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Screening and characterization of antioxidant, anti-aging, and anti-microbial activity of herbal extracts

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ABSTRACT

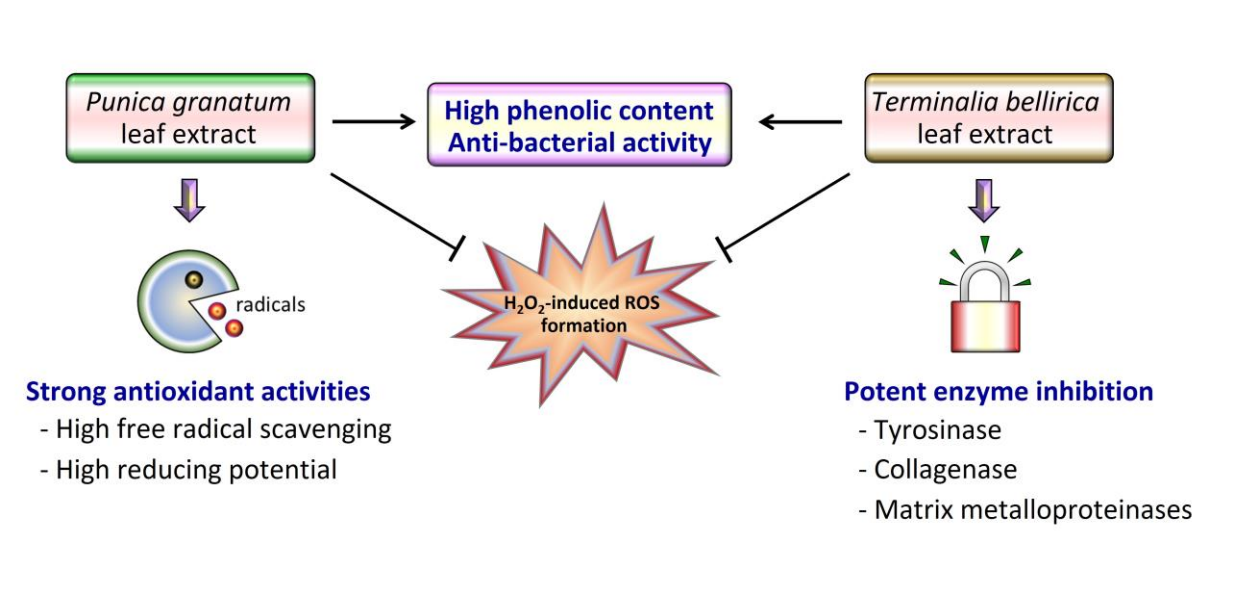
Background: Aging is a process caused by oxidants and aging-related enzymes. Therefore, the inhibition of these processes can exacerbate anti-aging agents. This study aimed to evaluate the antioxidant and anti-aging activities of leaf extracts from seven herbs used in traditional Thai herbal remedies.

Methods: Researchers assessed the total levels of phenolic content (TPC), antioxidant, anti-collagenase, and anti-tyrosinase activity using colorimetric methods. Cytotoxicity effects were determined using MTT assays, and matrix metalloproteinases(MMPs) secretion was assessed through gelatin zymography. In addition, inhibitory effects on the growth of microorganisms were examined using the disc diffusion and broth microdilution method.

Results: TPC ranged between 48.68–440.91 mg GAE/g of ethanolic leaf extracts. High antioxidant activities against ABTS radicals were detected in *P. granatum*, *P. emblica*, *P. guajava*, *T. bellirica*, and *T. chebula*, while high DPPH neutralization appeared in *M. coreia*, *P. guajava*, and *P. granatum*. FRAP assays significantly reduced the power of *T. chebula* and *P. granatum*. *T. chebula* and *P. guajava* exhibited the highest inhibitory effect on H_2O_2induced ROS production. *P. emblica*, *P. guajava*, *T. bellirica*, and *E. hygrophilus* reduced tyrosinase and collagenase activities. *P. gaujava*, *T. chebula,* and *T. bellirica* were shown to inhibit the secretion of MMP-2 from fibroblast cells. All concentrations of leaf extracts were non-toxic to fibroblast cells. *P.granatum* and *T. bellirica* could inhibit the growth of *P. acnes*, *E. coli*, and *S. aureus*.

Conclusion: Preliminary studies showed that *P. granatum* and *T. bellirica* leaf extracts have antioxidants, anti-aging, and anti-bacterial activities and have potential for use as active ingredients for the development of functional foods and cosmetic products.

Key words: antioxidants, anti-aging, leaf extracts



INTRODUCTION

Aging is caused by degenerative changes and further perpetuated by intrinsic factors, especially senescence. Aging is also induced by extrinsic factors, including poor nutrition, air pollution and sun exposure. One of the most potent accelerating factors is an excess of reactive oxygen species (ROS) [1,2]. An imbalance of ROS and antioxidants contributes to the dysfunction of biomolecules [3]. Currently, ROS-mediated degeneration has been linked to skin aging [4,5]. Accumulated evidence has revealed that UV radiation decreases the synthesis of collagen and elastic fiber via ROS production and upregulation of collagenase, as well as matrix metalloproteinases (MMPs) through the activation of receptors for MAPK-cJun-AP1 signaling [6,7]. Sun exposure manifests as brown/dark spots [8]. This is caused by the accumulation of melanin pigment in the epidermis due to tyrosinase activity. Increased melanogenesis is closely related to an upregulation of tyrosinase, contributing to hyperpigmentation. Moreover, the formation of melanin also generates ROS, especially H_2O_2.

