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Matrix metalloproteinase-2 inhibition activity of *Plukenetia volubilis* L. leaves extract for anti-aging application

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Abstract

Plukenetia volubilis L. leaves were part of the traditional diets in many countries. *P. volubilis* leaves were used to make tea and sold as local products in Thailand. There is less information on the composition of *P. volubilis* leaves. Previous study revealed that roasted leaves extract with hot water showed the highest antioxidant activity and the antioxidant property might be due to the presence of flavonoid. The present study was carried out to determine the quercetin content in *P. volubilis* leaves extract and evaluate the anti-aging potential activities including MMP-2 inhibition activity and telomerase stimulation activity. *P. volubilis* leaves were roasted in hot air oven and extracted with hot water. The extract was investigated for quercetin content by high-performance liquid chromatography (HPLC). In vitro cytotoxicity, MMP-2 inhibition activity and telomerase stimulation activity were determined for anti-aging properties. The results revealed that *P. volubilis* leaves contained quercetin 50.50±4.78 mg/g DW. The extract showed no cytotoxicity on human skin fibroblast with cell viability of 96.76-120.83%. It demonstrated the potential of MMP-2 inhibition (8.74±2.84%) activity but lower than ascorbic acid. *P. volubilis* leave extract did not have telomerase stimulation activity on the human Hela cell line. However, the results from this study have indicated the possibility of anti-aging potential of *P. volubilis* leaves extract.

1. Introduction

The accumulation of reactive oxygen species (ROS) and free radical can lead to impaired physiological function through cellular damage of DNA, proteins, lipids, and other macromolecules, which can lead to pathogenesis of a variety of human diseases and potentially important contributors to the aging process. ROS lead to a strong degradation of the extracellular matrix (ECM) which composed mainly of collagen, elastin and fibronectin by inducing expression of matrix metalloproteinases (MMPs) (Kim *et al*., 2004). MMPs are zinc-containing endopeptidases responsible for remodeling the ECM via cleavage of internal peptide bonds. More than 20 MMPs have been identified. MMPs play a significant role in development, tissue repair and remodeling but are also directly associated with chronic inflammation, arthritis, osteoporosis, periodontal diseases, tumor invasion, cancer progression, pathological processes, oxidative stress and wrinkle formation (Bae *et al*., 2016; Tajhya *et al*., 2017). MMP-2 (gelatinase A, EC 3.4.24.24) was originally described as type IV collagenases because of their ability to promote the hydrolysis of collagen IV, a major component of basement membranes (Manosroi *et al*., 2012). Increasing MMP-2 expression is involved with collagen degradation in the aged human skin leading to wrinkling and loss of skin elasticity (Yu *et al*., 2013). Therefore, the use of antioxidants and agents that inhibit MMP-2 activity may have beneficial effects to maintain healthy skin by preventing collagen degradation and anti-aging.

Telomerase is a ribonucleoprotein enzyme that responsible for adding telomeric repeats to the ends of chromosomes, prevents progressive telomere loss and maintains telomere length (Jafri *et al*., 2016). Telomeres are the specialized DNA sequences of 2-20 kbp length that are repeated (TTAGGG repeats) at the end of the linear chromosomes which act as caps that serve to maintain the integrity of chromosome stability during replication (Hornsby, 2007). Telomere length shortened each cell division during the process of DNA replication. When telomeres lose their length critically, cell division can no longer occur which causes cells to enter

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senescence. Telomeres are sensitive to ROS which can cause telomere shortening. The shortening of telomeres is one of the major factors that accelerate aging and cause degeneration of various systems (Raguraman and Subramaniam, 2016). Therefore, the regulation of telomerase activity in aged cells is very important. Some previous studies reported the potential of plant extracts such as *Withania somnifera* and *Astragalus membranaceus* on increasing telomerase activity in the Hela cell line (Raguraman and Subramaniam, 2016).

