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Contributed Paper

In vitro Anti-aging Activities of *Centotheca lappacea* (L) desv. (Ya Repair) Extract

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ABSTRACT

Centotheca lappacea extracts were prepared by maceration with 95% (v/v) ethanol or decoction with distilled water. The extracts were tested for *in-vitro* anti-aging activities including DPPH radical scavenging, metal ion chelating, lipid peroxidation inhibition, tyrosinase inhibition, collagenase inhibition, gelatinolytic activity (MMP-2 inhibition activity) on human skin fibroblasts and estrogenic effect in human breast cancer cell line (MCF-7). The ethanolic extract exhibited better activities than the aqueous extract on DPPH scavenging, metal chelating and lipid peroxidation inhibition with the SC_{50}, CC_{50} and IPC_{50} values of 1.39±0.50, 366.95±13.47 and 0.99±0.26 mg/ml, respectively, as well as mild tyrosinase inhibition activity (IC_{50} value of 3,756.71±169.14 mg/ml). This ethanolic extract also gave strong collagenase inhibition with the IC_{50} value of 1.26±1.01 mg/ml which was close to ascorbic acid (IC_{50} value of 1.24±0.25 mg/ml). For the MMP-2 inhibition, the extract gave the percentage of pro MMP-2 inhibition of 21.49±2.60% which was almost the same as that of ascorbic acid (24.19±1.60%). The extract at the concentration of 1000 μg/ml also showed estrogenic activity by increasing the MCF-7 cell proliferation of 107.05±17.36% of the control. The results from this study have suggested the development potential as an anti-aging product of the ethanolic extract from *C. lappacea* and the confirmation of the traditional use of *C. lappacea* for rejuvenile in women after laboring.

Keywords: *Centotheca lappacea*, antioxidation, tyrosinase inhibition, cytotoxicity, MMP-2, estrogenic activity, anti-aging

1. INTRODUCTION

In general, aged skin shows a decrease in epidermal thickness, with a flattening of the dermal-epidermal junction. In fact, dermis decreases in thickness by about 1% per year throughout adult life [1]. For the skin condition of post-menopausal women, the decrease of

collagen (of about 2% per year) [2], and the reduction of other matrix and cellular components of the skin have been reported [3]. Estrogen insufficiency in the menopause women can result in the decrease of the defense against the oxidative stress, causing the skin becoming thinner with less collagen, decreasing elasticity, increasing wrinkle and dryness as well as the reduction of vascularity resulting in atrophic skin changes and acceleration of skin aging [4]. After parturition, the fast losing weight from laboring can cause skin around the belly to be ended up with saggy and wrinkled as well as wider, dryness and soreness of the vagina. However, plants which are used in women's health after parturition have not been previously reported. Nowadays, trends of anti-aging market value are increasing continuously. Alternative medicines, especially herbs from folklore wisdoms appear to be more interesting than drugs or hormones which usually cause side effects. Recently, phytocosmetics have been popularly investigated. Several Thai medicinal plants have been claimed for anti-aging activity, such as White Kaow Krua (*Pueraria mirifica* Airy Shaw et Suvatab) and Asiatic Pennywort (*Centella asiatica* L.). *P. mirifica* consisting of various active phytoestrogens has been reported to have antioxidative capacity and strong estrogenic activity in human breast cancer cell line (MCF-7) [5]. Yingngam et al. have reported that several Thai plants exhibited estrogen-like effect in MCF-7 cells including *Pueraria mirifica*, *Linum usitatissimum*, *Glycine max*, *Curcuma aeruginosa*, *Cissus quadrangularis*, *Tadehagi godefroyanum*, *Curcuma comosa*, *Butea superba*, *Trigonella foenum-graecum* and *Punica granatum* [6]. These extracts were candidates as potential active ingredients for anti-skin aging in post-menopausal women.