Therefore, compounds that foment progressive skin aging through ROS neutralization, suppression of ECM-digested enzymes, and inhibition of melanogenesis are sought after as cosmetic ingredients and used for the treatment of human skin aging. Synthetic antioxidants, such as ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), and idebenone, are typically used as cosmetic ingredients. They are

generally considered to be more effective than natural antioxidants. However, they frequently cause allergic reactions in hypersensitive individuals [9-11].

Cosmetics are often exposed to microbial environments, and they are easily contaminated. Effective and adequate protection is needed to maintain the economic value of cosmetics [12]. There is an increasing demand for natural antimicrobials as natural preservatives in food and cosmetics [13]. Therefore, there may be great value in developing and utilizing natural plant materials with antibacterial activities. Importantly, the use of natural ingredients for delaying skin aging is also demanded for safety reasons. In this preliminary study, several plant extracts were screened for antioxidant and enzyme inhibitory activities, with the aim of the researchers being to assess any beneficial effects for the treatment of skin aging.

MATERIALS and METHODS

Reagents and Chemicals: L-Ascorbic acid, gallic acid, kojic acid, Folin-Ciocalteu reagent, 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), ferrous sulfate heptahydrate, hydrogen peroxide (H2O2), 2',7'-dichlorofluorescin diacetate (DCF-DA), mushroom tyrosinase were obtained from Sigma-Aldrich (St. Louis, MO, USA). DQ gelatin was supplied by Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS), penicillin–streptomycin, and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) derived from Thermo Fisher Scientific (Burlington, ON, Canada). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA).

Plant materials and preparation of extracts: The primary focus of the screening was 7 plants collected from Phayao province, Thailand (Tambon Baan Tham, Amphur Dok Kham Tai). The voucher specimens were collected and verified by the Queen Sirikit Botanic Garden Herbarium (QBG), Chiang Mai, Thailand, including leaves from *Elaeocarpus hygrophilus*, *Morinda coreia*, *Phyllanthus emblica*, *Psidium guajava*, *Punica granatum*, *Terminalia bellirica*, *Terminalia chebula* with the codes QBG—119044, -119043, -119045, -115051, -115050, -119047, and -119046 respectively. The plant materials were extracted using a 70% ethanol solution which was stirred for 4 h and consequently separated through centrifugation at 5,800xg for 10 min. Crude extracts were lyophilized and stored at -20°C and suspended in dimethyl sulfoxide (DMSO) before use.

Determination of total phenolic content (TPC): TPC levels were estimated spectrophotometrically using Folin-Ciocalteu assays with minor modifications [14,15]. Briefly, Briefly, in the total volume of 200 µl, the reaction mixture contained 3% Na_2CO_3, 5% Folin–Ciocalteu reagent and various concentrations of the leaf extracts. After incubation for 30 min, A_{765} was detected. TPC was reported in mg GAE/g extract that was calculated by equation of gallic acid calibration curve y = 0.495X + 0.0141 (R^2 = 0.0996). Each sample was analyzed in triplicate.

Evaluation of in vitro antioxidant capacity

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity: DPPH decolorization assay was used to determine antioxidant capacity of the extract [16]. The reaction mixtures contained 20 µL of various concentration extracts and 180 µL of DPPH solution. The mixtures were incubated in the darkness for 30 min, followed by A_{540} measurement with AccuReader microplate reader (MeterTech Taiwan), comparing with ascorbic acid, as the positive control. The values were represented as 50% DPPH decolorization (IC_{50}). The

ability of DPPH scavenging was calculated as the following:

DPPH inhibition (%) = ($A_{control}$ - A_{test}) / $A_{control}$ x 100:

The antioxidant activities were represented by the IC_{50} value which defined the concentration (µg/mL) of extract at 50% inhibition.

2,2'-azino-bis (3-ethylbenzthiazoline-6- sulphonic acid) (ABTS) radical scavenging activity:

The scavenging activities of free radical cation of the plant extracts was investigated using ABTS assays with a slight modification, according to [17]. Generation of ABTS cation radicals was performed by diluting ABTS in potassium persulfate, followed by keeping this in darkness for 12 h. The $ABTS^{\cdot+}$ solution was further dissolved with dH_2O to obtain A_{734} about 0.70. The working ABTS solution was added to different concentrations of each extract and further kept for 6 min in the darkness. A positive control was a reaction containing ascorbic acid. The ability of radical neutralization was calculated as the following:

ABTS inhibition (%) = ($A_{control}$ - A_{test}) / $A_{control}$ x 100:

The antioxidant activities were represented by the IC_{50} value which defined the concentration (µg/mL) of extract at 50% inhibition.

Ferric reducing capacity: The reducing capacity of the extracts was estimated by ferric reducing power (FRAP) assays [18]. To conduct a reaction, the mixture of 40 µL of the extracts and FRAP reagent were mixed and subsequently kept at 37°C for 4 min, followed by A_{593} measurements. Distilled water was used instead of extracts in a blank reaction. Different concentrations of $FeSO_4 \bullet 7H_2O$ were used to plot a standard curve. Results were exhibited as mg Fe (II) equivalent/1 gram extract.

Cytotoxicity assay: The fibroblast and RAW264.7 cells were acquired from the American Type Culture Collection (ATCC). The DMEM solution containing 10% FBS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B were used for cell growth at 37ºC in 5% CO_2 incubator. Cells were plated in microplate ($5x10^5$ cells per well for fibroblast and $5x10^3$ cells per well for RAW264.7). Cells were treated with 0-200 µg/mL of extract for 24 h. Then, the MTT solution at 5 mg/mL was added and further incubation was carried out at 37ºC for 4 h. DMSO was used dissolving of the formazan crystals. A_{540} was measured. Cell viability was presented as a relative percentage of control.

