncol Rep. 41 (2019) 387-396. https://doi.org/https://doi.org/10.3892/or.2018.6789.

21. W. Zheng, S.Y. Wang, Antioxidant activity and phenolic compounds in selected herbs, J Agric Food Chem. 49 (2001) 5165-5170. https://doi.org/https://doi.org/10.1021/jf010697n.

22. T.W. Kim, S. Paveen, Y.H. Lee, Y.S. Lee, Comparison of cytotoxic effects of pentagalloylglucose, gallic acid, and its derivatives against human cancer MCF-7 and MDA MB-231 cells, B. Korean Chem. Soc. 35 (2014) 987-988. https://doi.org/10.5012/bkcs.2014.35.4.987.

23. T.A. Slotkin, F.J. Seidler, Antimitotic and cytotoxic effects of theophylline in MDA-MB-231 human breast cancer cells, Breast Cancer Res. Tr. 64 (2000) 259-267. https://doi.org/10.1023/a:1026508605951.

24. P. Chaikul, J. Manosroi, W. Manosroi, A. Manosroi, Melanogenesis enhancement of saturated fatty acid methyl esters in B16F10 melanoma cells, J. Comput. Theor. Nanosci. 17 (2012) 251-256. https://doi.org/10.1166/asl.2012.4254.

25. K.A. Naidu, Vitamin C in human health and disease is still a mystery? An overview, Nutr. J. 2 (2003) 7. https://doi.org/10.1186/1475-2891-2-7.

26. J. Rocha, The evolutionary history of human skin pigmentation, J Mol Evol. 88 (2020) 77-87. https://doi.org/https://doi.org/10.1007/s00239-019-09902-7.

27. Y. Yamaguchi, M. Brenner, V.J. Hearing, The regulation of skin pigmentation, J Biol Chem. 282 (2007) 27557-27561. https://doi.org/https://doi.org/10.1074/jbc.R700026200

28. S. Venkatesh, M.B.C. Maymone, N.A. Vashi, Aging in skin of color, Clin. Dermatol. 37 (2019) 351-357. https://doi.org/10.1016/j.clindermatol.2019.04.010.

29. M. Kanlayavattanakul, N. Lourith, P. Chaikul, Jasmine rice panicle: a safe and efficient natural ingredient for skin aging treatments, J. Ethnopharmacol. 193 (2016) 607-616. https://doi.org/10.1016/j.jep.2016.10.013.

30. K. Sato, M. Toriyama, Depigmenting effect of catechins, Molecules. 14 (2009) 4425-4432. https://doi.org/10.3390/molecules14114425.

31. P. Chaikul, N. Khat-udomkiri, K. Iangthanarat, J. Manosroi, A. Manosroi, Characteristics and *in vitro* anti-skin aging activity of gallic acid loaded in cationic CTAB niosome, Eur. J. Pharm. Sci. 131 (2019) 39-49. https://doi.org/10.1016/j.ejps.2019.02.008.

32. F. Hu, Theophylline and melanocyte-stimulating hormone effects on gamma-glutamyl transpeptidase and DOPA reactions in cultured melanoma cells, J. Invest. Dermatol. 79 (1982) 57-62. https://doi.org/10.1111/1523-1747.ep12510659.

33.	J. Cabanes, S. Chazarra, F. Garcia-Carmona, Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase, J. Pharm. Pharmacol. 46 (1994) 982-985. https://doi.org/10.1111/j.2042-7158.1994.tb03253.x.

34.	E.R. Whittemore, D.T. Loo, J.A. Watt, C.W. Cotmans, A detailed analysis of hydrogen peroxide-induced cell death in primary neuronal culture, Neuroscience. 67 (1995) 921-932. https://doi.org/10.1016/0306-4522(95)00108-u.

35.	C.-B. Ong, M.S.M. Annuar, Polyphenolic composition and *in vitro* antioxidant activities of native- and tannase-treated green tea extracts, Int J Food Sci Tech. 52 (2017) 748-756. https://doi.org/https://doi.org/10.1111/ijfs.13331.

36.	C.R. Kliment, J.M. Englert, L.P. Crum, T.D. Oury, A novel method for accurate collagen and biochemical assessment of pulmonary tissue utilizing one animal, Int. J. Clin. Exp. Patho. 4 (2011) 349-355.

37.	G. Park, B.S. Yoon, J.-H. Moon, B. Kim, E.K. Jun, S. Oh, H. Kim, H.J. Song, J.Y. Noh, C. Oh, S. You, Green tea polyphenol epigallocatechin-3-gallate suppresses collagen production and proliferation in keloid fibroblasts via inhibition of the STAT3-signaling pathway, J Invest Dermatol. 128 (2008) 2429-2441.

