https://doi.org/10.48048/tis.2023.6548

Phytochemicals, Cytotoxicity, Inhibitory Effect on Nitric Oxide Production and Alpha-Glucosidase Activity, and DPPH Scavenging Activity of Leaf Extract from *Gymnema inodorum* (Lour.) Decne

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Received:26 December 2022, Revised: 27 January 2023, Accepted: 6 February 2023, Published: 19 February 2023

Abstract

Investigation of phytochemicals, cyto-toxicity, inhibitory effect on nitric oxide (NO) production and α-glucosidase activity, and DPPH scavenging activity of leaf extract from *Gymnema inodorum* (Lour.) Decne (LEGI) revealed that GC-MS analysis demonstrated the presence of 83 phytochemicals with 5 main compounds i.e. Phytol (18.14 %), n-Hexadecanoic acid (12.45 %), 9,12-Octadecadienoic acid (Z,Z)-(3.92 %), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-(10.39 %) and Betulin (8.56 %), and 78 trace compounds in LEGI. MTT assay to investigate cytotoxicity showed that LEGI exhibited low cytotoxicity to the RAW 264.7 macrophages with IC50 of 128.77±2.82 µg/mL. Treatment with the LEGI significantly inhibited nitric oxide (NO) production, an inflammatory mediator, in the LPS-stimulated RAW 264.7 cells. At the concentration of 50 mg/mL, LEGI inhibited NO production by 16.73±1.52 % which was less than Diclofenac did (27.07±1.81 %). The α-glucosidase inhibitory activity to determine the antidiabetic activity showed that LEGI exhibited the inhibition on α-glucosidase activity with IC_{50} of 0.34 mg/mL, which was less potent than Acarbose (IC_{50} of 0.20 mg/mL). Assay of 2,2-diphenyl-2-picrylhydrazyl- hydrate (DPPH) radical scavenging to examine antioxidant activity revealed that LEGI demonstrated less potent DPPH scavenging activity compared to Butylated hydroxytoluene, BHT with IC_{50} of 0.43 vs 0.36 mg/mL, respectively. These results indicate that the leaf extract from *G. inodorum* possesses anti-inflamatory, antidiabetic and antioxidant activities that can be developed and used as natural agents for the tréamatent of diseases related to these activities.

Keywords: *Gymnema inodorum*, Chemical components, α-glucosidase, DPPH, NO production, Phytochemicals, Inflammatory mediator

Introduction

Gymnema inodorum (Lour.) Decne (GI) belongs to the Family Asclepiadaceae. GI is indigenous in Southeast Asia including Thailand [1]. It has been popularly consumed as vegetable, used as herbal remedy and commercial herb tea products especially in the northern and northeastern parts of Thailand [2,3,4]. GI has high nutritional values. Apart from nutritional value, its leaves have therapeutic value in diabetes mellitus, rheumatic arthritis and gout [5]. It is also used to treat various ailments for example asthma, eye irritation, chronic cough, trouble breathing, constipation, and abdominal pain due to indigestion. In addition, it has been reported to possess anti-microbial activities, anti-hypercholesterolemic activity, and anti-inflammatory properties [6].

GI leaves contain some nutritional values such as protein, fat, and fiber contents [7] and many phytochemicals including phenolics, flavonoids, terpenoids, and glycoside [1,8,9]. Total phenolic content was found in both the narrow and board leaves of GI [7]. Pregnane glycosides, gyminosides A and B, tinctoroside B, tinctoroside C, and gymnepregoside F were isolated from the GI leaves [9,10]. GI juice has high amounts of vitamin E and β-carotene [11,12]. GI extract has been reported to have triterpene glycoside, (3β, 16β)-16,28-dihydroxyolean-12-en-3-yl-O-β-D-gluco-pyranosyl-β-D glucopyranosiduronic acid (GIA1) [13] and 16 volatile compounds [4]. Total phenolic and flavonoid contents were more intensive in the GI ethanolic extracts [12].

MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay revealed that GI extract at a concentration of 62.5 μg/mL exhibited noncytotoxicity in Caco-2 cells [13] and may not be toxic to the liver and kidney in mice [1]. GI tea did not affect hepatic and renal functions [14]. Moreover, long-term consumption of GI for 28 days did not cause hepatotoxicity [1].

GI leaf extract exhibited anti-inflammatory effects by inhibiting the NO production in LPS-activated RAW 264.7 macrophages [12] and suppressing nitric oxide (NO) and downregulation of the expression of inducible nitric oxide synthase (iNOS) in LPS plus IFN-γ-induced RAW264.7 cells [4].

GI has been reported to possess a hypoglycemic effect. Its leaves are used as an effective herbal medicine for the prevention and treatment of diabetes [12] and used as an antidiabetic functional food or tea [15]. GI water leaf extract decreased blood glucose in alloxan-induced diabetic rats [16,17]. Consumption of GI leaf extract can significantly decrease peak plasma glucose [3]. Phenolic content and some bioactive compounds containing in its leaves can control blood sugar levels [7]. GI extract exhibited antidiabetic activity in high-fat diet and STZ diabetic rats as well [18]. Gymnepregoside F, gyminosides A and B, tinctoroside B, and tinctoroside C isolated from GI leaves can inhibit α-glucosidase activity [9].

The methanolic leaf extract of GI showed the highest level of antioxidant activity of 43 edible plants belonging to 8 families in Thailand [8]. The ethanolic GI leaf extract exhibited free radical scavenging effect on the DPPH assay [12]. PCL assay also demonstrated the antioxidant property of GI extract [19].