Measurement of intracellular formation of ROS: Intracellular ROS by H_2O_2-induced was monitored through the assessment of the oxidation of 2',7'-dichlorofluorescin diacetate (DCF-DA) to fluorescent 2',7'-dichlorofluorescein (DCF) [19]. The RAW 264.7 cells growing in black 96-well plates were treated with non-toxic doses of the extracts (0-100 µg/ml) or antioxidant control, 250 µM L-ascorbic acid for 1 h. Cells were then treated with DCFH-DA solution with a final concentration of 40 mM followed by 5 mM H_2O_2 for 30 minutes. The fluorescence was measured using a Cytation5 multi-mode microplate reader and Gen5 software (Agilent, Santa Clara, CA, USA) at an excitation of 480 nm and emission of 525 nm.

Evaluation of inhibitory activity for aging-related enzymes in 96-well plate format

Tyrosinase activity: The tyrosinase inhibitory activity was assessed using a modified Dopachrome method [20]. Kojic acid was determined as a positive control.

100 U/mL mushroom tyrosinase was mixed with the extracts, followed by the addition of L-DOPA. After incubation for 10 min, the reaction was spectrophotometrically measured at 490 nm every 1.5 min for 15 min using a micro-plate reader (Synergy H4 Hybrid, Biotek, Vermont, USA). The % inhibition was quantify using the formula:

Inhibition (%) = [(A-B) / A] x100

Where A was the change in A_{490} without a plant extract, and B was the change in A_{490} with an extract.

Collagenase activity: The collagenase activity was studied using fluorogenic DQ-gelatin assay [21]. Briefly, 100 µg/mL of each extract were mixed with collagenase derived from *Clostridium histolyticum* (1 U/ml) was added in each well (100 µL/well), and then loaded with fluorescein-conjugated gelatin (15 µg/mL) and incubated for 10 min. After that, the fluorescence was measured using a micro-plate reader (Synergy H4 Hybrid, Biotek, Vermont, USA) at A_{485} (excitation wavelength) and A_{528} (emission wavelength) every 2 min for 20 min of reaction. Collagenase activity was analyzed from the slope between time and absorbance over a 2-6 min period.

Metalloproteinase activity: Gelatin zymography was used to assess the metalloproteinase activity assessed [22]. Fibroblast cells were treated with extracts and culture supernatants were loaded in a 10% polyacrylamide gel in the presence of 1% gelatin. Gels were washed with triton-x-100 (2.5%) after electrophoresis. Then, incubate with gelatinase buffer for 24 h. After Coomassie blue staining, gels were washed, and digestive areas relative to gelatinolytic activity were measured using ImageJ software (NIH).

Evaluation of antibacterial activity

Disc diffusion method: The antibacterial activity of the extracts was evaluated using a disc diffusion method. The bacterial culture was adjusted to obtain turbidity equal to that of 0.5 McFarland standard ($1.5x10^8$ cell/mL). The bacterial suspensions were uniformly spread on each appropriated agar medium using sterile cotton swabs. *Propionibacterium acnes* was spread on a brain–heart infusion (BHI) agar medium with 1% glucose and *Staphylococcus aureus* and *Escherichia coli* were spread on Mueller–Hinton (MH) agar plates. Three sterile paper discs impregnated with the extracts at different concentrations (250-1,000 µg/mL, and one disc of a tetracyclin (30 µg)), antimicrobial agents were used as a positive control and were placed on each suitable agar plate. The inoculated plates were incubated at 37ºC for 48-72 h under anaerobic conditions for *P. acnes*. While *S. aureus* and *E. coli* were incubated at 37ºC for 24 h in an aerobic environment. After incubation, the inhibition zone diameter was measured in millimeters using calipers.

Minimum inhibitory concentration (MIC) of the extracts: A broth microdilution method was performed to measure the MIC values of the extracts against microorganisms. The cultures containing 1×10^5 cells/mL of *P. acnes* were conducted in a BHI medium containing 1% glucose, while *S. aureus* and *E. coli* were prepared in a Mueller–Hinton (MH) broth medium. A total of 10 µL of bacterial cells was adjusted to 100 µL by two-fold serially; diluted plant extracts (250 to 1000 µg/mL) were dissolved in appropriated broth. After that, a plate containing *P. acnes* was incubated for 48-72 h at 37ºC

under anaerobic conditions. While a plate containing *S. aureus* and *E. coli* were incubated at 37ºC for 24 h in an aerobic environment. The MIC value was elucidated as the lowest concentration of plant extract, where the microorganisms do not exhibit any visible growth. Triplicates of each extract were used, and the average MIC values were collected.

Statistical analysis: Data was shown as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) for the three independent experiments. The statistical analysis was determined with Prism version 9.0 software using a one-way ANOVA. The significant differences at the level of $p< 0.05$, 0.01, and 0.001 were determined using Turkey's HSD (Honestly Significant Difference) multiple comparison test and Pearson's correlation test.

RESULTS

Extraction yield: Seven herbal plants grown locally in Phayao province were selected for the screening. Their common names and scientific names are shown in Table 1. The herbarium specimen code number was also identified. The percentage yield of the leaf extracts from 100 g of dried materials ranged from 12.5 - 23.8.