Plukenetia volubilis L. is a perennial, oleaginous plant from Euphorbiaceae family native to the Amazon region of South America. *P. volubilis* seed oil is a rich source of fatty acids such as linoleic acid, linolenic acid, palmitic acid, stearic acid and oleic acid (Guillén *et al*., 2003; Wuttisin, 2017). *P. volubilis* oil has been used for cosmetic, nutritional, and pharmaceutical production. *P. volubilis* leaves were used to make tea and sold as local products in Thailand. Fresh leaves, dried leaves, roasted leaves and commercial tea leaves were extracted with hot water and screened for phytochemical contents. The extracts revealed the presence of phenols, flavonoids, tannin, cardiac glycosides, steroids, and terpenoids (Wuttisin *et al*, 2021). The previous study also found that roasted leaves extract with hot water exhibited the highest phenolic content (21.36±1.90 µg GAE/mg), flavonoid content (8.65±0.16 µg QE/mg), flavonol content (0.249±0.004 µg QE/mg) and exhibited the most potent antioxidant activity by DPPH assay (IC_{50} = 135.97±6.71 µg/mL) and ABTS assay (IC_{50} = 37.53±3.87 µg/mL). Flavonoid was found to has a positive correlation with antioxidant activities (Wuttisin *et al*, 2021). Quercetin is the major flavonoid in a subclass of flavonol which has antioxidant and free radical scavenging properties. Quercetin exerts both the scavenging effect of DPPH radical and the inhibitory effect of lipid peroxidation (Pereira *et al*., 2018). It has also been demonstrated the anti-carcinogenic, anti-inflammatory, anti-bacterial, and vasodilatory properties (Ang *et al*., 2014). Several studies demonstrate that quercetin plays an important role in cancer treatment and prevention by inhibiting telomerase activity and inducing apoptosis (Ganesan and Xu, 2018). However, there have also been contrasting reports of quercetin possessing no inhibition of telomerase activity in human nasopharyngeal cancer cells in culture (Chen *et al*., 2011). Then, this study aimed to determine the quercetin content in *P. volubilis* leaves by HPLC and evaluate the anti-aging potential activity including MMP-2 inhibition activity and telomerase stimulation activity. The data might be useful for supporting the benefit of *P. volubilis* leaves for anti-aging in the future.

2. Materials and methods

2.1 Chemical and reagents

Quercetin, 2,4,6-tripyridyl-s-triazine, ascorbic acid, sulphorodamine B (SRB), and Sulforhodamine B (SRB) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO., U.S.A.). Dulbecco's modified Eagles's culture medium (DMEM), antibiotics (penicillin and streptomycin), fetal bovine serum (FBS) and trypsin were purchased from HyClone (Logan, UT, USA). Other chemicals and reagents used in this study were analytical grades.

2.2 Plant Materials

Plukenetia volubilis plant was grown in Chiang San, Chiang Rai, Thailand. The leaves (5 months old) were collected during May 2019. The leaves were roasted in a hot air oven (60°C, 48 hrs) and blended in the blender (Sharp/ EM-11). The leaves powder was extracted with hot water (90°C) for 30 mins in a ratio of 1:10 (w/v). The extracts were then filtered through Whatman® paper No.1 and dried using freeze dryer (Labconco).

2.3 Determination of quercetin content by HPLC method

Plukenetia volubilis leaves extract was determined for quercetin content by HPLC analysis. HPLC was performed using an ACE Generix 5 C18 column (Shimazu, Japan). The injection volume was 20 µL with a flow rate of 1 mL/min. The mobile phase was acetonitrile and 2% v/v acetic acid (pH 2.60) (40:60 v/v). Quercetin at 0.015, 0.031, 0.063, 0.125 and 0.25 mg/mL were used to prepare a standard calibration curve. The extract was prepared at the concentration of 2.5 mg/mL in the mobile phase. HPLC analysis was performed using a tungsten lamp 370 nm detector (Ang *et al*., 2014).

2.4 Normal human fibroblasts cytotoxicity by sulforhodamine B (SRB) assay

2.4.1 Cell cultures

The normal human skin fibroblasts were cultured under the standard conditions in the complete culture medium containing DMEM medium supplemented with 10% (v/v) FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL). Cells were incubated in a temperature-controlled and humidified incubator (Shel Lab, USA) with 5% CO_2 at 37°C (Manosroi *et al*., 2014).