The biological activities including anti-inflammatory, antidiabetic and antioxidant activities of GI leaves and leaf extract have been investigated. However, mechanisms of action on these activities have not much been reported. The present study was therefore undertaken *in vitro* to investigate phytochemicals using GC-MS, anti-inflammatory activity by measuring NO production, antidiabetic activity using α-glucosidase inhibitory activity, and antioxidant activity using DPPH radical scavenging activity and to elucidate the underlying mechanism involved the activities of ethanol leaf extract from *G. inodorum*. And also, determination of cytotoxicity using MTT assay was carried out to ensure for safety applications.

Materials and methods

Plant materials

The plant used in the present study was fresh mature and healthy leaves of *Gymnema inodorum* (Lour.) Decne and was purchased from a local garden in Chiang Mai province, Thailand. The plant leaves were identified and authenticated by Dr. Panarat Thongpoem, a lecturer in the Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathum Thani, Thailand. The authentic samples with reference SCI/BIO/GI 2020-001 have been submitted to the herbarium in the Department of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi (RMUTT).

Preparation of plant extract

The plant leaves were cleaned and removed all impurities by washing thoroughly with tap water. The leaf samples were cut into small pieces, dried in shade and further dried in a hot air oven at 60 °C. The dried leaves were ground to fine powder using an electric grinder, and extracted. Extraction of the plant samples was performed using the macerating process according to Buddhakala and Talubmook [20]. The powder sample was mixed with 95 % ethanol at a ratio of 100 g : 400 mL. The maceration of the mixture was carried out at room temperature for 7 days with intermittent stirring. After the maceration period, the mixture was filtered through Whatman No. 1 filter paper. The extraction process was repeated for a second time by adding 400 mL of 95 % ethanol to the mixture residue. The filtrates were pooled and concentrated using a rotary evaporator (Heidolph Laborota 4000, Germany) at 50 °C followed by oven-dried at 40 °C until paste-like mass extract was formed. The leaf extract of *G. inodorum* (LEGI) was kept in an air-tight glass container at −20 °C before the commencement of the experiments.

Determination of phytochemicals

Phytochemicals in LEGI were determined by using a gas chromatography-mass spectrometry (GC-MS) instrument (Agilent7890B GC System) equipped with a capillary column (Agilent HP-5MS UI; 30 m length × 0.25 mm internal diameter and 0.25 μm film thickness). For GC-MS detection, an electron ionization system was operated in the electron impact mode with an ionization energy of 70 eV. The temperature process of the column was initially at 80 °C for 6 min, followed by an increase of 5 °C/min to 200 °C and then 20 °C/min to 250 °C, and the ion-source temperature was 200 °C. The oven temperature was programmed from 45 °C (2 min), with an increase of 7 °C/min to 250 °C and maintained for 25 min. The carrier gas was helium with a flow rate of 1.0 mL/min. The mass spectrometer was operated in electron ionization with an acquisition mode scan of 35 - 500 amu. The ion source and transfer line temperatures were kept at 250 °C. The injection volume of 1 μL of the sample (0.1 % in absolute methanol) was split at a ratio of 250 : 1. The phytochemical components of LEGI were identified by comparison with standards, on the basis of their mass spectral fragmentation using the National Institute of Standards and Technology (NIST) and Wiley GC-MS libraries [13]. Identification of essential oil components by Gas Chromatography/mass Spectrometry. Allured Publishing Corporation. Carol Stream, IL, USA).

Determination of cytotoxicity

The macrophage cells, RAW 264.7 cells, were purchased from the American Type Culture Collection, ATCC®TIB-71™, USA. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC® 30-2002™ USA) supplemented with 5 % fetal bovine serum and 1 % antibiotics penicillin-streptomycin at 37 °C in a 5 % CO_2 incubator. The cells at a density of 1×10^5 cells/mL were prepared in a 25 cm^3 flask and incubated in a 5 % CO_2 incubator at 37 °C. After 24 h, the cultured medium was removed and the cells were washed twice with PBS. DMEM in a volume of 2 - 3 mL was added to the flask. The cells were counted and diluted to a density of 1×10^5 cells/ml in a 96-well plate for the determination of cell viability.

Cell viability was carried out using 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay, a colorimetric assay which is based on the conversion of MTT solution into formazan crystals by living cells. In this study, MTT assay was conducted to determine the viability of the Raw 264.7 cells after treatment with LEGI solutions (25, 50, 100, 150, 200, 250, 500, 750 and 1000 μg/mL). The RAW 264.7 cells (1×10^5 cells/well) were cultured in the 96-well plates containing DMEM cultured medium. The plates were incubated overnight at 37 °C in a 5 % CO_2 incubator for 24 - 48 h. The cultured medium was removed from each well and washed with PBS. LEGI solutions prepared by dissolving in dimethyl sulfoxide (DMSO) with a final DMSO concentration < 0.1 % (v/v), LPS (1 μg/mL), positive control (10 % DMSO), and negative control (Blank) in a volume of 200 μL/well were added to each well and incubated in a 5 % CO_2 incubator at 37 °C for 24 h. The cells were treated with various concentrations of LEGI solution for 24 h. The cultured medium was removed. A volume of 50 μL/well of 5 mg/mL MTT solution in PBS and a volume of 150 μL/well of fresh cultured medium were added to each well. The plates were incubated at 37 °C for 24 h. The solutions in the plates were removed. Then 100 μL of DMSO was added to each well and shaken gently for 15 min, in order to dissolve the formazan crystals. After gently shaking, cell viability was quantified by an absorbance measurement. The absorbance was measured at 570 nm using micro-plate reader (Multiskan Go, Thermo/Scientific, Netherlands). The percentage of the cell viability was computed using the following equation:

$$\% \text{ cell viability} = (\text{OD of treated sample}/\text{OD of untreated sample})\times100$$

Cytotoxicity of LEGI was expressed as LC_{50} (the concentration of LEGI required to produce 50 % cell viability of the RAW 264.7 macrophages).

