

PHYTONUTRIENT SCREENING BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY AND ANTI-AGING PROPERTIES OF *GLYCOSMIS PENTAPHYLLA* (RETZ.) DC. (SOM-CHUEN) EXTRACTS

THISAKORN DUMRONGPHUTTIDECHA¹, WARACHATE KHOBJAI², SURACHAI TECHAEI¹, KHEMJIRA JARMKOM¹, AND SURADWADEE THUNGMUNGMEE¹

¹Department of Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi. ²Department of Clinical Chemistry, Faculty of Medical Technology, Nation University, Lampang, Thailand. Email: thisakorn_d@rmutt.ac.th

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ABSTRACT

Objective: The aims of this study were to screen the phytonutrient constituents and investigate their anti-aging property of leaves and bark of *Glycosmis pentaphylla* (Retz.) DC. (GP).

Methods: GP is the medicinal plant which used as traditional medicine. *Glycosmis pentaphylla* leaves (GPL) and *Glycosmis pentaphylla* bark (GPB) of GP were extracted with ethanol. The gas chromatography–mass spectrometry was used for phytonutrients analysis. The anti-aging properties were performed using ABTS assay, deoxyribose degradation assay, bovine serum albumin (BSA)-fructose model, and matrix metalloproteinase 1 (MMP-1) inhibitory activity.

Results: The important phytonutrients of GPL were α -tocopherol, linolenic acid, squalene, stigmaterol, and β -amyryn and for GPB were α -tocopherol, phytol, campesterol, stigmaterol, dictamine, and γ -sitosterol. The percentage inhibition of GPL and GPB extracts at various concentrations was between 16.57–76.05 and 20.66–78.81 by ABTS assay, 68.24–90.06 and 73.83–96.64% by deoxyribose degradation assay, 4.24–99.98 and 54.81–99.94 by BSA-fructose model, and 6.31–81.55 and 1.06–74.45 by MMP-1 inhibitory activity, respectively.

Conclusion: Leaves and bark extracts of GP (Retz.) DC. are good sources of important phytonutrients and have the potential in anti-aging property.

Keywords: *Glycosmis pentaphylla* (Retz.) DC., Anti-aging, Phytonutrients, Anti-Glycation, Matrix Metalloproteinase-1.

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INTRODUCTION

Phytonutrients are bioactive compounds in plants that provide medical health benefits for the human body whether to maintain the immune function and prevent the body from various diseases. Some important bioactive phytonutrients were polyphenols, terpenoids, flavonoids, isoflavonoids, carotenoids, limonoids, glucosinolates, phytosterols, ω -3 fatty acids, and probiotics [1]. *Glycosmis pentaphylla* (Retz.) (GP) is the plant in the Rutaceae family and the native plant in East, South, and Southeast Asia. Fruits of GP are translucent pink and sweet taste. The leaves and stems are used as traditional medicine and the stems are used as a toothbrush for cleaning the teeth [2,3]. Previous studied, the phytonutrient constituents in GP extract were alkaloids, terpenoids, amides, and flavonoids such as arborine, acridone, carbazole, quinolone, β -sitosterol, glycosmicine, quinazoline, furoquinolines, arborine, stigmaterol, and γ -fagarine triterpenes [4]. The pharmaceutical properties were antioxidant, anti-inflammatory, antifungal, and wound healing [2].

Aging is a multifactorial phenomenon with the accumulation of cellular damage leading to elevate the risk of age-related diseases including diabetes, hypertension, cardiovascular diseases, neurological disease, Parkinson's disease, atherosclerosis, Alzheimer's disease, and cancer [5]. Tumor necrosis factor- α is one of the biological biomarkers of aging in the inflammatory process which tends to increase with age. It is triggered by oxidative stress in the body [6]. Oxidative stress occurs from the imbalance between free radicals and antioxidants leading to oxidative damage to lipid, protein, and DNA until the death of cells. Hence, the elevation of antioxidants can scavenge the excessive free radicals and protect cells from oxidative damage including slowing down the process of aging [7]. Consequently, this research was to

screen the phytonutrient constituents and investigate their anti-aging property of leaves and bark of GP (Retz.).

METHODS

Collection and extraction of GP (Retz) DC. leaves and bark

Glycosmis pentaphylla leaves and *Glycosmis pentaphylla* bark (GPB) were collected in the hot season at Phayao Province in the north of Thailand. After a harvest, they were washed and dried at 45°C and were pulverized into coarse powder and macerated with ethanol for 7 days. Then, they were filtrated and evaporated with a rotary evaporator.