Centotheca lappacea, a grass known in Thai as "Ya Hee Yoom" or "Ya Repair" has long been used in Thailand and many Asian countries to treat women at post labour, as it is believed to heal wounds and tears on the labia, and tighten the respective muscles as well as for rejuvenile and anti-aging. The traditional usage is from the smoke of the burning grass both in the dry or fresh plant with wood fuel. The postpartum women, wearing a Thai traditional dress called "Sarong" will stand over the pile of burning wood with the grass to be smoked on the treated area. Another way to use is by boiling the grass to extract its essence in the form of juice for drinking. However, no work has been reported on the *in vitro* anti-aging activity of the extract from this grass. Therefore, the aim of this study was to investigate the *in vitro* anti-aging potential of *C. lappacea* including antioxidative, tyrosinase inhibition, collagenase inhibition and gelatinolytic activity on MMP-2 in human skin fibroblasts and estrogenic effect in MCF-7 cell.

2. MATERIALS AND METHODS

2.1 Materials

Vitamin C (l-(+)-ascorbic acid, 99.5%), 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), ethylenediaminetetraacetic acid (EDTA), kojic acid (99.0%), ammonium thiocyanate (NH_4SCN), ferrozine and ferric chloride ($FeCl_2$), acrylamide (minimum 99%), glycerol, sulforhodamine B, β-estradiol, collagenase type V and Pz-peptide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulfate and glycine from BDH Limited Poole, England and trifluoroacetic acid and sodium chloride from Merck, Germany were used. Mushroom tyrosinase (4276 U/mg), l-tyrosine, N, N'-methylenebisacrylamide, TEMED (N, N, N', N'-tetramethyl ethylenediamine) and linoleic acid (99.0%) were from Fluka (Switzerland). Dulbecco's modified Eagle's culture medium (DMEM), antibiotics

(penicillin and streptomycin), fetal bovine serum (FBS) and trypsin were obtained from HyClone (Logan, UT, USA). Tris (hydroxymethyl) methylamine was purchased from Fisher Scientific UK Limited, UK, sodium dodecyl sulfate and coomassie brilliant blue R-250 from Bio-Rad Laboratories, UK, and bromophenol blue dye and ammonium persulfate from Amersco Inc., USA were used. All other chemicals and reagents were of analytical grade.

2.2 Preparation of the *C. lappacea* Extracts

The whole plant of *C. lappacea* was collected from Chaophraya Aphai Phu Bet Hospital in Prachin Buri and Suan Pa Samun Phrai Foundation, Phra Nakhon Si Ayutthaya in Thailand during 2014-2015, December to January and June to July. They were chopped into small pieces. For ethanol extraction, the plant was macerated with 95% (v/v) ethanol (1:10 w/v) at room temperature (27±2 °C) for 72 h. For aqueous extraction, the plant was boiled with distilled water (1:10 w/v) at 100±2 °C for 2 h. The extract was filtered through the Whatman no.1 filter paper connected with a vacuum pump. The residues were re-extracted by the same process for 2 times. All filtrates were collected, pooled and dried by a rotary evaporator. The percentage yields were calculated on the dry weight basis. The dried extracts were kept in an amber vial until use at 4 °C.

% yield = (weight of the extract/weight of the dry plant) × 100

2.3 Determination of the Total Phenolic Content of the Extracts

The total phenolic content of the extracts was determined by the modified Folin-Ciocalteu assay [7]. Briefly, 300 μl of the solution (extract dissolved in distilled water or ethanol at the concentration of 10 mg/ml) were mixed with 2.25 ml of Folin-Ciocalteu reagent. Then, 2.25 ml of sodium carbonate solution were added and mixed. The absorbance was measured at 725 nm. The total phenolic contents were quantified by the calibration standard curve obtained from various known concentrations of gallic acid. The concentrations were expressed as mg of Gallic Acid Equivalents (GAE)/g of the dried extracts.