2.4.2 Cytotoxicity assay

The extract was tested for cytotoxicity on the normal human skin fibroblasts by the SRB assay as previous described (Manosroi *et al*., 2012). Cells were seeded in 96-well plates at an amount of 1×10^4 cells/well and left overnight for cell attachment on the plate in 5% CO_2 at 37°C. Then, cells were exposed to various concentrations

of the extracts (0.0001-1 mg/mL) for 24 hrs. After incubation, the adherent cells were fixed by adding 50 µL of cold 50% w/v trichloroacetic acid and incubated for a further 1 hr at 4°C. Then, the cells were rinsed five times with distilled water, air-dried and stained with 50 µL of 0.4% SRB in 1% glacial acetic acid for 30 min at room temperature. The unbound SRB was removed by washing with 1% glacial acetic acid solution four times. After air-drying, 10 mM Tris base (100 µL per well) were added to dissolve the bound dye. After mixing, the absorbance was measured at 540 nm with a microplate reader (Biorad, Italy). The cells with no treatment were used as the negative control. Ascorbic acid (0.0001-1 mg/mL) was used as a positive control. The assays were done in triplicate. Cell viability (%) was calculated using the following equation:

$$\text{Cell viability (\%)} = (A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where A sample was the absorbance of the cells treated with extract or ascorbic acid and A control was the absorbance of non-treated cells.

2.5 MMP-2 Inhibiting activity assay by gelatinolytic activity (zymography)

Plukenetia volubilis leaves extract (1 mg/mL) which showed no toxicity on normal human skin fibroblasts was tested for gelatinolytic activity of MMP-2 inhibition in comparison to ascorbic acid (0.1 mg/mL) (Manosroi *et al*., 2012). The extract was sterilized through a 0.2 µm membrane and used for further analysis. The normal human skin fibroblasts cells were seeded in 6-well plates with a density of 5×10^5 cells/well. The monolayer of cells was maintained in the culture medium without FBS for 24 h then treated with the extract and incubated for 48 h. The culture supernatants were collected to assess the gelanolytic activities of MMP-2. Sodium dodecyl sulfate (SDS)-polyacrylamide gels electrophoresis (PAGE) zymography using gelatin as a substrate was performed. Briefly, 20 µl of the cell culture supernatant was suspended in the loading buffer [0.125 M Tris (pH 6.8), 4% (w/w) SDS and 0.04% bromophenol blue] and run on the 10% SDS-PAGE containing 0.1% (w/v) gelatin. 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 hrs at 37°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% (v/v) acetic acid at room temperature (27±2°C) to visualize the bands. The gelatinolytic activity was detected as a white band against a blue background of Coomassie blue staining. The gel was documented by a gel documentation system (Bio-Rad Laboratories, UK) and analysed by the Quantity 1-D analysis software. The area multiplied by the 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 (non-treated cells) were calculated by the following equation:

$$\text{MMP-2 inhibition (\%)} = 100 - [(\text{Area}_{\text{Sample}} / \text{Area}_{\text{Control}}) \times 100]$$

Where $\text{Area}_{\text{sample}}$ was the band intensity of cells treated with extract or positive control (ascorbic acid) and $\text{Area}_{\text{control}}$ was the band intensity of non-treated cells.

2.6 Telomerase stimulation activity in the human cervical carcinoma cell line (Hela cell line)

The human HeLa cell line was used to study telomerase stimulation activity of *P. volubilis* leaves extract. The extract was tested for cytotoxicity on the human HeLa cell line by the SRB assay as described in 2.4. *P. volubilis* leaves extract (1 mg/mL) which showed no toxicity on human HeLa cell line was used in this study. The extract was sterilized through a 0.2 µm membrane and used for further analysis. Cells were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator (37˚C and 5% CO_2). Cells (40-60% confluence) were treated with *P. volubilis* leaves extract and incubated for 72 hrs.

Detection of telomerase activity was based on the PCR-based telomeric repeat amplification protocol (TRAP assay) with some modifications (Raguraman and Subramaniam, 2016). Briefly, the total volume of the reaction mixture was 50 µL contained 0.5 µL of dNTP mix (2.5 mM), 5 µL of 10X TRAP Buffer, 0.15 µg TS [5'-AATCCGTCGAGCAGAGTT-3'] primer, 0.15 µg ACX [5'GCGCGGCTTACCCTTACCCTTACCCTAACC-3'] primer, 40 µL RNase-free H_2O, 0.5 µL of 5 U/µL Taq DNA polymerase and 2 µL (1000 cells) of cell lysate. The PCR mixture was incubated for 30 mins at room temperature. The PCR was then started at 94°C for 90 s followed by a 40-cycle amplification (94°C for 20 s, 50°C for 30 s, and 72°C for 90 s). The amplified PCR product of telomerase DNA was separated on 12% acrylamide-TBE gels and visualized by ethidium bromide staining in Gel documentation (Bio-Rad Laboratories, UK). The percentage of telomerase stimulation in the human Hela cell line was calculated by the following equation:

$$\text{Telomerase activity (\%)} = [(\text{Area}_{\text{extract}} / \text{Area}_{\text{control}}) \times 100]$$