Phytonutrients analysis by gas chromatography–mass spectrometry (GC–MS)

The GPL and GPB extracts were analyzed the phytonutrients using Agilent Technologies GC–MS with 30 m \times 250 μ m id \times 0.25 μ m HP-5MS column. Helium gas was used as the carrier gas with 0.1 ml/min of constant rate flow. The samples were injected for 2 μ l. The injection was split at the ratio 10:1 and the inlet temperature was 250°C and the transfer line was 280°C. The condition of oven was started from 110°C (held for 2 min), increased to 200°C (10°C/min, no held), and increased 5–280°C for 9 min. Mass spectra were taken at 70 eV. The total running time was 36 min.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

Free radicals scavenging activity of GPL and GPB was determined using ABTS radical cation decolorization assay. 7 mM of ABTS was mixed with 2.45 mM of potassium persulfate and kept the mixture for reaction in

the dark at room temperature for 12–6 h to produce ABTS radical cation (ABTS⁺). The ethanol was added to the ABTS⁺ stock solution for dilution at the ratio 89:1 v/v and measured the absorbance at 734 nm before assay. The absorbance of the ABTS⁺ solution should be 0.700±0.02. After that, 10 µl of samples were added to 1 ml of ABTS⁺ solution and incubated in the dark for 30 min. The mixtures were measured the absorbance at 734 nm using spectrophotometer.

Deoxyribose degradation assay

This method was used to evaluate the hydroxyl radical scavenging activity of GPL and GPB at various concentrations (10, 5, 2.5, 1.25, and 0.625 mg/ml). 200 µl of 15 mM 2-deoxyribose, 200 µl of 1 mM EDTA, 200 µl of 500 mM FeCl₃, 100 µl of 10 mM H₂O₂, and 100 µl of 1 mM ascorbic acid were added to 200 µl of 100 mM phosphate buffer, pH 7.4 and incubated at 37°C for 1 h. After incubation, 1 ml of 2.8% trichloroacetic acid was added for terminating the reaction and 1 ml of 1% 2-thiobarbituric acid used as chromogen which was added for color development. The mixtures were heated in boiled water for 20 min and then cooled in ice water for 15 min. The mixtures were measured the absorbance at 532 nm using a spectrophotometer.

Antiglycation assay using bovine serum albumin (BSA)-fructose model

BSA-fructose model used to evaluate all stage protein glycation. 1 ml of 1.5 M fructose was mixed with 1 ml at various concentrations of GPL and GPB (10, 5, 2.5, 1.25, and 0.625 mg/ml) and 1 ml of 0.5 M sodium phosphate buffer, pH 7.4 containing 0.02% of sodium azide. Then, the mixture was incubated at 37°C for 2 h. After incubation, 1 ml of 30 mg/ml BSA was added to the mixtures and incubated at 37°C for 7 days. 10 mM aminoguanidine was used as a positive control. The mixtures were measured with spectrofluorometer using excitation at 340 nm and emission at 420 nm.

Matrix metalloproteinase 1 (MMP-1) inhibitory activity

1 µL of N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid was diluted with 200 µL assay buffer consists of 50 mmol/L

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mmol/L CaCl₂, 0.05% Brij-35, and 1 mmol/L 5, 5'-dithiobis(2-nitrobenzoic acid). The substrate was diluted DMSO using 10 µl/well. The MMP-1 enzyme was diluted with assay buffer. GPL and GPB extracts at various concentrations (10, 5, 2.5, 1.25, and 0.625 mg/ml) were added to each well which contained 50 µl of assay buffer and 20 µl of MMP-1 enzyme. Then, the mixtures were incubated for 30 min at 37°C to allow inhibitor and enzyme interaction. After that, 10 µl of MMP-substrate was added to start the reaction. The mixtures were measured the absorbance at 412 nm at 1 min time interval for 10 min.

Statistical analysis

All measurements were performed in triplicates and the experiment results were reported as mean ± standard. Statistical analysis was performed using ANOVA. Differences were considered significant when p≤0.05.