38.	L. Wang, G. Yang, L. Yuan, Y. Yang, H. Zhao, C.-T. Ho, S. Li, Green tea catechins effectively altered hepatic fibrogenesis in rats by inhibiting ERK and Smad1/2 phosphorylation, J Agric Food Chem. 67 (2019) 5437-5445. https://doi.org/10.1021/acs.jafc.8b05179.

39.	J. Varga, J. Rosenbloom, S.A. Jimenez, Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts, Biochem J. 247 (1987) 597-604. https://doi.org/https://doi.org/10.1042/bj2470597.

40.	K.Y. Park, J. Kim, Synthesis and biological evaluation of ascorbyl-conjugated peptide derivatives as collagen synthesis stimulating agents in human skin fibroblasts, Int. J. Pept. Res. Ther. (2020) 1-8. https://doi.org/10.1007/s10989-020-10041-7.

41.	C.A. Papaharalambus, K.K. Griendling, Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury, Trends Cardiovas. Med. 17 (2007) 48-54. https://doi.org/10.1016/j.tcm.2006.11.005.

42.	M. Maeda-Yamamoto, H. Kawahara, N. Tahara, K. Tsuji, Y. Hara, M. Isemura, Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells, J. Agric. Food Chem. 47 (1999) 2350-2354. https://doi.org/10.1021/jf9811525.

43.	L. Wang, W. Lee, Y.R. Cui, G. Ahn, Y.-J. Jeon, Protective effect of green tea catechin against urban fine dust particle-induced skin aging by regulation of NF-κB, AP-1, and MAPKs signaling pathways, Environ Pollut. 252 (2019) 1318-1324. https://doi.org/https://doi.org/10.1016/j.envpol.2019.06.029.

44.	A. Bendich, L.J. Machlin, O. Scandurra, G.W. Burton, D.D.M. Wayner, The antioxidant role of vitamin C, Adv. Free Radic. Biol. Med. 2 (1986) 419-444. https://doi.org/10.1016/S8755-9668(86)80021-7.

45.	B.V. Nusgens, A.C. Colige, C.A. Lambert, C.M. Lapière, P. Humbert, A. Rougier, M. Haftek, A. Richard, P. Creidi, Topically applied vitamin C enhances the mRNA level of collagens I and III, their processing enzymes and tissue inhibitor of matrix metalloproteinase 1 in the human dermis, J. Invest. Dermatol. 116 (2001) 853-859. https://doi.org/10.1046/j.0022-202x.2001.01362.x.

46.	A. Gramza, J. Korczak, R. Amarowicz, Tea polyphenols-their antioxidant properties and biological activity, Pol. J. Food Nutr. Sci. 55 (2005) 219-235.

47.   D.-S. Kim, S.-H. Park, S.-B. Kwon, K. Li, S.-W. Youn, K.-C. Park, (−)-Epigallocatechin-3-gallate and hinokitiol reduce melanin synthesisvia decreased MITF production, Arch. Pharm. Res. 27 (2004) 334. https://doi.org/10.1007/BF02980069.

48.   H.S. Kim, M.H. Kim, M. Jeong, Y.S. Hwang, S.H. Lim, B.A. Shin, B.W. Ahn, Y.D. Jung, EGCG blocks tumor promoter-induced MMP-9 expression via suppression of MAPK and AP-1 activation in human gastric AGS cells, Anticancer Res. 24 (2004) 747-754.

49.   K. Dvorakova, R.T. Dorr, S. Valcic, B. Timmermann, D.S. Alberts, Pharmacokinetics of the green tea derivative, EGCG, by the topical route of administration in mouse and human skin, Cancer Chemother. Pharmacol. 43 (1999) 331-335. https://doi.org/10.1007/s002800050903.

50.   S.E. dal Belo, L.R. Gaspar, P.M.B.G. Maia Campos, J.P. Marty, Skin penetration of epigallocatechin-3-gallate and quercetin from green tea and *Ginkgo biloba* extracts vehiculated in cosmetic formulations, Skin Pharmacol. Physiol. 22 (2009) 299-304. https://doi.org/10.1159/000241299.