Where Area $_{extract}$ was the band intensity of cells treated with extract and Area $_{control}$ was the band intensity of non-treated cells.

2.7 Statistical analysis

All assays were performed triplicate in three independent experiments. The data were presented as mean ± standard deviations (SD). SPSS 23.0 was employed for all data analyses. The independent sample t -test was used to determine a significant difference between the group. The level of significance was at $P<0.05$.

3. Results and discussion

3.1 Determination of quercetin content by HPLC method

The present study is a preliminary HPLC analysis to determine the quercetin in *P. volubilis* leaves extract. The HPLC calibration curve of standard quercetin was prepared and presented in Figure 1. The HPLC chromatograms of *P. volubilis* leaves extract (2.5 mg/mL) reveal the quercetin peak at 1.598 mins as shown in Figure 2. When compared with the standard quercetin calibration curve, it can be calculated that *P. volubilis* leaves extract contained quercetin 50.50±4.78 mg/g DW. The phytochemical investigation, total phenolic contents and flavonoid contents of *P. volubilis* leaves extracts were previously reported (Nascimento *et al*., 2013). The extracts revealed the presence of phenols, flavonoids, tannin, cardiac glycosides, steroids, and terpenoids and exhibited potent antioxidant activity. Flavonoid was found to has a positive correlation with antioxidant activities (Wuttisin *et al*., 2021). Quercetin is the major flavonoid in a subclass of flavonol which has antioxidant and free radical scavenging properties then the antioxidant activity of *P. volubilis* leaves extract might be attributed to the presence of quercetin. Further studies are required to determine other chemical components in *P. volubilis* leaves.

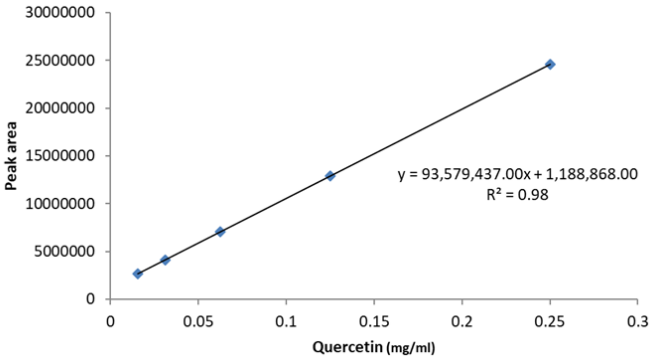

Figure 1. HPLC calibration curve of standard quercetin

3.2 Normal human fibroblasts cytotoxicity

The extract was tested for cytotoxicity on the normal human skin fibroblasts by the SRB assay. Figure 3 showed the percentage of cell viability after treated with *P. volubilis* leaves extract and vitamin C. It was found that *P. volubilis* leaves extracts at 0.0001 - 1 mg/mL were non-toxic to normal human fibroblast with the cell viability 96.76-120.83%. The cell viability after treatment with the extracts was above 90% then it can be considered that *P. volubilis* leaves extract was non-cytotoxic (Kim *et al*., 2018). The percent cell viability of *P. volubilis* leaves treated cell at 0.001 – 1 mg/mL were significant higher ($P<0.05$) than ascorbic acid-treated cell. The concentration-dependent cytotoxicity was observed in the ascorbic acid-treated cell when the concentrations of ascorbic acid were increased from 0.001 to 1 mg/mL. The cytotoxicity was found when treated with ascorbic acid at 1 mg/mL.

P. volubilis leaves extract at the concentration of 1 mg/mL and ascorbic acid at the concentration of 0.1 mg/mL were further used for MMP-2 Inhibiting activity assay by gelatin zymography.