2.4 Antioxidant Activities

2.4.1 DPPH radical scavenging assay

Free radical scavenging activity of *C. lappacea* extracts was determined by a modified DPPH assay [8]. Briefly, 50 μl of five serial concentrations of the extracts (0.001-10 mg/ml) dissolved in distilled water or ethanol, 50 μl of DPPH in ethanol solution were put in each well of a 96-well microplate (Nalge Nunc International, NY). The reaction mixtures were allowed to stand for 30 min at room temperature (30±2 °C), and the absorbance was measured at 515 nm by a microplate reader (Model 680, Bio-Rad Laboratories Ltd., Corston, UK) against the negative control (solution without the sample). Ascorbic acid (0.001-10 mg/ml) was used as a positive control. The experiments were done in triplicate. The free radical scavenging percentages were calculated as the following [8]: Scavenging (%) = $[(A-B)/A]\times 100$, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% of the activity (SC_{50}) was calculated from the graph plotted between the percentages of the scavenging activity and the sample concentrations.

2.4.2 Lipid peroxidation inhibition activity

The lipid peroxidation activity of

C. lappacea extracts was assayed by the modified ferric-thiocyanate method [9]. Fifty µl of five serial concentrations of the extracts (0.001-10 mg/ml) dissolved in distilled water or ethanol, added to 50 µl of linoleic acid in 50% (v/v) DMSO. The reaction was initiated by adding 50 µl of NH_4SCN (5 mM) and 50 µl of $FeCl_2$ (2 mM). The mixture was incubated at 37±2 °C in a 96-well microplate for 1 h and the absorbance was measured using a microplate reader at 490 nm. The solution without the sample was used as a negative control. Ascorbic acid (0.001-10 mg/ml) was used as a positive control. All determinations were performed in triplicate. The inhibition percentages of lipid peroxidation of linoleic acid were calculated by the following equation [9]: Inhibition of lipid peroxidation (%) = $[(A-B)/A]\times 100$, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% inhibition of lipid peroxidation (IPC_{50}) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

2.4.3 Metal ion chelating assay

The metal ion chelating activity of *C. lappacea* extracts was assayed by the modified ferrous ion chelating method [10]. Briefly, 100 µl of five serial concentrations of the extracts (0.01-10 mg/ml) dissolved in distilled water or ethanol were added to the solution of 2 mM $FeCl_2$ (50 µl) in distilled water. The reaction was initiated by adding 5 mM ferrozine (50 µl) and the total volume was adjusted to 300 µl by distilled water. Then, the mixture was left at room temperature (30±2 °C) for 15 min. Absorbance of the resulting solution was then measured at 570 nm by a microplate reader. EDTA (0.001-10 mg/ml) was used as a positive control. The negative control contained $FeCl_2$ and ferrozine which was the complex formation molecules. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe^{2+} complex formation were calculated by the following equation [10]: Metal chelating activity (%) = $[(A-B)/A]\times 100$, where A was the absorbance of the negative control and B was the absorbance of the sample. The sample concentration providing 50% metal chelating activity (CC_{50}) was calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

2.5 Tyrosinase Inhibition Assay

The tyrosinase inhibition activity of the *C. lappacea* extract was assayed by the modified dopachrome method using tyrosine as a substrate [11]. Briefly, 50 µl of five serial concentrations of the extracts (0.001-10 mg/ml) dissolved in ethanol, 50 µl of 100 units mushroom tyrosinase solution in 0.1 M phosphate buffer, 50 µl of 1 mg/ml tyrosine solution in 0.1 M phosphate buffer, and 50 µl of 0.1 M phosphate buffer were added into each well of a 96-well plate. The mixture was incubated at 37±2 °C for 60 min and the absorbance at 450 nm was measured. The absorbance was measured before and after incubation. Kojic acid (0.001-10 mg/ml) was used as a positive control. The solution without the extracts was used as a negative control. All experiments were performed in triplicate. The percentages of tyrosinase inhibition were calculated according to the following equation [11]: Tyrosinase inhibition activity (%) = $[(A-B)-(C-D)/(A-B)]\times 100$, where A was the absorbance of the blank after incubation, B was the absorbance of the blank before incubation, C was the absorbance of the sample after incubation, and D was the absorbance of the sample

before incubation. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted between the percentages of tyrosinase inhibition activity and the sample concentrations.