Wordcount: Manuscript 4985 words (included abstract and references); Figure 6 figures, 274 words; Table 1 table, 60 words

Figures

Fig. 1 HPLC chromatograms of (a) standard mixture and (b) green tea. GA, GC, EGC, C, EC, EGCG, CF, GCG and ECG were gallic acid, gallocatechin, epigallocatechin, catechin, epicatechin, epigallocatechin gallate, caffeine, gallocatechin gallate and epicatechin gallate, respectively.
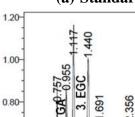
Fig. 2 Cytotoxicity assay in (a) B16F10 melanoma cells and (b) human skin fibroblasts treated with green tea (GT), theophylline (TP), kojic acid (KJ), and vitamin C (VC). * indicates a significant difference from the control (*$p<0.05$,**$p<0.001$).

Fig. 3 Melanogenesis assay in B16F10 melanoma cells treated with 0.5% green tea (GT), 0.1 mg/mL theophylline (TP), and kojic acid (KJ). * indicates a significant difference from the control (*$p<0.05$, **$p<0.001$).
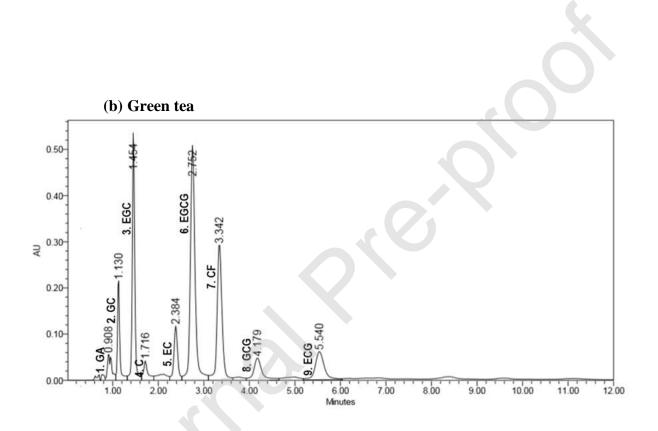
Fig. 4 Antioxidant activity assay in human skin fibroblasts treated with green tea (GT), and vitamin C (VC). Ctr and H_2O_2 were the solvent control and the oxidative control, respectively. * indicates a significant difference from the control (*$p<0.05$, **$p<0.001$).