The concentrations of LEGI which showed more than 80 % viable cells were used for determination of NO production.

Determination of NO production

NO production was determined by measuring nitrite concentration in the cultured medium. The nitrite concentration was quantified using the Griess assay, a simple technique that is widely used for the quantification/detection of NO [21]. The basic reaction involves reacting the Griess reagent, sulphanilamide and N-1-napthylethylenediamine dihydrochloride (NED), to form a stable azo compound.

The RAW 264.7 cells were cultured in DMEM and incubated at 37 °C in 5 % CO_2. The viable cells were counted and diluted with a medium to give a concentration of 1×10^5 cells/mL. These cells were cultured in each well of a 96-well plate and allowed to adhere for 1 h at 37 °C under 5 % CO_2. After that,

the cultured medium was removed from each well and washed with PBS. The LEGI solutions, positive control (Diclofenac) and LPS were added to the well and incubated in a 5 % CO_2 at 37 °C for 24 h. After the incubation period, the cultured medium was collected for the determination of NO production. The cultured medium treated with LEGI in a volume of 50 µL/well was added to the well. Sulfanilamide solution (50 µL) and NED solution (50 µL) were also added to the well and incubated at room temperature in dark place for 5 - 10 min. Absorbance (A) of the mixture solution was measured at 570 nm using micro-plate reader. Percentage (%) nitric oxide (NO) production was calculated using the following equation and compared to a positive control (Diclofenac):

% NO production = (A extract + LPS / A control + LPS)×100

Determination of α-glucosidase inhibitory activity

An assay of α-glucosidase inhibitory activity was carried out to determine the antidiabetic activity of LEGI. Inhibition on α-glucosidase activity was assessed according to [22], with slight modifications. Briefly, 60 µL of sample solution and 50 µL of 0.1 M phosphate buffer containing α-glucosidase solution (0.2 U/mL) were incubated in 96-well plate at 37 °C for 20 min. After pre-incubation, 50 µL of the substrate (5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) solution in 0.1 M phosphate buffer, (pH 6.8) was added to each well and incubated at 37 °C for another 20 min. A volume of 160 µL of 0.2 M Na_2CO_3 was then added to the well to terminate the reaction. The absorbance reading was recorded at 405 nm using the micro-plate reader and compared to the control which had 60 µL of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), the enzyme solution was replaced by buffer solution, and absorbance was recorded. Acarbose, a positive control of α-glucosidase inhibitor was also assayed. The % inhibition on α-glucosidase activity was calculated using the following equation:

% inhibition = [(Abs blank-Abs sample)/Abs blank]×100

where Abs blank is the absorbance of the control without the test solution, and Abs sample is the absorbance of the sample with the test solution.

IC_{50} value, the concentration of the LEGI required to inhibit 50 % of α-glucosidase activity was determined and compared to Acarbose.

Determination of DPPH scavenging activity

DPPH (2, 2-diphenyl-2-picrylhydrazylhydrate) scavenging activity of LEGI was determined spectrophotometrically. Various concentrations of LEGI (25, 50, 100, 200 and 400 µg/mL) prepared by dissolving LEGI in methanol were added to solution of DPPH in methanol. The mixture was shaken vigorously and left to stand at room temperature in a dark place for 30 min. After that, the absorbance at 517 nm, the maximum absorbance of DPPH, was recorded as Abs sample using an ultraviolet/visible (U/V) spectrophotometer. A blank experiment was also carried out using the same procedure, and the absorbance was recorded as Abs blank. The experiments were carried out in triplicates [8]. The percentage DPPH radical scavenging activity was calculated using the following equation:

% DPPH radical scavenging = [(Abs blank − Abs sample)/Abs blank]×100

where Abs blank is the absorption of blank sample, and Abs sample is the absorption of the LEGI tested solution.

DPPH radical scavenging activity of LEGI was expressed as LC50 (the concentration of LEGI required to inhibit 50 % of DPPH radical scavenging) and compared to that of butylated hydroxytoluene (BHT), an antioxidant compound.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). The data were first tested for normality and homogeneity. A comparison of the control and treatment groups was performed using one-way analysis of variance (ANOVA). Least Significant Difference (LSD) was performed to verify differences among treatment groups. Differences of $p < 0.05$ were considered statistically significant.

Results and discussion

Phytochemicals

GC-MS analysis demonstrated the presence of 83 phytochemicals with 5 main chemical components and 78 other prevalent compounds in LEGI. Retention time, compound name, molecular formula, and % of total of the chemical compounds were presented in **Figure 1** and **Table 1**. The first compound identified with less retention time (5.1833) was Z-8-Methyl-9-tetradecenoic acid whereas Betulin was the last compound identified which took the longest retention time (53.4554) for identification.