2.6 Collagenase Inhibition Activity

The collagenase activity of the *C. lappacea* extract was determined by the procedure previously described [12]. Briefly, collagenase (5 μg) and Pz-peptide (0.5 mg) in 0.1 M Tris buffer (pH 7.1) containing 20 mM $CaCl_2$ in five serial concentrations of the extract (0.001-10 mg/ml) were mixed and incubated at 37±2 °C for 30 min, and 25 mM citric acid solution was added to terminate the reaction. Thereafter, ethyl acetate was immediately added, vortexed and centrifuged. The absorbance of the organic layer was measured at 320 nm. Ascorbic acid (0.001-10 mg/ml) was used as a positive control. The solution without the sample was used as a negative control. The experiments were done in triplicate. The percentages of collagenase inhibition were calculated according to the following equation [12]: % collagenase inhibition activity = $[(A-B)-(C-D)/(A-B)]\times 100$, where A was the absorbance of the control with collagenase, B was the absorbance of the control without collagenase, C was the absorbance of the sample with collagenase, and D was the absorbance of the sample without collagenase. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted between the percentages of collagenase inhibition activity and the sample concentrations.

2.7 Cytotoxicity in Human Skin Fibroblasts

Human skin fibroblasts at the 4th passage from American Type Culture Collection, USA were used. The cells were seeded in 96-well plates at an amount of 1×10^4 cells/well and allowed to attach overnight. Then, the cells were exposed to various concentrations (0.1-1000 μg/ml) of the extract for 24 h. After incubation, the adherent cells were fixed by adding cold 50%w/v trichloroacetic acid and further incubated for 1 h at 4 °C. Then, the cells were rinsed with distilled water, air-dried and stained with 0.4%SRB in 1%glacial acetic acid for 30 min at room temperature (27±2 °C). The unbound SRB was removed by washing with 1% glacial acetic acid solution for four times. After air-drying, 100 μl per well of 10 mM Tris base were added to dissolve the bound stain. After mixing, the absorbance was measured at 540 nm with a microplate reader (Biorad, Milan, Italy). The untreated cells were used as a negative control. Cell viability (%) was calculated by the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{treated cells}} / \text{Absorbance}_{\text{untreated cells}}) \times 100$$

2.8 Gelatinolytic Activity on MMP-2 Inhibition Zymography

Human skin fibroblasts at the 4th passage were seeded in 6-well plates at an amount of 5×10^5 cells/well. The monolayer of the cells maintained in the culture medium without FBS for 24 h, was treated with the extract and incubated for 48 h. The culture supernatants were collected to assess for the gelanolytic activities of MMP-2 in the culture media. The SDS-PAGE zymography using gelatin as a substrate was performed. Briefly, 20 μl of the cell culture supernatant were suspended in the loading buffer (0.125M Tris (pH 6.8), 4% SDS and 0.04% bromophenol blue), run on the 10% SDS polyacrylamide gel containing gelatin (1 mg/ml). After electrophoresis, gels were washed to remove SDS and incubated for 20 min in

the renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 2.5% Triton X-100). The gels were then incubated for 24 h at 27 °C in the developing buffer [50 mM Tris (pH 7.5), 5 mM $CaCl_2$, 0.02% NaN_3 and 1%Triton X-100]. Gels were subsequently stained with 0.5%Coomassie brilliant blue G-250 and de-stained in 30% methanol and 10% acetic acid (v/v) to detect gelatinolytic activity. The gel was documented by a gel documentation system (Bio-Rad Laboratories, UK) and analyzed by the Quantity 1-D analysis software. The area multiplied by intensity (mm^2) of the bands on the gel was determined as the relative MMP-2 content. The percentages of MMP-2 inhibition in comparing to the control (the untreated systems) were calculated using the following equation [13]:

MMP-2 inhibition (%)=100 - [(MMP-2 content of the sample/MMP-2 content of the control) × 100]

The assays were done in three independent separate experiments. The potency of MMP-2 inhibition of the extracts was compared with the positive control (ascorbic acid at 100 μg/ml).