Fig. 5 Collagen content analysis in human skin fibroblasts treated with green tea (GT) and vitamin C (VC). * indicates a significant difference from the control (*$p<0.05$, **$p<0.001$).
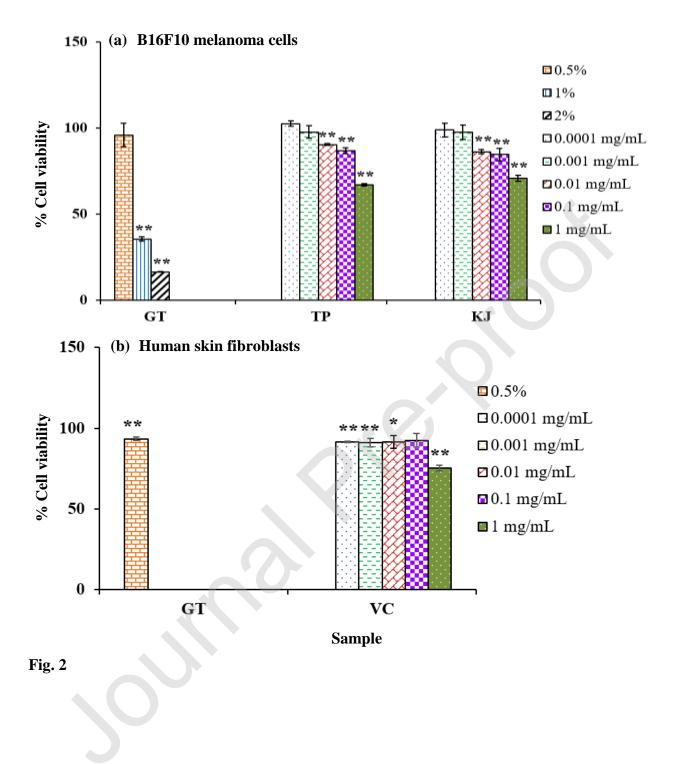
Fig. 6 MMP-2 inhibitory assay in human skin fibroblasts treated with green tea (GT), and vitamin C (VC): (a) zymogram and (b) MMP-2 inhibitory activity. * indicates a significant difference from the control (*$p<0.05$, **$p<0.001$).
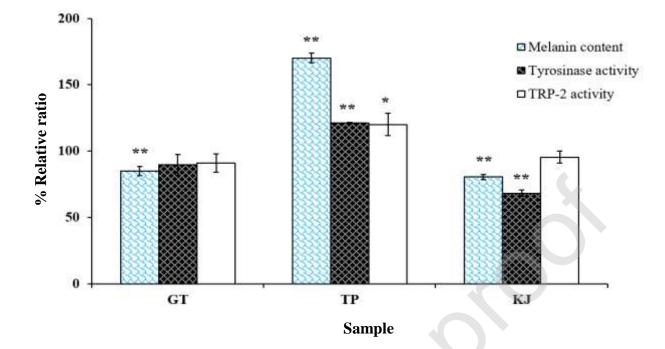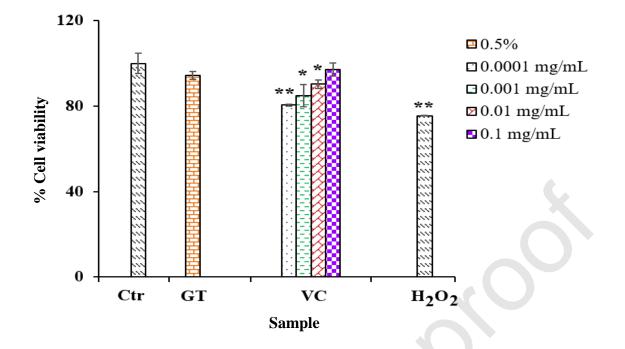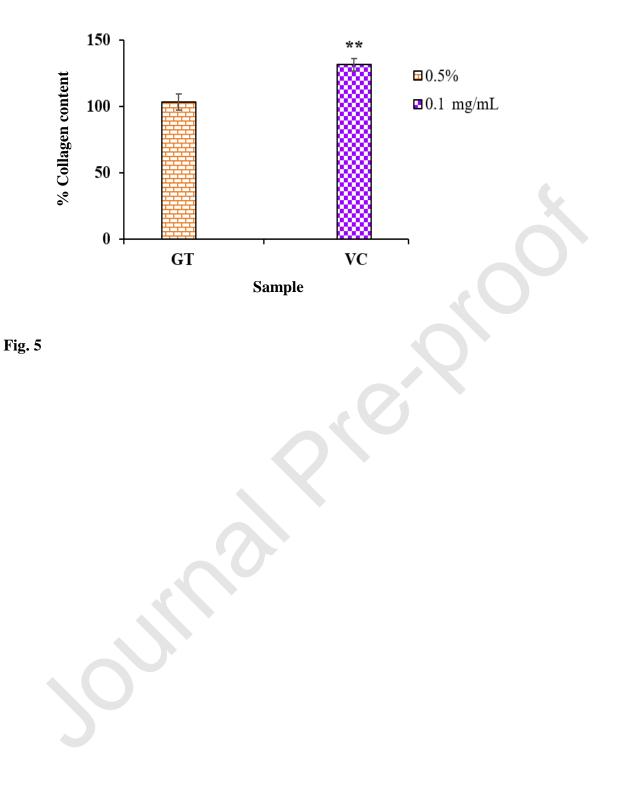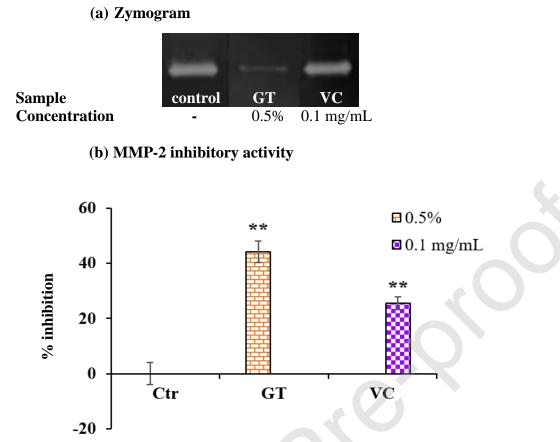
(a) Standard mixture

(b) Green tea

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

(a) Zymogram

Sample	control	GT	VC
Concentration	-	0.5%	0.1 mg/mL

(b) MMP-2 inhibitory activity

Fig. 6

Table 1 Standardisation of phenolic profile by HPLC

Compound	Content (µg/mL)
Gallic acid (GA)	155.66 ± 3.79
Gallocatechin (GC)	303.43 ± 6.91
Epigallocatechin (EGC)	930.24 ± 27.04
Catechin (C)	149.75 ± 2.55
Epicatechin (EC)	331.39 ± 4.32
Epigallocatechin gallate (EGCG)	1,903.77 ± 33.59
Caffeine (CF)	1,281.85 ± 19.44
Gallocatechin gallate (GCG)	316.09 ± 8.37
Epicatechin gallate (ECG)	607.84 ± 22.65