Total phenolic content of leaf extracts: As represented in Table 2, the TPC ranged between 48.68–440.91 mg GAE/g per ethanolic leaf extract. It was found that *P. emblica* had the highest phenolic content, followed by *P. granatum*, and *T. bellirica*. These findings showed that all the leaf extracts contained phenolic compounds and, consequently, they were investigated for their antioxidant capacities.

Table 1. Plants and percentage yields of investigated specimens from ethanolic leaf extracts.

Plants name	Bionomial name	Yield (%)	Key chemical constituents
Olive	*Elaeocarpus hygrophilus* Kurz	14.1	Myricitrin [23]
Hairy noni	*Morinda coreia* Buch.-Ham	18.4	Iridoid glucosides, phenolic glycoside [24]
Amla	*Phyllanthus emblica* L.	21.3	Rutin [25]
Pomegranate	*Punica granatum* L.	23.8	Gallic acid, Ellagic acid [26]
Guava	*Psidium guajava* L.	16.1	Quercetin [27]
Beleric myrobalan	*Terminalia bellirica* (Gaertn.) Roxb.	16.9	Ellagitannins, Proanthocyanidins [28]
Myrobalan	*Terminalia chebula* Retz.	12.5	Punicalin, Punicalagin, Terflavins [29]

Plants were collected in northern areas of Thailand. The herbarium specimen code number was identified.

Antioxidant activity of leaf extracts: The IC_{50} values of the extracts against DPPH and ABTS radicals was found to be 3.01–18.98 µg/mL, and 2.2 – 37.25 µg/mL, respectively. Furthermore, results from FRAP assays showed a value between 0.09 – 3.91 mg Fe (II)E/g extract (Table 2). Moreover, to determine the correlation between TPC and antioxidant ability, we constructed a scatter plot of seven leaf extracts and examined the relationship using the Pearson correlation test. Among the antioxidant assays, only the FRAP assay showed a positive correlation ($r = 0.849$) with the phenolic contents as presented in Figure 1. These findings suggested that these extracts could be examined further for anti-aging activities, which relate to antioxidant properties.

Table 2 Total phenolic content and antioxidant activities of the extracts

Traditional plants	TPC	ABTS	DPPH	FRAP
	(mg.GE/g)	(IC_{50}; µg/mL)	(IC_{50}; µg/mL)	(mg.Fe(II)E/g)
E. hygrophilus	347.56 ± 2.27 ^{c}	52.79 ± 1.96 ^{d}	17.40 ± 2.75 ^{d}	2.09 ± 0.02 ^{c}
M. coreia	48.68 ± 1.55 ^{a}	47.65 ± 0.40 ^{c}	3.01 ± 0.06 ^{a}	0.09 ± 0.00 ^{a}
P. emblica	440.91 ± 3.17 ^{f}	5.62 ± 0.16 ^{b}	8.47 ± 0.08 ^{b}	2.65 ± 0.05 ^{d}
P. guajava	310.98 ± 2.94 ^{b}	4.41 ± 0.06 ^{b}	5.54 ± 0.47 ^{a}	1.89 ± 0.01 ^{b}
P. granatum	411.40 ± 4.15 ^{e}	2.47 ± 0.03 ^{a}	7.14 ± 0.22 ^{b}	3.00 ± 0.04 ^{f}
T. bellirica	404.75 ± 3.55 ^{e}	5.05 ± 0.32 ^{b}	16.87 ± 0.03 ^{d}	2.78 ± 0.04 ^{e}
T. chebula	359.32 ± 2.07 ^{d}	5.55 ± 0.09 ^{b}	11.22 ± 0.27 ^{c}	3.91 ± 0.03 ^{g}
Vitamin C	ND	2.3 ± 0.06 ^{a}	4.32 ± 0.08 ^{a}	ND
Trolox	ND	3.0 ± 0.07 ^{a}	6.95 ± 0.19 ^{b}	ND

Data is presented as mean ± SD (n = 3). ND= Not determined. Different letters show a significant difference (p<0.05)

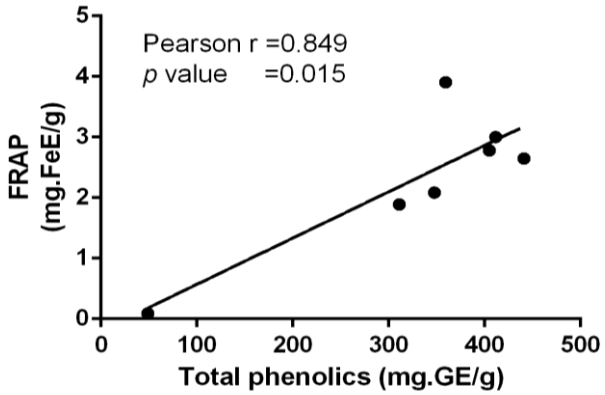


Figure 1. The relationship between TPC and reducing power from FRAP assays of 7 leaf extracts using Pearson's correlation coefficient (r)

Effects of leaf extracts on cell cytotoxicity: The cytotoxicity were assessed using MTT assays. Fibroblast cells and RAW264.7 cells were treated with 0-200 µg/mL of the extracts. The results indicated that the concentration of the extracts up to 200 µg/ml and 100 µg/ml had no toxic effect on the fibroblast cells and RAW264.7 cells, respectively, as presented in Figure 2. Therefore, this concentration could also be used in furthur experiments.