2.9 Estrogenic Activity by the MCF-7 Cell Proliferation Assay

Human breast cancer cell line (MCF-7) was obtained from American Type Culture Collection (ATCC HTB-22), USA. The extract was investigated for anti-proliferative activity by the SRB assay as previous described in section 2.7. β-estradiol was used as a positive control [14]. The percentages of cell growth (G) were determined by the following equation:

$$G\ (\%) = (T_{treat} - T_0/C - T_0) \times 100$$

where T_{treat} was the absorbance of the treated plate, T_0 was the absorbance of the reference plate (the incubated plate at the first day before the treatment) and C was the absorbance of the control plate.

2.10 Statistical Analysis

Data were expressed as mean ± SD. Statistical analysis was carried out using the ANOVA using the software SPSS 13.0 for Windows and p at less than 0.05 was considered as statistical significance.

3. RESULTS AND DISCUSSION

3.1 Percentage Yields and Total Phenolic Contents

The percentage yields of the ethanolic and aqueous extracts from *C. lappacea* were 1.52±0.13 and 1.10±0.05%, respectively. The different yields were obtained because of the different polarity between ethanol and water which can extract and dissolve differently the compound containing in the plant [15]. Phenolic compounds are among the most widely occurring secondary metabolites in the plant kingdom including simple phenols, phenolic acids (benzoic, gallic acid and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins [16]. Folin-Ciocalteu which is also called the gallic acid equivalence method, has been used to determine the total phenolic content in this study. Thus, gallic acid was used as a standard and the total phenolic contents containing in the extract was determined. The aqueous extract (43.26 mg GAE/g dried extract) gave higher total phenolic content than the ethanolic extract (39.22 mg GAE/g dried extract) of 1.10 times. This may be due to the higher polarity of water which can extract the phenolic content more than the lower polarity of ethanol. The aqueous extracts of *C. lappacea* contained several

types of phenolic compounds including catechin, catechin gallate, rosemarinic acid, epigallocatechin gallate and gallic acid [17].

3.2 Antioxidant Activities

The antioxidant activities of the *C. lappacea* extracts were shown in Table 1. The ethanolic extract exhibited DPPH scavenging, metal chelating and lipid peroxidation inhibition activities with the SC_{50}, CC_{50} and IPC_{50} values of 1.39±0.50, 366.95±13.47 and 0.99±0.26 mg/ml, respectively. The ethanolic extract gave higher antioxidant activities than the aqueous extract. This may be due to the different polarity of the extracted solvent that is contributed to different phytochemical compositions of the extracts, resulting in different biological activities. Ethanol which is a moderate polar solvent (polarity index = 5.2) may give more abundant phytochemical substances than water which is a high polar solvent (polarity index = 9) [18]. However, antioxidant activities of the ethanolic extract were lower than ascorbic acid (SC_{50} value of 0.05±0.01 mg/ml), EDTA (CC_{50} value of 0.01±0.001 mg/ml) and ascorbic acid (IPC_{50} value of 0.18±0.03 mg/ml) of about 27.8, 36,695 and 5.5 times, respectively.

Table 1. Antioxidative activities of the *C. lappacea* extracts and standards.

Samples	SC_{50} of DPPH radical scavenging activity (mg/ml)	CC_{50} of chelating activity (mg/ml)	IPC_{50} of lipid peroxidation inhibition activity (mg/ml)
Ethanolic extract	1.39±0.50	366.95±13.47	0.99±0.26
Aqueous extract	2.17±0.31	> 1000	1.06±0.15
Standards			
Ascorbic acid	0.05±0.01	ND	0.18±0.03
EDTA	ND	0.01±0.001	ND
Kojic acid	ND	ND	ND

Note: ND represented not determined. SC_{50} value (mg/ml) was the concentration of the sample that scavenged 50% of the DPPH radicals. IPC_{50} value (mg/ml) was the concentration of the sample that inhibited 50% of the lipid peroxidation. CC_{50} value (mg/ml) was the concentration of the sample that chelated 50% of the metal ion.