RESULTS AND DISCUSSION

Phytonutrients analysis

Phytonutrient constituents of GPL and GPB extracts were analyzed by GC-MS with different retention times. The spectrum of various phytonutrients that are constituents in GPL extract is shown in Fig. 1 and GPB extract is shown in Fig. 2. The main phytonutrient constituents in GPL extract were α-tocopherol, hexadecanoic acid, ethyl ester, ethyl isoallocholate, phytol, ethyl 9.cis.,11.trans.-octadecadienoate, and quinolinium. In addition, there were important phytonutrients in GPL extract what play the important role in antioxidant and anti-inflammatory such as linolenic acid, squalene, stigmaterol, and β-amyryn, as shown in Table 1. For the main phytonutrients in GPB extract were hexadecanoic acid, ethyl ester, ethyl oleate, and ethyl ester and there were some important phytonutrients such as α-tocopherol, phytol, campesterol, stigmaterol, dictamine, and γ-sitosterol, as shown in Table 2.

Anti-aging properties of GPL and GPB extracts

Aging is the progressive deterioration of unrepaired damaged cells and accumulative in human body. According to the free radical theory of

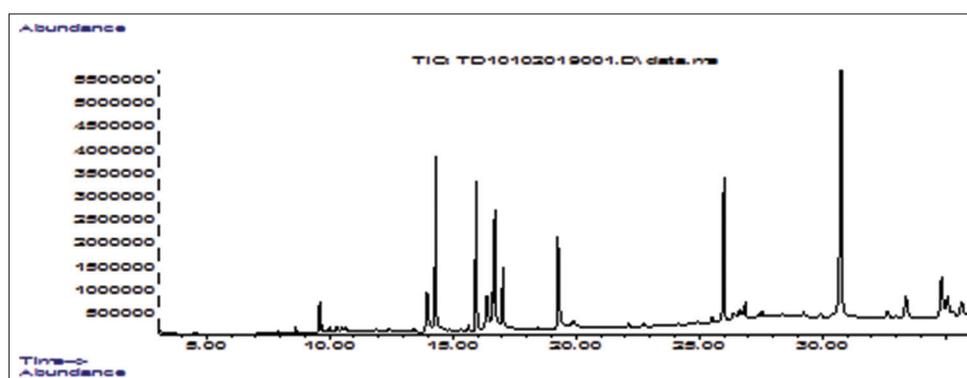


Fig. 1: Spectrum of various phytonutrients in *Glycosmis pentaphylla* leaves extract

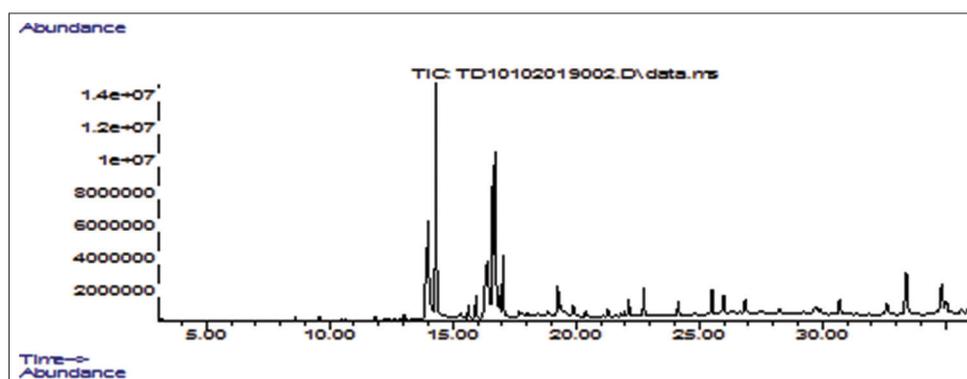


Fig. 2: Spectrum of various phytonutrients in *Glycosmis pentaphylla* bark extract

aging identified that free radicals especially reactive oxygen species (ROS) are caused the age-related damage at the cellular and tissue levels and leading to cells and tissues loss their ability functions normally. ROS are generated by both endogenous and exogenous

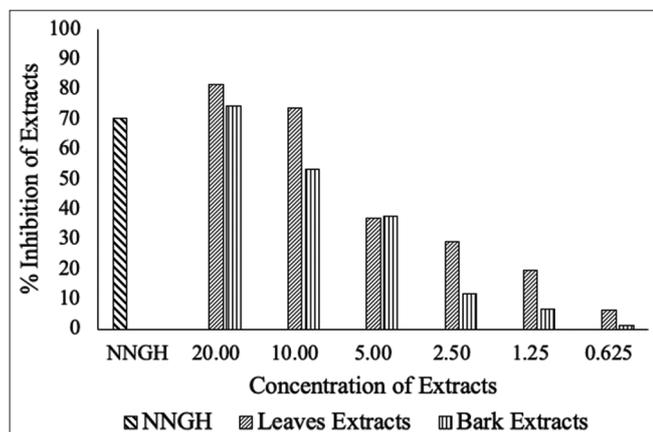


Fig. 3: The percentage inhibition of matrix metalloproteinase 1 of *Glycosmis pentaphylla* leaves and *Glycosmis pentaphylla* bark extracts