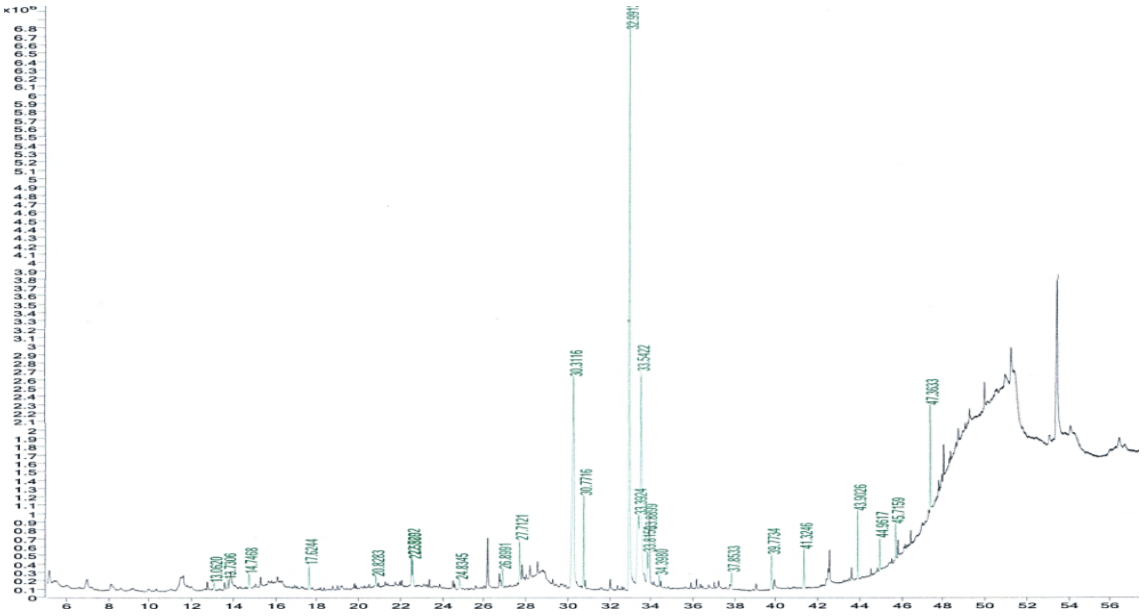

Figure 1 GC/MS chromatogram of phytochemicals found in LEGI.

Table 1 GC-MS of 83 chemical compounds found in LEGI.

No.	TR (min)	Compound name	Molecular formula	% of Total
1	5.1833	Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$	0.60
2	6.9859	Undecane, 2,6-dimethyl-	$C_{13}H_{28}$	0.61
3	8.1733	2-Pyrrolidinone	C_4H_7NO	0.53
4	8.6226	Benzenamine, N,N-dimethyl-	$C_8H_{11}N$	0.13
5	10.3181	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	0.11
6	11.6018	Benzoic acid	$C_7H_6O_2$	0.13
7	12.7411	Benzofuran, 2,3-dihydro-	C_8H_8O	0.26
8	13.0620	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	$C_7H_9NO_2$	0.22
9	13.5648	2-Piperidinemethanol, .alpha.-ethyl-	$C_8H_{17}NO$	0.23
10	13.7306	2,5-Pyrrolidinedione, 3-ethyl-3-methyl-	$C_2H_{11}NO_2$	0.32
11	13.8964	Acetic acid, phenyl-	$C_8H_8O_2$	1.24
12	14.7468	Indole	C_8H_7N	0.41
13	15.3138	Ethanone,1-(2-hydroxy-5methylphenyl)-	$C_9H_{10}O_2$	0.29
14	16.1108	2-Cyclopenten-1-one, 2-methyl-	C_6H_8O	0.19
15	16.9398	Benzeneacetic acid, alpha.-oxo-, ethyl ester	$C_{10}H_{10}O_3$	0.07
16	17.6422	Tetradecane	$C_{14}H_{30}$	0.63
17	18.3305	alpha-Ionone	$C_{13}H_{20}O$	0.05
18	18.7477	2-ethoxycarbonyl-5-oxo pyrrolidine	$C_7H_{11}NO_3$	0.09

No.	TR (min)	Compound name	Molecular formula	% of Total
19	18.9777	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	$C_{13}H_{22}O$	0.10
20	19.8067	3-Buten-2-one, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	$C_{13}H_{20}O$	0.12
21	20.5127	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	0.08
22	20.8283	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7atrimethyl-, (R)-	$C_{11}H_{16}O_2$	0.29
23	21.4006	Disulfide, di-tert-dodecyl	$C_{24}H_{50}S_2$	0.07
24	21.9783	Fumaric acid, ethyl 2-methylallyl ester	$C_{10}H_{14}O_4$	0.12
25	22.0746	3-Methyl-4-phenyl-1H-pyrrole	$C_{11}H_{11}N$	0.16
26	22.5292	Hexadecane	$C_{16}H_{34}$	0.71
27	22.5880	2-Dimethylaminobenzoic acid	$C_9H_{11}NO_2$	0.72
28	23.3903	2,6,8-Trimethylbicyclo[4.2.0]oct-2-ene-1,8-diol	$C_{11}H_{18}O_2$	0.16
29	23.8771	2,3-Dioxabicyclo[2.2.2]oct-5-ene, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{16}O_2$	0.09
30	24.5189	3-Buten-2-one, 4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)	$C_{13}H_{20}O_3$	0.24
31	24.5992	3-Hydroxy-7,8-dihydro-.beta.-ionol	$C_{13}H_{20}O_2$	0.30
32	24.8345	4-(3-Hydroxybutyl)-3,5,5-trimethyl-2-cyclohexen-1-one	$C_{13}H_{22}O_2$	0.30
33	25.6047	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a tetra hydrobenzofuran-2(4H)-one	$C_{11}H_{16}O_3$	0.09
34	26.1877	Myristic acid	$C_{14}H_{28}O_2$	1.69
35	26.7386	(S,E)-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	$C_{13}H_{18}O_3$	0.37
36	26.7921	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	0.17
37	26.8991	Octadecane	$C_{18}H_{38}$	0.42
38	27.7121	Neophytadiene	$C_{20}H_{38}$	1.07
39	27.8298	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	0.59
40	28.2042	9-Octadecenoic acid (Z)-	$C_{18}H_{34}O_2$	0.46
41	28.5679	Phytol, acetate	$C_{22}H_{42}O_2$	0.33
42	29.2900	5,9,13-Pentadecatrien-2-one,6,10,14-trimethyl, (E,E)-	$C_{18}H_{30}O$	0.11
43	29.7767	Palmitoleic acid	$C_{16}H_{30}O_2$	0.17
44	30.3116	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	12.45
45	30.7716	Hexadecanoic acid, ethyl ester	$C_{16}H_{36}O_2$	2.14
46	30.8571	Icosane	$C_{20}H_{42}$	0.16
47	31.6327	Cyclopentadecanone, 2-hydroxy-	$C_{15}H_{28}O_2$	0.12
48	32.0499	Heptadecanoic acid	$C_{17}H_{34}O_2$	0.36
49	32.4029	1-Tetradecanol	$C_{14}H_{30}O$	0.11
50	32.6169	Heptadecanoic acid, ethyl ester	$C_{19}H_{38}O_2$	0.08
51	32.7238	10-Heptadecen-8-ynoic acid, methyl ester, (E)-	$C_{18}H_{30}O_2$	0.08
52	32.9913	Phytol	$C_{20}H_{40}O$	18.14
53	33.3924	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	3.92
54	33.5422	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	10.39
55	33.8150	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	0.70
56	33.8899	Octadecanoic acid	$C_{18}H_{36}O_2$	1.16
57	33.9327	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	$C_{20}H_{34}O_2$	1.74
58	34.3980	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	0.26
59	34.4782	Octadecane	$C_{18}H_{38}$	0.14