Effects of leaf extracts on H_2O_2-induced ROS production: H_2O_2 induces intracellular ROS production, which leads to skin aging through the upregulation of collagenase, tyrosinase and MMPs [6]. Hence, we set out to determine whether or not the leaf extracts would affect H_2O_2-induced ROS generation in RAW264.7 cells. Our results revealed that the generation of intracellular ROS was decreased by the treatment of the positive

control (vitamin C 250 µM) and some plant extracts (100 µg/mL) (Figure 3). In particular, *T. chebula* and *P. guajava* exhibited the highest inhibitory effect on H_2O_2-induced ROS formation at 15.26±3.19 and 28.36±3.14 % reduction, respectively.

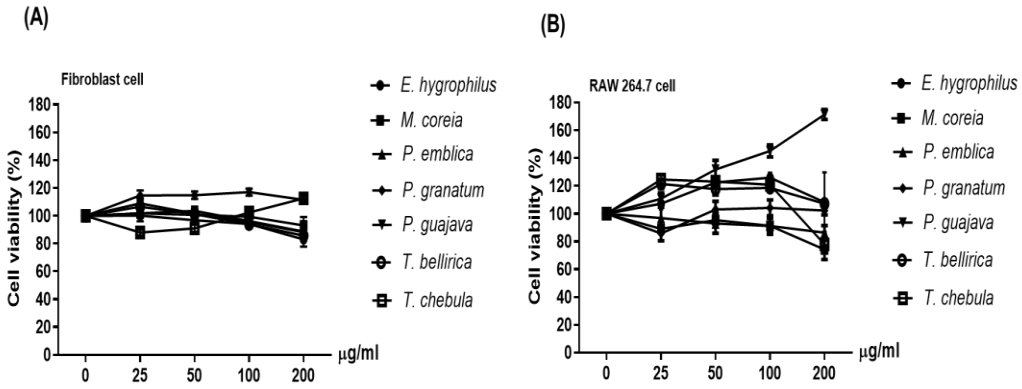


Figure 2. Cytotoxicity test of leaf extracts on (A) Fibroblast cells and (B) RAW264.7 cells. Cells were treated with different concentration of the extracts (25, 50, 100, and 200 µg/mL) for 24 h. Cell viability was examined using an MTT assay and represented as a percentage of untreated control (n=3). Data was shown as mean ± SEM.

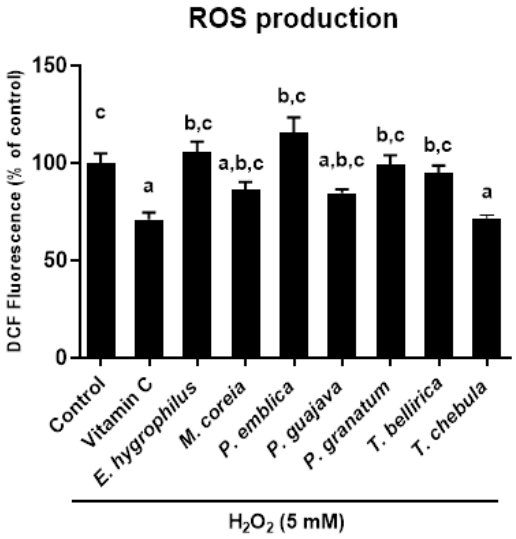


Figure 3: Determination of H_2O_2-induced ROS production by the extracts. The RAW 264.7 cells were treated with 100 µg/mL of leaf extract for 1 hr. Then, cells were added with 40 mM DCFH-DA, followed by 5 mM H_2O_2 and incubated for 30 min. The green fluorescence intensity was measured (n=3). Vitamin C (250 µM) was used as antioxidant control. Data is shown as mean ± SEM of three independent experiments. Statistical significance was considered with $p< 0.05$.

Effects of leaf extracts on tyrosinase activity: To evaluate the anti-aging ability of the leaf extracts, the inhibitory effect of tyrosinase activity, which is involved in the pigmented mechanisms, was investigated. Kojic acid, a natural tyrosinase inhibitor, was used as a positive control. Our results indicated that the 100 µg/mL of the extracts showed inhibitory effects against tyrosinase activity ranging between 12-55% of inhibition, as presented in Figure 4. However, kojic acid accounted for 95% of inhibition, it had a significantly higher inhibitory effect than leaf extracts. The data from this study implies that these leaf extracts can produce

moderate anti-tyrosinase activity and would affect melanin biosynthesis. Importantly, for the current study, M coreia Buch.-Ham shows the lowest anti tyrosinase activity, which means it likely has less anti-aging properties, therefore, we exclude M coreia leaf extract from the anti-aging experiments that followed.

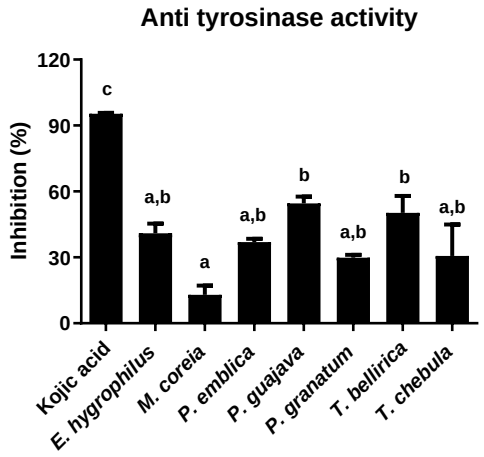


Figure 4: Determination of tyrosinase inhibition of the extracts. 100 µg/mL of the leaf extracts were incubated with tyrosinase, and the percentage of enzyme inhibition was determined using the colorimetric method (n=3). Kojic acid was used as a positive control. Data was shown as mean ± SEM. Different letters show significant differences (p<0.05).