Table 1: The important phytonutrient constituents in *Glycosmis pentaphylla* leaves extract

Peak	RT	Phytonutrients	MF	MW
1	15.937	Phytol	C ₂₀ H ₄₀ O	296
2	16.380	Linolenic acid	C ₁₈ H ₃₀ O ₂	278
3	25.998	Squalene	C ₃₀ H ₅₀	410
4	30.751	α-tocopherol	C ₂₉ H ₅₀ O ₂	430
5	33.387	Stigmasterol	C ₂₉ H ₄₈ O	412
6	35.074	β-amyrin	C ₃₀ H ₅₀ O	426

RT: Retention time, MF: Molecular formula, MW: Molecular weight

Table 2: The important phytonutrient constituents in *Glycosmis pentaphylla* bark extract

Peak	RT	Phytonutrients	MF	MW
1	13.905	Dictamnine	C ₁₂ H ₉ NO ₂	199
2	15.937	Phytol	C ₂₀ H ₄₀ O	296
3	16.405	Linoleic acid	C ₁₈ H ₃₂ O ₂	280
4	30.690	α-tocopherol	C ₂₉ H ₅₀ O ₂	430
5	32.623	Campesterol	C ₂₈ H ₄₈ O	400
6	33.399	Stigmasterol	C ₂₉ H ₄₈ O	412
7	34.840	γ-sitosterol	C ₂₉ H ₅₀ O	414

RT: Retention time, MF: Molecular formula, MW: Molecular weight

Table 3: Antioxidant capacity of *Glycosmis pentaphylla* leaves and *Glycosmis pentaphylla* bark extracts in various methods

Test sample	Concentration of samples	Antioxidant activities		
		ABTS assay	Deoxyribose degradation assay	Antiglycation assay
<i>Glycosmis pentaphylla</i> leaves	0.625	20.66±0.57	68.24±1.72	NA
	1.25	28.76±1.26	72.96±0.82	4.28±2.365
	2.50	29.20±1.03	75.73±0.58	35.19±2.841
	5.00	35.82±4.26	80.91±0.42	82.79±0.480
	10.00	77.03±2.81	84.52±0.29	99.35±0.021
	20.00	78.81±1.52	90.06±0.40	99.98±0.001
<i>Glycosmis pentaphylla</i> bark	0.625	16.57±0.38	73.83±0.74	NA
	1.25	21.72 ± 1.00	82.10±0.48	NA
	2.50	22.16±0.92	88.46±0.38	NA
	5.00	29.72±2.12	91.71±0.11	54.81±1.270
	10.00	73.26±3.44	91.84±0.21	95.94±0.474
	20.00	76.05±1.54	96.64±1.05	99.94±0.008

NA: Not available in inhibition

factors (toxic chemicals, air pollutions, etc.). In normal physiological process, the body will keep the equilibrium between free radicals and the endogenous antioxidants but the excessive of free radicals disturb the balance of free radicals and the endogenous antioxidants which is the cause of oxidative stress which may contribute to age-related diseases including aging [8]. There are several studies shown the consumption of antioxidant foods could protect the body against oxidative stress such as tea, mulberry, cruciferous vegetables, and others. The GPL and GPB extracts were evaluated the antioxidant capacity using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, deoxyribose degradation assay, and antiglycation assay using BSA-fructose model. ABTS assay was used to determine antioxidant capacity to scavenge the ABTS•+ of GPL and GPB extracts in which ABTS•+ was generated by the reaction between ABTS and potassium persulfate [9]. Ascorbic acid was used as a positive control. This method was used to evaluate the antioxidant activity of extracts due to its simple, rapid, and sensitive. Both GPL and GPB extracts had antioxidant properties at various concentrations. The half maximal inhibitory concentration (IC₅₀) of GPL and GPB was 5.81 and 5.10 mg/ml, respectively and percentage of inhibition as shown in Table 3. Therefore, ABTS•+ was gave electrons by GPL and GPB extracts to neutralized their positive charges to form the stable molecules because their phytonutrients constituents in both extracts such as α-tocopherol, linolenic acid, phytol, campesterol, stigmasterol, and other [10]. Since 1987, deoxyribose degradation assay has been used for hydroxyl radical (•OH) scavenging activity of various extracts, foods, and other medicinal plants. •OH is the most powerful free radical in the group of ROS and it is a major ROS in the living organisms [11]. •OH was generated through Fenton reaction and attached to 2-deoxyribose which made up the cyclic furan ring in generated the malondialdehyde (MDA) [12]. MDA can occur by food consumption and lipid peroxidation. The high level of •OH causes the overproduction of MDA and leading to oxidative stress. The measurement of the level of oxidative stress usually uses MDA as the biomarker [13]. The GPL and GPB extracts were tested at different concentrations (0.625–20 mg/ml). The %inhibition of GPB extracts was higher than GPL extracts, as shown in Table 3. 20 mg/ml of GPB extract can inhibit the formation of •OH for 96.94% and the percentage inhibition of 20 mg/ml of GPL extract was 90.06 that means both GPL and GPB extracts had the ability to decrease the MDA generation. The advanced glycation end products (AGEs), irreversible heterogeneous compounds, are generated by glycation reaction. It is a non-enzymatic reaction between the carboxyl group of reducing sugars and free amino group of proteins. The formation of AGEs can generate free radicals which has the important role in aging and diabetes [14,15]. BSA-fructose model was used to determine the ability of GPL and GPB extracts in inhibitory activity of AGEs formation through glycation reaction. As the results shown in Table 3, GPL extracts at concentration below 1.25 mg/ml did not inhibit the formation of AGEs but 1.25, 2.5, 5, 10, and 20 mg/ml exhibited good ability to inhibit the formation of