No.	TR (min)	Compound name	Molecular formula	% of Total
60	34.8312	Phytol, acetate	$C_{22}H_{42}O_2$	0.04
61	35.9117	Octanoic acid, 2-dimethylaminoethyl ester	$C_{12}H_{25}NO_2$	0.14
62	36.1791	Pentacosane	$C_{25}H_{52}$	0.25
63	36.3717	Palmitoyl chloride	$C_{16}H_{31}ClO$	0.16
64	37.0403	4,8,12,16-Tetramethylheptadecan-4-olide	$C_{21}H_{40}O_2$	0.13
65	37.2489	Eicosanoic acid	$C_{20}H_{40}O_2$	0.20
66	37.7784	Eicosanoic acid, ethyl ester	$C_{22}H_{44}O_2$	0.11
67	37.8533	Tetracosane	$C_{24}H_{50}$	0.47
68	39.7734	Pentacosane	$C_{25}H_{52}$	1.01
69	39.9072	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	0.33
70	41.3246	Hexacosane	$C_{26}H_{54}$	0.96
71	42.5120	Ethyl (9Z,12Z)-9,12 Octadecanoate	$C_{20}H_{36}O_2$	0.26
72	42.5708	Heptacosane	$C_{27}H_{56}$	0.66
73	43.6192	Tricosane	$C_{23}H_{48}$	0.25
74	43.9026	Squalene	$C_{30}H_{50}$	1.25
75	44.5498	Pentatriacontane	$C_{35}H_{72}$	0.13
76	44.9617	2,8-Dimethyl-2-(4,8,12-trimethyl tridecyl)-6-chromanol	$C_{27}H_{46}O_2$	0.61
77	45.8282	gamma.-Tocopherol	$C_{28}H_{48}O_2$	0.25
78	46.4486	Vitamin E	$C_{29}H_{50}O_2$	0.21
79	47.3633	Stigmasterol	$C_{29}H_{48}O$	1.98
80	47.7591	Stigmast-5-en-3-ol	$C_{29}H_{50}O$	0.20
81	48.0105	beta-Amyrin	$C_{30}H_{50}O$	0.54
82	49.9734	3 beta.-Myristoylolean-12-en-16.beta.-ol	$C_{44}H_{76}O_3$	0.58
83	53.4554	Betulin	$C_{30}H_{50}O_2$	8.56

The 5 main chemical components found in LEGI were Phytol (18.14 %), n-Hexadecanoic acid (12.45%), 9, 12-Octadecadienoic acid (Z,Z)-(3.92%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-(10.39 %), and Betulin (8.56 %) as shown in **Table 2**.

Table 2 The 5 main chemical compounds found in LEGI.

Compound name	Molecular formula	Groups/activities
n-Hexadecanoic acid (12.45%)	$C_{16}H_{32}O_2$	Saturated fatty acid Anti-inflammatory and antioxidant [23-26]
Phytol (18.14 %)	$C_{20}H_{40}O$	Diterpene compound Antioxidant, anti-inflammatory and antihyperglycemic [26-32]
9,12-Octadecadienoic acid, (*Z,Z*)- (3.92%)	$C_{18}H_{32}O_2$	Unsaturated fatty acid (Essential fatty acid) Inhibitory effect on α-glucosidase and antioxidant [34]
9,12,15-Octadecatrienoic acid, (*Z,Z,Z*)- (10.39 %)	$C_{18}H_{30}O_2$	Unsaturated fatty acid (Essential fatty acid) Antioxidant and hypoglycemic [35]
Betulin (8.56 %)	$C_{30}H_{50}NO_2$	Triterpene Anti-inflammatory and antidiabetic [37,38]

Based on the results in **Table 2**, the 5 main compounds found in LEGI were fatty acid both saturated (n-Hexadecanoic acid) and unsaturated fatty acid (9,12-Octadecadienoic acid, (Z,Z)- and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-), diterpene (Phytol), and triterpene (Betulin). Wongnoppavich *et al.*[12] found that total phenolic and flavonoid contents were more intensive in GI ethanolic extracts. Moreover, polyphenols and flavonoids are the major compounds in GI leaf extract [4].