Effects of leaf extracts on collagenase activity: Collagenase is a critical enzyme that participates in collagen degradation in the skin, which leads to the wrinkling process. To evaluate anti-aging potential, we determined the inhibitory effects of 100 µg/mL of leaf extract on collagenase activity. The results revealed that most of the extracts inhibited enzyme activity by more than 80%, while *P. granutum* L. produced only a 60% irate of inhibition (Figure 5). The results indicated a potent inhibitory effect on collagenase activity that was expected to reduce collagen degradation and improve signs of skin aging. According to this investigation, these extracts could aid in limiting collagenase activity during the *in vitro* trial.

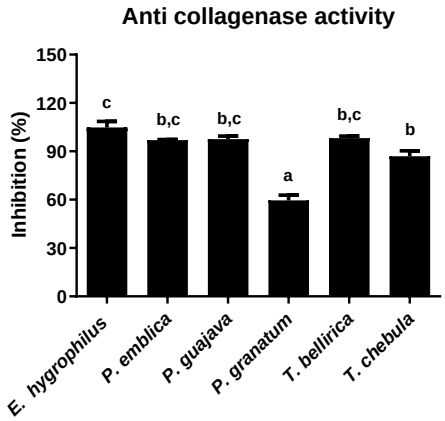


Figure 5 Determination of collagenase inhibition of the extracts. The extracts (100 µg/mL) were incubated with collagenase and the percentage of enzyme inhibition was determined (n=3). Data is shown as mean ± SEM. Different letters show significant differences (p<0.05).

Effects of leaf extracts on MMP secretion: MMPs are extracellular proteases that can degrade various components of the extracellular matrix, especially collagen. This study used gelatin zymography to examine the anti-enzymatic activities of the extracts. The results show that *P. gaujava*, *T. chebula* and *T. bellirica* significantly decreased the digestive band on polyacrylamide gel containing gelatin at 200 µg/mL in comparison with the untreated group as presented in Figure 6. This data suggests that the *P. gaujava, T. chebula,* and *T. bellirica* leaf extracts can inhibit protease secretion (MMPs), which might decrease the destruction of the extracellular matrix in the skin.

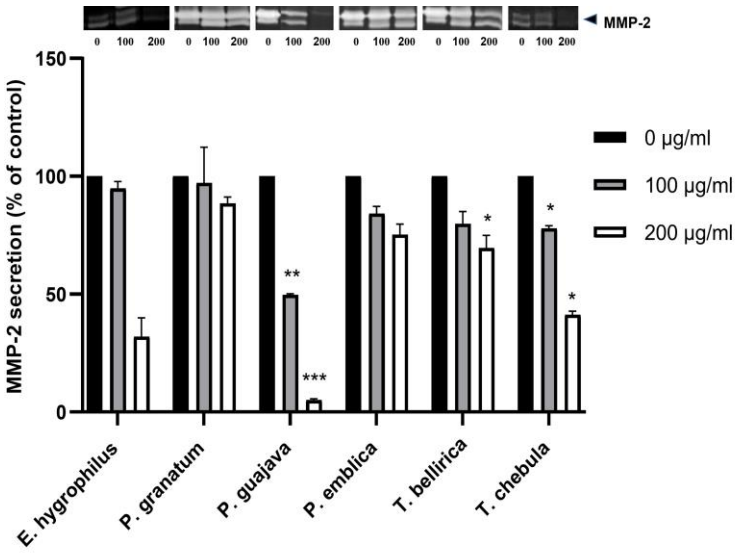


Figure 6. Determination of the inhibition of MMP-2 secretion of the extracts. Fibroblast cells were treated with various concentrations of the extracts. Culture supernatants were used to analyze the MMP-2 secretion by gelatin zymography (n=3). Each value shows the mean ± SD. **p*<0.05, ***p*<0.01 and ****p*<0.001 compared with the control (0 µg/ml).

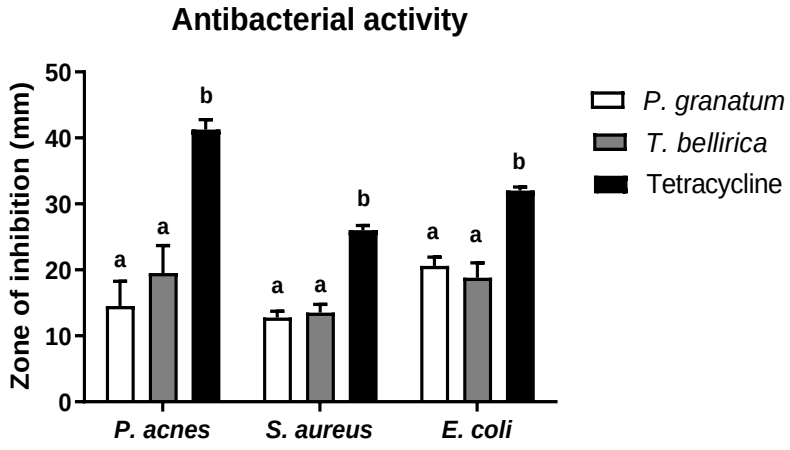


Figure 7: Antibacterial activity of leaf extracts. The inhibition zone of extracts against microorganisms was compared to a positive control tetracycline (n=3). Error bars were indicated as mean ± SEM. Different letters for each bacteria type show significant difference (*p*<0.05).