3.3 MMP-2 Inhibiting activity

The MMP-2 inhibition activity of *P. volubilis* leaves extract was presented in Figure 4. The *P. volubilis* leaves extract at 1 mg/mL indicated the MMP-2 inhibition of

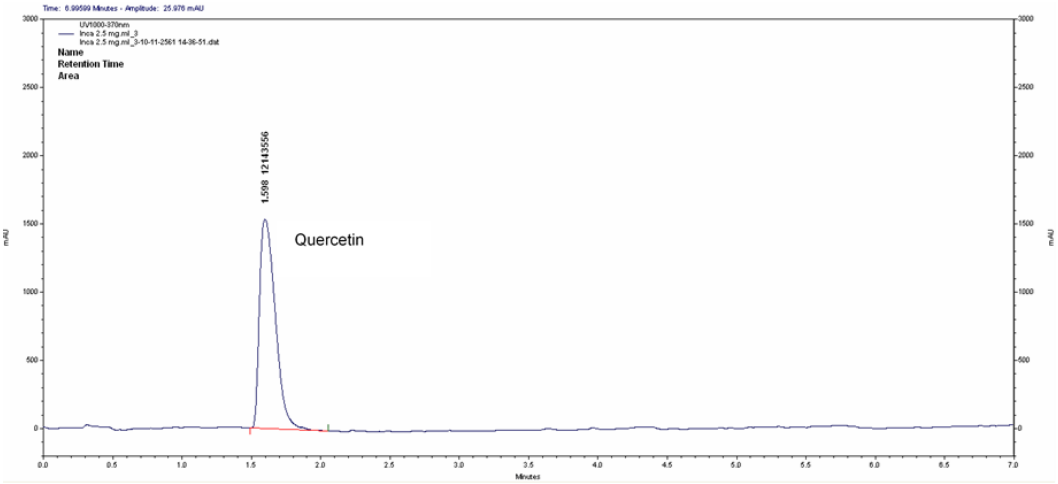

Figure 2. HPLC chromatogram of *P. volubilis* leaves (2.5 mg/mL) containing quercetin

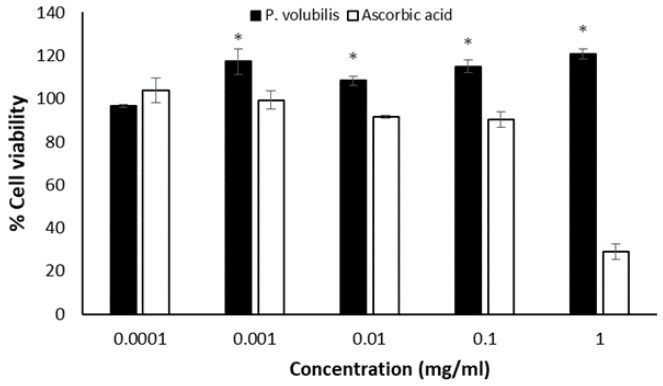

Figure 3. Percentage cell viability of normal human fibroblast treated with *P. volubilis* leaves extract and ascorbic acid. *Significant difference (P<0.05) in comparing to ascorbic acid.

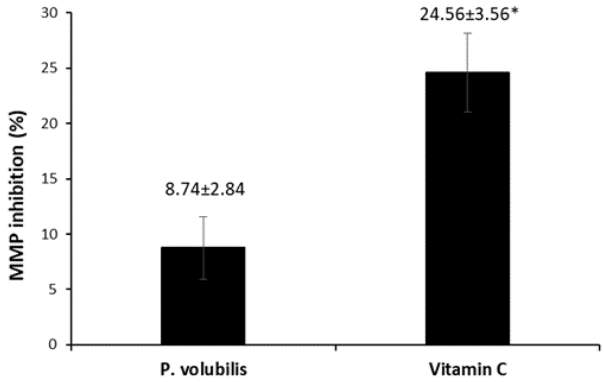

Figure 4. MMP-2 inhibition activity of *P. volubilis* leaves extract (1 mg/mL) and ascorbic acid (0.1 mg/mL). *Significant difference (P<0.05)