Pharmacological activities of active compounds have been reported. n-Hexadecanoic acid has anti-inflammatory and antioxidant activities [23-26]. Phytol has antioxidant, anti-inflammatory and antihyperglycemic activities [26-33]. 9,12-Octadecadienoic acid, (Z,Z)- has inhibitory effect on α-glucosidase and antioxidant activities [34]. 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- has anti-oxidant and hypoglycemic activities [35-36]. Betulin has anti-inflammatory and antidiabetic activities [37,38].

The results from the present study suggest that LEGI are rich with various bioactive compounds including the anti-inflammatory compound such as n-Hexadecanoic acid and Phytol, the hypoglycemic/anti-hyperflycemic/anti- diabetic compounds such as Phytol, 9,12-Octadecadienoic acid, (Z,Z)- and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- and the antioxidant compounds such as n-Hexadecanoic acid, phytol, 9,12-Octadecadienoic acid, (Z,Z)- and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-.

Cytotoxicity

Investigation on cytotoxicity of LEGI in the RAW 264.7 cells after treating with various concentrations of LEGI (25 to 1,000 µg/mL) using MTT assay revealed that cytotoxicity was found when LEGI at the concentrations higher than 100 µg/mL were applied. The cell viability of the RAW 264.7 cells were 97.45±1.67, 77.97±2.13, 64.19±2.82, 25.83±3.62, 8.06±1.26, 5.11±0.74, 2.13±0.02, 2.10±0.02, and 2.12±0.02 % after treating with LEGI at the concentrations of 25, 50, 100, 150, 200, 250, 500, 750, and 1000 µg/mL, respectively (**Table 3**).

Table 3 Cell viability of RAW 246.7 cells after treating with LEGI.

Concentrations (µg/mL)	Cell viability of RAW 246.7 cells LEGI + LPS 1 µg/mL	
	(%)	(IC_{50})
25	128.82±1.97^{g}	128.77±2.82 µg/mL
50	115.46±2.39^{f}	
100	83.27±2.62^{e}	
150	46.19±2.82^{d}	
200	25.83±3.62^{c}	
250	8.06±1.26^{b}	
500	5.97±0.74^{b}	
750	2.26±0.12^{a}	
1000	2.02±0.57^{a}	

Values are expressed as mean ± SEM of 3 independent experiments. Mean ± SEM followed by different superscripts$^{(a,b,c)}$ in the same column indicate significant differences at $p < 0.05$.

LEGI at the concentrations of 25, 50 and 100 µg/mL exhibited the cell viability values of the RAW 246.7 cells more than 80%. Thus they were used further to investigate NO production. LEGI exhibited cytotoxicity on the RAW 246.7 cells with IC50 of 128.77±2.82 µg/mL (**Table 4**). This result was not consistent with the published reports. No adverse reactions were found in a long-term study of insulin-dependent diabetic patients who consumed or received GI in any form [39,40]. In an acute toxicity study in mice, alterations of gross behavioral, neurological, or autonomic effects did not occur. The acute LD_{50} was 3990 mg/kg. The safety ratio (LD_{50} /ED_{50}) was 11 and 16 in normal and diabetic rats, respectively [39, 41,42]. No side effects were found upon the administration of GI at doses of 504 - 563 mg/kg/d in men [43]. GI extract revealed non-toxic in mice liver and kidney [1]. The reliable toxic dose of GI has not been found. The LD_{50} in mice and rats is greater than 5 g/kg [44].

NO production

NO production quantified by measuring nitrite concentration revealed that LEGI at the concentrations of 25, 50 and 100 mg/mL inhibited NO production in the LPS-stimulated RAW 264.7 cells concentration-dependently by 14.07±1.82, 16.73±1.52 and 28.30±1.24 %, respectively. At the concentration of 50 µg/mL,

LEGI inhibited NO production in the LPS-stimulated RAW264.7 cells less potent than Diclofenac did (16.73±1.52 % vs. 27.07±1.81 %) as shown in **Table 4**.

During inflammation, free radical molecule such as NO is generated by inflammatory cells. LEGI inhibited NO production in the LPS-stimulated RAW 264.7 cells suggesting the anti-inflammatory activity of LEGI. This result is concomitant with the study by Wongnoppavich *et al*. [12] who found that GI leaf extract possessed anti-inflammatory effects by inhibiting the NO production in the LPS-activated RAW 264.7 macrophages.

Table 4 Inhibition on NO production (%) in the LPS-stimulated RAW 246.7 cells.

Concentrations (µg/ml)	Inhibition on NO production (%)	
	LEGI	Diclofenac
25	14.07 ± 1.82^{a}	-
50	16.73 ± 1.52^{aA}	27.07 ± 1.81^{B}
100	28.30 ± 1.24^{b}	-

Values are expressed as mean ± SEM of 3 independent experiments. Mean ± SEM followed by different superscripts $^{(a,b,c)}$ in the same column and different superscripts$^{(A,B)}$ in the same row indicate significant differences at $p < 0.05$.