Effects of leaf extracts on antibacterial activity: The antimicrobial properties of the leaf extracts were determined using the disc diffusion method. We assessed this effect by selecting extracts of *T. bellirica* and *P. granatum*; as described above, these extracts produced the highest TPC, antioxidant activities and were also shown to inhibit MMP activity. The results of this experiment are shown in Figure 7. Notably, 250 ug/mL of *P. granatum* leaf extract produced an inhibition zone diameter in *P. acnes* (14.50±3.79 mm), *S. aureus* (12.75±0.96 mm), and *E. coli* (20.60±1.34 mm). In the same test, *T. bellirica* leaf extract produced 19.50±4.20, 13.50±1.29, and 18.80±2.28 mm, respectively. These two extracts were shown to be effective anti-bacterial agents, despite having produced lower levels of activity from the positive control tetracycline. Moreover, a broth Microdilution method was performed to measure the MIC values of the 2 extracts. The MIC value of *T. bellirica* extract in opposition to test *P. acnes*, *S. aureus*, and *E. coli* was found to be 3.91 mg/ml, 7.82 mg/ml, and 62.5 mg/ml, respectively. While the antimicrobial activity of *P. granatum* extract was observed to have an MIC value of 1.95 mg/ml, 3.91 mg/ml, and 125 mg/ml, respectively. These results suggest that these two extracts can produce anti-bacterial activity.

DISCUSSION

The phenolic compounds from several extracts were examined using colorimetric methods. According to our results, *P. emblica*, *P. granatum*, and *T. bellirica*, were ranked as the highest three (Table 2). *P. emblica* (amla) is widely used in Thai traditional medicine. Although *P. emblica* fruits have been reported as containing a source of phenolic compounds, our results reveal that a high amount of phenolic content can exist in ethanolic *P. emblica* leaf extract [30]. Previous studies have demonstrated that rutin, caffeic acid, gallic acid, ellagic acid, and various secondary metabolites have been detected in *P. emblica* leaf extract, and that they produce antioxidant activities [25,31]. *P. granatum* leaf extract has also been shown to present a high TPC. A quantitative analysis of phenolic compounds demonstrated that gallic and ellagic acids can be high in *P. granatum* leaves [26,32]. In another report, a phenolic profiling of *P. granatum* leaves was more varied, it showed they consist of cyanidin, catechin, luteolin, tyrosol, 5-pentadecylresorcinol, ferulic acid, sesamin, matairesinol, resveratrol, apigenin, rutin, and hydrolysable tannins [33-35]. *T. bellirica*, a deciduous tree, has been said to possess medicinal potential [36]. The corilagin, chebulagic acid, galloylpunicalagin, and digalloyl-hexahydroxydiphenoyl-hexoside belong to ellagitannins and proanthocyanidins that were found to be the major phenolics in *T. bellirica* leaves [28]. An amount of phenolic content is an important characteristic for the selection of cosmeceutical ingredients, because of its bioactivities. We investigated antioxidant properties, anti-tyrosinase, anti-collagenase, anti-MMP, and antibacterial activities to aid in appropriate selections.

Assessment of antioxidant capacity was carried out using a radical scavenging assay. An ABTS radical assay was used to target oxygen radicals for estimating neutralization of ROS, whereas DPPH was used to target nitrogen radicals, especially reactive nitrogen species (RNS) that are well-known to be pro-inflammatory mediators [37,38]. In the ABTS assay, *P. granatum* was shown to exhibit the highest radical neutralizing activities, followed by *P. emblica*, *P. guajava*, *T. bellirica*, and *T. chebula*, which were not significantly different. The result of ROS production was that *T. chebula* exhibited the highest levels of ROS inhibition, which correlated with ABTS results. For the DPPH assay, *M.*

coreia and *P. guajava* showed significantly high activity, followed by *P. granatum* and *P. emblica*. Although antioxidant activities were not correlated with a TPC ranking, our results showed that *P. granatum* and *P. guajava* leaf extracts were potent in the eradication of both ROS and RNS. We were confident that *P. granatum* not only contained a high TPC, but also that it possessed a strong antioxidant capacity equal to vitamin C and Trolox when investigated using an ABTS and DPPH assay. It has been reported by Sreedevi and colleagues that a variety of antioxidant phytochemicals are contained within *P. granatum* leaves [39]. Accumulated evidence has indicated that ethanolic *P. granatum* leaf extract is rich in various active compounds, especially the phenolic acids, flavonoids, alkaloids, and hydrolysable tannins that participate in antioxidation [33-35]. However, we found that *P. guajava* leaf extract showed TPC (310.98 ± 2.94 mg GAE/g) but exhibited strong scavenging properties in opposition to DPPH radicals. *P. guajava* leaves contain seven major flavonoids, including quercetin, kaempferol, hesperetin, quercitrin, rutin, catechin, and apigenin which are responsible for antioxidant properties [40]. Among these flavonoids, quercetin is the most abundant [27]. However, it might be that the synergistic actions of these active compounds can produce a strong neutralization of DPPH radicals obtained from polyphenols with a higher antioxidant power [41].