C. lappacea belongs to grasses in Poaceae (Gramineae) family in which many important useful plants such as wheat, rice, maize, sorghum, millet, barley and rye are in this family. As previously reported, species in this family contain bioactive components including flavonoids (such as C-glycosides of apigenin, luteolin and tricin), phenolic acids (such as ferulic acid, caffeic acid and p-hydroxybenzoic acid), and triterpenes, saponins and sterols [19]. HariBabu and Savithramma have demonstrated that the major compounds of the grass species were phenolic and flavonoid compounds [20]. Also, some grass species have been reported to have strong antioxidant properties. Corn (*Zea mays*), wheat (*Triticum aestivum)*, oats (*Avena sativa)* and rice (*Oryza sativa*) gave the antioxidant activity of 181.42±0.86, 76.70±1.38, 74.67±1.49 and 55.77±1.62 μmol of vitamin C equiv/g of grain), respectively [21]. Thus, the antioxidant activities of the

C. lappacea extract might be from the phenolic compounds that possess phenol groups which have sufficient antioxidant activity to protect oxidative stress [22]. The antioxidant activities of the extracts were not only from the phenolic compounds (TPC), but also from other compounds such as flavonoids, fatty acids, triterpenes and phytosterols [23].

The ethanolic extract showed higher antioxidant activities than the aqueous extract. Hence, the ethanolic extract was selected to further investigate for other activities.

3.3 Tyrosinase Inhibition Activity

The *C. lappacea* ethanolic extract exhibited mild tyrosinase inhibition activity at the IC_{50} value of 3,756.71±169.14 mg/ml with lower activity than kojic acid (IC_{50} value of 0.025±0.005 mg/ml). In fact, tyrosinase inhibition activity of the *C. lappacea* extract has never been previously reported. This activity might be from the hydroxyl groups of the phenolic compounds existing in the extract that could bind to the active site of the tyrosinase enzyme, leading to the inhibition of enzymatic activity. Some tyrosinase inhibitors can act through the binding to the active site of the enzyme, resulting in the inhibition of the enzyme activity by the steric hindrance or conformation change. Also, antioxidant activity may be one of the important mechanisms for tyrosinase inhibitory activity. The metal chelating activity may be related to the tyrosinase enzyme inhibition activity, since tyrosinase is a metalloenzyme with two copper ions in the active site. Thus, the tyrosinase inhibitory activity of antioxidants is related to its ability to bind these two metal ions [24]. Other mechanisms might be from the aromatic aldehyde, aromatic acid or polyphenol of the active constituents since these compounds consist of hydrophobic parts that could act as the competitive inhibitors in melanin synthesis [25].

3.4 Collagenase Inhibition Activity

The *C. lappacea* ethanolic extract showed the dose-dependent inhibition of collagenase. The extract gave collagenase inhibition activity at the IC_{50} value of 1.26±1.01 mg/ml which was close to ascorbic acid (IC_{50} value of 1.24±0.25 mg/ml). It has been reported that collagenase cleaves the amino acid bond of collagen and breaks down the collagen and elastin which possibly leads to the prolonged skin damage or wrinkle formation [26]. This collagenase inhibition might be from the metal chelating activity of the extract. The previous study has reported that collagenase can be inhibited by metal chelating agents such as cysteine, EDTA or o-phenanthroline [27]. Hence, as collagenase is a zinc-containing metalloproteinase, phenolic compounds in the extract which are also known to be metal chelator compounds, may bind to Zn^{2+} ion in the enzyme, and prevent it from binding with the substrate. Many plant phenolic compounds have been reported to inhibit the collagenase activity. The high collagenase inhibition activity of the *C. lappacea* extract may be from the conformational change in collagenase induced by the synergistic effect of polyphenols. Madhan et al. have demonstrated that the conformational change in collagenase induced by green tea polyphenols is responsible for the inhibition of *Clostridium histolyticum* collagenase I. This was due to not only the bindng of the hydroxyl part of polyphenols to the functional groups of collagenase, but also the benzene group of the polyphenols can be involved in the hydrophobic interactions with collagenase as well [28].