Inhibition on α- glucosidase activity

The *in vitro* enzymatic model revealed that LEGI exhibited significant inhibitory activity against α-glucosidase concentration-dependently by 16.50 ± 0.02, 23.12 ± 0.02, 38.35 ± 0.04, 51.34 ± 0.05, 59.86 ± 0.02, and 67.47 ± 0.03 % at the concentrations of 0.04, 0.08, 0.16, 0.31, 0.63 and 1.25mg/mL, respectively with IC_{50} of 0.31 mg/mL. However, at the same concentrations, its activity was less potent than that of Acarbose, a positive control which was 19.40±0.04, 30.38±0.00, 48.85±0.01, 63.60±0.20, 71.39±0.21 and 82.80±0.01% respectively with IC_{50} of 0.20 mg/mL. The results are summarized in **Table 5** and **Figures 2** and **6**. The results are in line with the study by Srinuanchai *et al.* [13] who found that the ethanolic extract of GI exhibited an inhibitory effect on α-glucosidase. The GI hypoglycemic effect of GI has been documented, such as consumption of GI tea with a meal or 15 min after a meal can significantly decrease peak plasma glucose [3]. GI exhibited hypoglycemic effect caused by triterpenoid saponins or gymnemic acids (GA) which inhibited glucose absorption from the intestinal tract and suppress the increase in blood glucose level in oral glucose tolerance tests in rats [47]. GA is thought to help the body maintain healthy blood glucose levels [45], can improve and restore the blood vessel which damaged by hyperglycemia in diabetes mellitus [46].

GI ethanol extract significantly delayed glycemic absorption by lowering α-glucosidase activity [11]. The antidiabetic or hypoglycemic effect of GI is a property of a substance called gymnemic acid [45]. The component of GI extract inhibited the increase in the blood glucose level by interfering with intestinal glucose absorption [47,48]. The GI leaf extract administered to the patient stimulates the pancreas and increases the release of insulin [49]. However, Chiabchalard *et al.* found that the hypoglycemic effect of GI does not relate to increase insulin secretion or inhibition of the α-glucosidase enzyme [3]. Anti-glycemic properties of the GI leaf extract were dependent on ethanol concentrations [11]. Moreover, the hypoglycemic effect of GI was dose-dependent. Nevertheless, GI tea had no effect on blood sugar in type 2 diabetic patients, this might be due to insufficient doses [14].

Table 5 Inhibitory effect of LEGI on α-glucosidase activity.

Samples	Concentrations (mg/mL)	Inhibitory effect on α-glucosidase activity	
		% Inhibition	IC_{50} (mg/mL)
LEGI	0.04	16.50 ± 0.02^{a}	0.31^{b}
	0.08	23.12 ± 0.02^{b}	
	0.16	38.35 ± 0.04^{c}	
	0.31	51.34 ± 0.05^{d}	
	0.63	59.86 ± 0.02^{e}	
	1.25	67.47 ± 0.03^{f}	

Samples	Concentrations (mg/mL)	Inhibitory effect on α-glucosidase activity	
		% Inhibition	IC_{50} (mg/mL)
Acarbose	0.04	19.40 ± 0.04[a]	0.20[a]
	0.08	30.38 ± 0.00[c]	
	0.16	48.85 ± 0.01[d]	
	0.31	63.60 ± 0.20[f]	
	0.63	71.39 ± 0.21[g]	
	1.25	82.80 ± 0.01[h]	

Values are expressed as mean ± SEM of 3 independent experiments. Mean ± SEM followed by different superscripts[(a,b,c..)] in the same column indicate significant differences at $p < 0.05$.

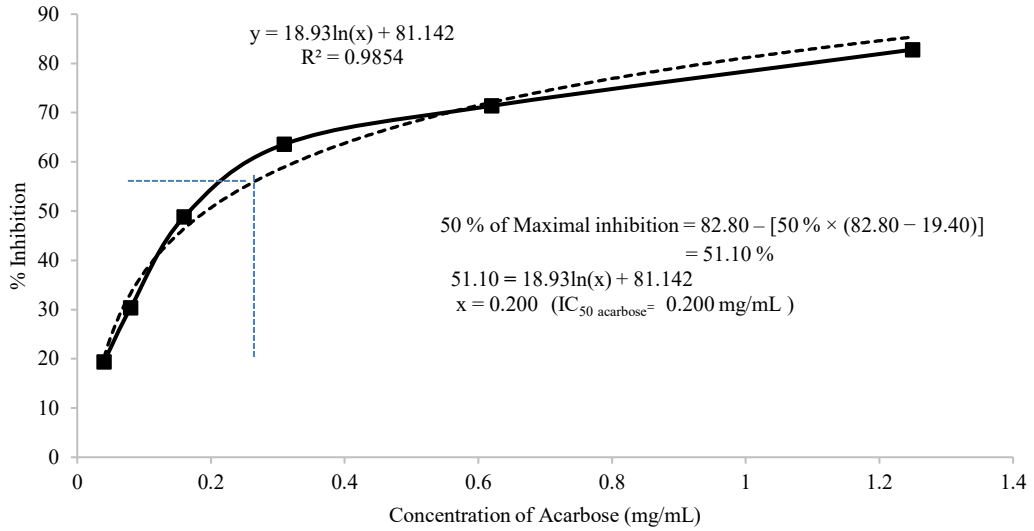

Figure 2 Inhibitory effect of Acarbose on α-glucosidase activity (IC_{50}).