8.74±2.84% but significant (P<0.05) lower than ascorbic acid (24.56±3.86%). *P. volubilis* leaves extract indicated the MMP-2 inhibition determined by gelatin zymography. Gelatin zymography is a technique developed to identify gelatinolytic activity in biological samples using SDS-PAGE impregnated with gelatin (Woessner 1995). The ability of MMP-2 to degrade the gelatin can be detected by a cleared zone after staining the gel (Toth and Fridman, 2001). MMPs are known to be a part of different important pathways including metastasis, oxidative stress and fibrosis (Reuter *et al*., 2010). MMPs play a key role in regulating matrix remodeling, as they are considered responsible for the degradation of collagen and proteoglycan (Sharma and Maffulli, 2006; Riley 2008). Increasing MMP-2 expression is involved with collagen degradation in the aged human skin leading to wrinkling and loss of skin elasticity (Manosroi *et al*., 2012; Yu *et al*., 2013). The inhibition of MMP-2 expression of the *P. volubilis* leaves might be related to its free radical scavenging activities (Manosroi *et al*., 2012). *P. volubilis* leaves extract composed of phenolic compounds and flavonoids such as quercetin which were reported to possess anti-MMP bioactivity (Adhami *et al*., 2003; Ende and Gebhardt, 2004; Vijayababu *et al*., 2006). The effect of quercetin on MMP-2 and MMP-9 activities were examined in prostate cancer cells (PC-3) and human fibrosarcoma cells (HT1080). It was found that quercetin can inhibit the expressions of both MMP-2 and MMP-9 (Vijayababu *et al*., 2006; Park *et al*., 2011). Previous studies suggested that inhibitory effects of quercetin on MMP-2 and MMP-9 expressions are associated with the inactivation of p38, a MAP Kinase, as well as the increased level of TIMP-1, a MMP inhibitor (Park *et al*., 2011). It appears that *P. volubilis* leaves will provide valuable insights for its utilization as a functional food due to its antioxidant and anti-aging via MMP-2 inhibiting activity.

3.4 Telomerase stimulation activity in the human Hela cell line

The human HeLa cell line was used due to the reported to have telomerase activity (Hou *et al*., 2001). Figure 5 shows the agarose gel electrophoresis of telomerase DNA from the human HeLa cell line treated with *P. volubilis* leaves extract. The arrows represent the amplification of a 36 bp internal standard and 50 bp product due to telomerase activity. It was found that *P. volubilis* leaves extract did not have telomerase stimulation activity on the human Hela cell line (telomerase activity 99.77±1.01%) when compared to untreated cell (telomerase activity 100%). Further studies are required for the determination of other activities of *P. volubilis* leaves.

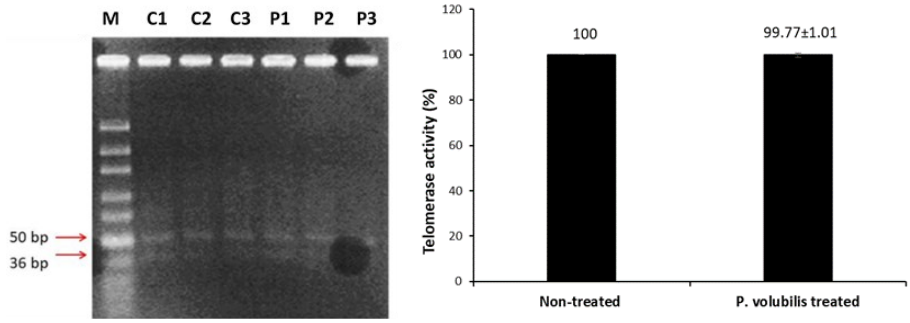

Figure 5. Agarose gel electrophoresis of telomerase DNA from human HeLa cell line treated with *P. volubilis* leaves extract (1 mg/mL). M = DNA ladder, C1 C2 C3 = non-treated cell, P1 P2 P3 = *P. volubilis* leaves extract treated cell. The arrows represent the amplification of a 36 bp internal standard and 50 bp product due to telomerase activity.

4. Conclusion

The present study is a preliminary HPLC analysis to determine the quercetin content in *P. volubilis* leaves. The extract showed no cytotoxicity on human skin fibroblast. It demonstrated the MMP-2 inhibition activity but did not have telomerase stimulation activity. However, the results from this study have indicated the possibility of anti-aging potential of *P. volubilis* leaves extract.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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