3.5 Cytotoxicity of the Extract on Human Skin Fibroblasts

The cell viability percentages of human skin fibroblasts treated with the *C. lappacea* ethanolic extract were shown in Table 2. The extract at the concentration range of 0.1-1000 μg/ml gave no cytotoxicity with the cell viability of more than 90%. When, the concentrations of ascorbic acid increased from the range of 0.1 to 100 μg/ml, the cell viability was increased. The extract at 1000 μg/ml that exhibited the lowest cytotoxicity with the cell viability of 93.22±4.34% was observed. Thus, this concentration of the extract was selected for the study of MMP-2 inhibition on human skin fibroblasts.

Table 2. Viability percentages of human skin fibroblasts treated with the *C. lappacea* ethanolic extract.

Samples / concentration (μg/ml)	Viability percentages of human skin fibroblasts (% of control)				
	0.1	1	10	100	1000
C. lappacea ethanolic extract	92.44±4.75	92.33±4.95	95.29±4.95	94.43±6.55	93.22±4.34
Ascorbic acid	101.25±4.05	103.29±1.61	107.38±5.91	109.59±6.68	ND

Note: ND represented not determined.

3.6 Gelatinolytic Activity

Figure 1 presented the gelatinolytic activity of MMP-2 inhibition on the 4th passage human skin fibroblasts of the *C. lappacea* ethanolic extract at 1000 μg/ml compared with the positive control (ascorbic acid at 100 μg/ml). The cells treated with the extract indicated the pro MMP-2 inhibition of 21.49±2.60%, and the active MMP-2 inhibition of 100±0.00%. However, MMP-2 inhibition of the extract was almost the same as that of ascorbic acid (pro and active MMP-2 inhibition = 24.19±1.60 and 100±0.00%, respectively). One possible MMP-2 inhibition mechanism of the extract is the antioxidant activity. It has been reported that the exogenous hydrogen peroxide and endogenous ROS can induce MMP expression in the endothelial cells, cardiac fibroblasts, macrophages and breast cancer cells [29]. Since MMPs are upregulated by the increased formation of the reactive oxygen species (ROS), antioxidant approaches can thus decrease the MMP-2 upregulation [30]. The inhibition of MMP-2 expression of the extract appeared to relate to its DPPH radical scavenging activity. The extracts from Hemp (*Cannabis sativa* L.) leaves which composed of polar compounds [31] with numerous double bonds and hydroxyl groups have been reported to donate the electrons through resonance to stabilize the free radicals [32]. In addition, ROS suppression is a by pass to inhibit MMP-2 activation. Therefore, the extract from the whole plant (including leaves of *C. lappacea*) may inhibit the MMP-2 synthesis and secretion steps or interrupt the activation processes by converting the latent form of MMP-2 (pro MMP-2) to an active form (active MMP-2), resulting in the decrease of the area and intensity of the active MMP-2 on the zymogram. Thus, antioxidant activities including DPPH radical scavenging, metal ion chelating and lipid peroxidation inhibition may be the possible mechanisms responsible for this MMP-2 expression inhibition.

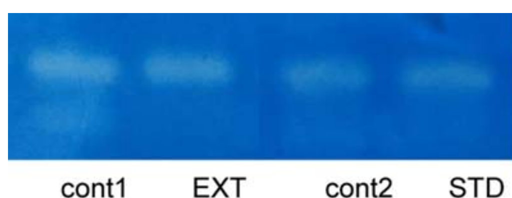

Figure 1 Zymograms of MMP-2 inhibition on human skin fibroblasts of the *C. lappacea* ethanolic extract at 1000 μg/ml and ascorbic acid at 100 μg/ml (positive control), cont1 = 10% v/v ethanol in DMEM medium, cont2 = DMEM medium, EXT = *C. lappacea* ethanolic extract and STD = ascorbic acid.