DPPH scavenging activity

DPPH radical scavenging assay showing in **Table 6** demonstrated that LEGI at the concentrations of 0.2, 0.4 and 0.8 mg/mL exhibited concentration-dependently DPPH radical scavenging activity (**Figure 3**) by 29.24±1.06, 43.43±3.04 and 54.39±2.10 %, respectively with IC_{50} of 0.41 mg/mL. At the same concentrations, LEGI exhibited DPPH radical scavenging activity significantly ($p < 0.05$) less than BHT did (47.80±0.63, 60.14±0.77 and 80.69±0.64 %, respectively (**Figure 3**) with IC_{50} of 0.36 mg/mL). This result is in agreement with that in previously published literature. Wongnoppavich *et al.* [12] reported the ethanolic extract from GI leaves exhibited free radical scavenging effect on the DPPH assay.

Many published data highlight the antioxidant activity of GI and the phytochemical compounds which may act as antioxidants. The chemicals such as vitamin C, vitamin E, carotenoids, and phenolic compounds are contributors to the antioxidant activity in GI [8]. GI juice exhibited the strongest antioxidant activity, partly due to the presence of high amount of vitamin E and β-carotene [10]. GI exhibited the highest antioxidant activity when compared to *Piper sarmentosum* and *Mentha arvensis* [8]. Total phenolic and flavonoid contents in the GI ethanolic leaf extract exhibited DPPH radical scavenging effect [12]. Water and ethanol extracts of GI leaves showed high antioxidant activity with polyphenols as the major antioxidant [16]. Moreover, antioxidant potential may be due to the conditions of extraction such as the extracted solvents and the process of extraction. The fresh juice of GI had the highest antioxidant activity compared to GI water extract and 50 % ethanol extract [2]. The antioxidant activity using DPPH assay was significantly different in GI tea process [50]. Additionally, antioxidant capacity of GI was mainly owing to the presence of water-soluble compounds [19].

Table 6 Effect of LEGI on DPPH scavenging activity (%).

Samples	Concentrations (mg/mL)	% DPPH scavenging	IC_{50} (mg/mL)
LEGI	0.2	29.24 ± 1.06[a]	0.41
	0.4	43.43 ± 3.04[b]	
	0.8	54.39 ± 2.10[c]	
BHT	0.2	47.80 ± 0.63[b]	0.36
	0.4	60.14 ± 0.77[d]	
	0.8	80.69 ± 0.64[e]	

Values are expressed as mean ± SEM of 3 independent experiments. Mean ± SEM followed by different superscripts[(a,b,c)] in the same column indicate significant differences at $p < 0.05$.

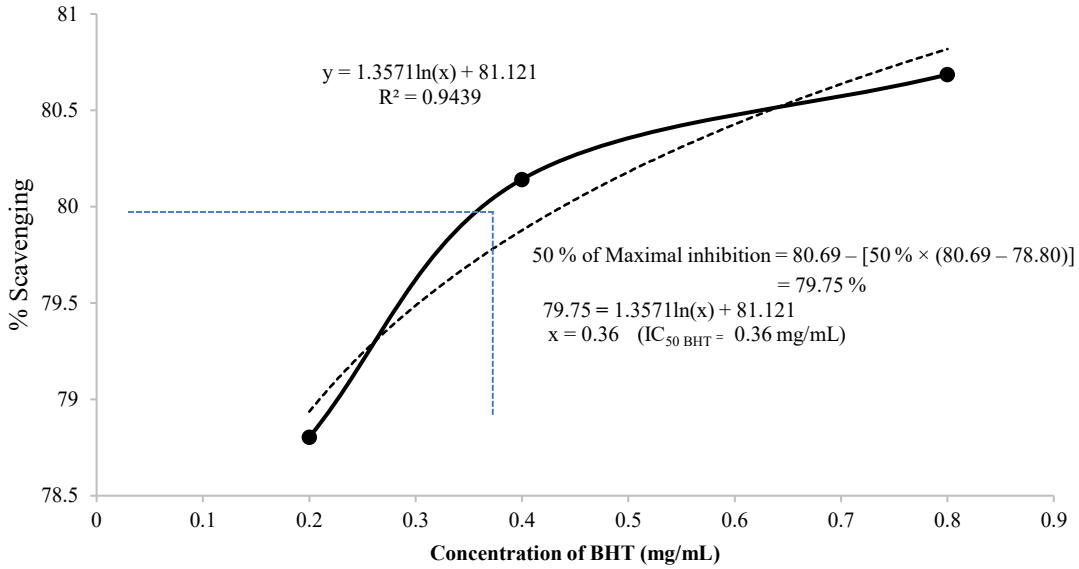

Figure 3 Effect of BHT on DPPH scavenging activity (IC_{50}).

The chemicals compounds with antioxidant activity, such as n-hexadecanoic acid, hexadecanoic acid ethyl ester and 9, 12, 15-octadecatrienoic acid, ethyl ester (Z,Z,Z) were found in LEGI. Therefore, the antioxidant activity of LEGI may be due to the synergistic effects of these compounds.

Conclusions

This study, the ethanolic leaf extract of *G. inodorum* exhibits anti-inflammatory, antidiabetic and antioxidant activities. The most predominant compound is n-Hexadecanoic acid followed by Phytol. The extract exhibits anti-inflammatory activity by inhibition of NO production, displays antidiabetic activity via inhibition on α-glucosidase activity, and shows antioxidant activity by DPPH radical scavenging. Besides, it exerts relatively low cytotoxicity to the RAW 264.7 cells, by MTT assay. Thus, the ethanolic leaf extract of *G. inodorum* could be considered as a good natural source for the development of novel anti-inflammatory, antidiabetic and antioxidant agents. Due to these activities of the extract has not been conducted directly, the mechanism of actions especially in in vivo and the active compounds which are responsible to these activities are needed to perform.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi (RMUTT), Pathum Thani, Thailand.

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