Evaluation of antioxidant efficiency involved consideration of radical scavenging, as well as a focus on the reduction potential that was shown by conducting FRAP assays. Our results showed that *T. chebula*, *P. granatum*, and *T. bellirica* were the top three in terms of high reduction potential (Table 2). Chemically, the reduction potential is obtained from a part of the molecule, which easily donates an electron, especially the hydrogen atom. Polyphenols, including phenolic acids, flavonoids, stilbenes, and lignans, have several hydrogen atoms. However, molecular stability is still a limitation for mechanisms of antioxidant action. *T. chebula* was shown to have the highest reduction power, meanwhile the TPC was 359.32 ± 2.07mg GAE/g, assuming an activity from other non-polyphenols. Previously, Tarig and Reyaz (2012) [42] demonstrated that the hydroalcohol extract of *T. chebula* contained a high content of reducing sugar up to 7.12mg/g dry weight. It is possible that the highest reduction potential of *T. chebula* in our study might be a result of reducing sugar. Interestingly, *P. granatum* leaf extract still ranked in the top 3 for high reduction potential. These results demonstrate that *P. granatum* leaf extract has a high TPC and a high antioxidant efficiency.

Skin aging is mostly characterized by progressive increase in pigmentation, extensibility, and reduction in elasticity. Aging skin undergoes progressive structural and functional degeneration by aging-related enzymes, especially tyrosinase, collagenase, and MMPs [8,43]. Our results revealed that *P. guajava*, *T. bellirica*, and *E. hygrophilus* leaf extracts predominantly inhibited tyrosinase and collagenase activities as did *P. emblica* (Figure 4 and 5). However, *P. granatum* exhibited low inhibitory activity. Investigation by gelatin zymography exhibited an inhibitory MMP-2 activity of *P. guajava*, *T. bellirica*, and *T. chebula* (Figure 6). Thirty types of flavonoids have been detected in *P. guajava* leaf, and these flavonoids express both anti-tyrosinase and anti-collagenase activities [44-46]. It is possible that strong antiprotease activities and MMP secretion mainly adopt the synergistic effects of various flavonoids. However, *P. guajava* leaf extract increased the cell viability of RAW264.7, and that might promote cell proliferation and trigger an immune response, leading to an allergic reaction. We suggest that these might be a limitation of *P. guajava* leaf extract, and it was denied for

antibacterial activities. *T. bellirica* leaf extracts showed a high TPC (404.75 ± 3.55 mg.GE/g) that was confirmed by Galav and colleagues (2019) [47]. Phytochemical profiling illustrated 50 identified compounds in *T. bellirica* leaves. Therefore, a variety of phenolic compounds results in the inhibition of tyrosinase collagenase activities, and MMP secretion. The chemical profile of EtOH extract of *E. hygrophilus* leaves has been shown to have 26 identified compounds that mainly belong to flavonoids and flavonol glycoside, with myricitrin being the major component. Dimethyl phthalate, a hazardous substance, was unfortunately detected in *E. hygrophilus* leaves [23]. We suggest that *E. hygrophilus* leaf extract is unsafe. Although our results found that *T. chebula* produced the highest inhibition of ROS production and could inhibit MMP activity, its low anti-tyrosinase activity was a limitation for its selection as an extract for evaluating antibacterial activities. However, the results showed no correlations between antioxidant capacity and antiprotease activities that could be observed from activities of *E. hygrophilus* leaf extract. We speculated that some phenolic compounds might possess a low antioxidant activity level, but that they are strong in enzyme inhibition.

According to the amount of TPC and antiprotease activities, *P. granatum* and *T. bellirica* leaf extracts were chosen to evaluate their antibacterial activities, when compared with tetracycline. They were shown to inhibit the growth of *P. acnes*, *S. aureus*, and *E. coli*. (Figure 7). Flavonoids and tannins are known to possess antibacterial compounds through their ability to bind to molecular structures like proteins or glycoproteins [48-50]. It has been reported that various flavonoids and tannins are found to be rich in *P. granatum* [26,51]. However, gallic and ellagic acids, major phytoconstituents of leaves, reportedly work as antibacterial agents [52]. Previous evidence indicated in vitro antibacterial activity of *T. bellirica* fruit extracts [53]. However, we found that leaves showed inhibitory effects on *P. acnes*, *S. aureus*, and *E. coli*. Ellagitannins such as corilagin, chebulagic acid, and galloylpunicalagin are a major phytoconstituent in *T. bellirica* leaves, and various flavonoid glycosides [28] that have been reported to have antimicrobial activities [54,55]. Cosmetic products require long-term preservation against microbial contamination to guarantee consumer safety and to increase their shelf-life. Currently, these polyphenols are accepted as active ingredients for cosmetic products [56]. Moreover, functional foods from FFC remained that it is natural or processed foods that consist of active compounds [57] which are non-toxic, exhibit the bioactivities, improve health, and provide a clinically proven and documented health benefit [58-59]. Hence, our extracts can act as functional food for skin. Therefore, we suggest that *P. granatum* and *T. bellirica* leaf extracts may possibly be applied as functional foods and cosmetic ingredients.

CONCLUSIONS

The value of phytochemicals is determined by bioactivities, especially antioxidation, antiprotease, and antimicrobial. This study found that *P. granatum* leaf extract predominantly exhibited antioxidant activities. *T. bellirica* leaf extract was potent in its ability to inhibit tyrosinase, collagenase, and MMP activities. Moreover, antibacterial activities were detected in both extracts. Therefore, we suggest that *P. granatum* and *T. bellirica* leaf extracts are attentive as active ingredients for the development of functional foods and cosmetic products and that their mechanisms should be investigated in further studies.

Abbreviations: ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), DCF-DA: 2',7'-dichlorofluorescin diacetate, DPPH: 2,2-diphenyl-2-picrylhydrazyl hydrate, FRAP: ferric reducing/antioxidant power, MIC: minimum inhibitory concentration, MMPs: matrix metalloproteinases, MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide, ROS: reactive oxygen species, TPC: total phenolic content.

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