3.7 Estrogenic Activity

Natural estrogens promote cell proliferation and hypertrophy of female secondary sex organs. Currently, the assay methods using cells that are responded to estrogen are widely used in several studies [33-35]. Both proliferation and differentiation of ovarian and mammalian cells responding to particular hormones are often used in endocrine disruption assay. Also, the established human breast cancer cell lines, such as MCF-7 have been used to study for mechanism of action of exogenous estrogens and antiestrogens on cell proliferation. Thus, estrogenic effect can be related to MCF-7 cell proliferation.

The proliferation percentages of MCF-7 cells treated with the *C. lappacea* ethanolic extract were shown in Table 3. The concentration-dependent manner was observed. The extract showed proliferation in MCF-7 cell at 1000 μg/ml of 107.05± 17.36% of the control, while β-estradiol exhibited cell proliferation at 1 and 10 μg/ml of 115.34±17.88 and 121.08± 9.83% of the control, respectively. However, β-estradiol gave cytotoxic effects at high concentrations (100 and 1000 μg/ml).

Table 3. Estrogenic activity of the *C. lappacea* ethanolic extract.

Samples / concentration (μg/ml)	Percentages of MCF-7 cell proliferation (% of control)				
	0.1	1	10	100	1000
C. lappacea ethanolic extract	81.15±12.35	81.61±14.30	88.35±14.61	99.69±10.62	107.05±17.36
β-estradiol	91.80±16.63	115.34±17.88	121.08±9.83	84.04±16.61	76.98±2.80

Phytoestrogens are defined as naturally occurring compounds that are structurally and functionally similar to β-estradiol that can give the estrogenic effects. These compounds mainly belong to a large group of substituted phenolic compound known as flavonoids. Phytoestrogens are from cereals, legumes and grasses. Compounds that are most likely to be responsible for the estrogenic activity of plants and plant products are isoflavones, coumestanes and resorcyclic acid lactones. There are several reports on isoflavones in barley (*Hordeum velgare*), wheat (*Triticum aestivum*) and triticale (*Triticale hexaploide* Lart.) which belong to the Poaceae family [36-37]. Estrogenic activity has been reported in several plants of the grasses family. For examples, α-citral from *Cymbopogon citrates* (DC.) Stapf exhibited estrogenic properties in the estrogen-inducible yeast screen and displaced ^{3}H-estradiol from neuronal estrogen α and β receptors [38]. Jawaid et al. have demonstrated that the hydroalcoholic leaf extract of *Bambusa arundinaceae* in the grasses family gave the estrogenic activity by increasing the uterine weight and vaginal

opening in the ovariectomized Wistar rats [38]. The estrogenic activity of the extract from the whole plant of *C. lappacea* was presented in this study.

4. Conclusions

This study has demonstrated that the *C. lappacea* ethanolic extract exhibited higher antioxidant activities than the aqueous extract of 1.56 and 1.07 times for DPPH scavenging and lipid peroxidation inhibition activities, respectively. Although, this extract exhibited mild tyrosinase inhibition, it gave high collagenase inhibition which is close to ascorbic acid. The extract at the concentration range of 0.1-1000 μg/ml gave no cytotoxicity in human skin fibroblasts with cell viability of more than 90%. This extract indicated pro MMP-2 inhibition in human skin fibroblasts of 21.49±2.60% and active MMP-2 inhibition of 100±0.00% which was almost the same as that of ascorbic acid. In addition, the extract showed proliferation in MCF-7 cell at 1000 μg/ml of 107.05±17.36% of the control. Therefore, this study has not only demonstrated that the *C. lappacea* ethanolic extract can be further developed for pharmaceutical and cosmeceutical application as anti-aging products, but also confirmed the traditional use of this plant for rejuvenile of post labored women as well. For the future study, the anti-aging mechanisms of compounds in the *C. lappacea* extract are suggested.

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