AGEs with the percentage inhibition at 4.28, 35.19, 82.79, 99.35, and 99.98, respectively. Another one, GPB extracts at the concentration below 5 mg/ml did not inhibit the formation of AGEs but 5, 10, and 20 mg/ml also exhibited good ability to inhibit the formation of AGEs with the percentage inhibition at 1.13, 54.81, 95.94, and 99.94, respectively. The ability of GPL and GPB extracts in inhibition on AGEs formation may occur from the α -tocopherol in the extracts that can inhibit the glycation and formation of AGEs [16]. The glycation and AGEs formation inhibition of GPL and GPB extracts reveal protective effects from diabetes, age-related diseases such as retinopathy, cataracts, arteriosclerosis, and renal dysfunction [17].

MMP-1 inhibitory activity

MMP is enzymes that degrade almost extracellular matrix and basement membrane components [18]. Tissue inhibitors of metalloproteinase play the important roles in counteraction the activity of MMP-1. The imbalance between TIMOs and MMP causes pathologic conditions such as cancer, rheumatoid arthritis, and periodontitis [19]. Amount of MMP-1 increases by inducing of UV light and degrades the fibrillar collagen Type I and III. After collagen degradation by MMP-1, it is degraded by MMP-3 and MMP-9 which also increase by UV irradiation. The excessive MMP-1 can affect the integrity of the dermis and leading to the remodeling of the skin and photoaging [20]. GPL and GPB extracts exhibited anti-MMP-1 properties. The percentage inhibition of GPL extracts at 0.625, 1.25, 2.5, 5, 10, and 20 mg/ml was 6.31, 19.44, 29.02, 36.83, 73.74, and 81.55, respectively, and the percentage inhibition of GPB extracts at 0.625, 1.25, 2.5, 5, 10, and 20 mg/ml was 1.06, 6.74, 11.70, 37.54, 53.15, and 74.45, respectively, as shown in Fig. 3.

CONCLUSION

The screening phytonutrients in leaves and bark of GP (Retz.) DC. in Thailand by GC-MS were found the important phytonutrients such as α -tocopherol, linolenic acid, squalene, stigmasterol, β -amyryn, campesterol, and dictamine. Both leaves and bark had anti-aging properties that exhibited to scavenge free radicals, inhibit all stages of glycation and MMP-1 enzyme. As the results, GP (Retz.) DC. could be developed as functional food/ingredient or nutraceutical for the prevention of age-related conditions.

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AUTHORS' CONTRIBUTIONS

Thisakorn Dumrongphuttidecha performed to evaluate the ABTS assay, deoxyribose degradation assay, antiglycation assay using BSA-fructose model, and MMP-1 activity. Warachate Khobjai performed the phytonutrients screening of extracts and was consultant in deoxyribose degradation assay, antiglycation assay, and MMP-1 activity. Surachai Techaeoi performed antiglycation assay using BSA-fructose model and phytonutrients screening of extracts. Khemjira Jarmkom performed ABTS assay. Suradwadee Thungmungmee performed the plant extraction.

CONFLICTS OF INTEREST

The authors declare that there were no conflicts of interest.

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