European Journal of Medicinal Plants
17(4): 1-9, 2016; Article no.EJMP.31403
ISSN: 2231-0894, NLM ID: 101583475

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HPLC/MS/MS Study of Phenolic Compounds of *Leucaena leucocephala* Legumes Monitored with Their *in vitro* Antihyperglycemic Activity

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Authors' contributions

This work was carried out in collaboration between both authors. They designed the study and wrote the protocol. Author NMA collected the plant material, carried out phytochemical procedures, analyses and interpretation of the identified compounds while author GMA carried out in vitro screening of the antihyperglycemic activity of different extracts and fractions, performed statistical data and interpretation of the gained results. Both authors managed the literature, wrote the first draft and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2016/31403
Editor(s):
(1) Thomas Efferth, Chair, Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Germany.
(2) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.
Reviewers:
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(4) Catalina Leos Rivas, Universidad Autonoma de Nuevo Leon, México.
Complete Peer review History: http://www.sciencedomain.org/review-history/17867

Original Research Article

Received 4th January 2017
Accepted 11th February 2017
Published 16th February 2017

ABSTRACT

High pressure liquid chromatography coupled with mass spectrometry (HPLC/MS/MS) is considered as one of most the sensitive modern techniques used for determination of phenolic compounds, hence in this research it was utilized for characterization of a variety of phenolic compounds in *Leucaena leucocephala* legumes growing in Egypt.
HPLC gradient elution analysis using water and acetonitrile, both containing 0.1% formic acid was carried out for standard solutions of the available phenolic and flavonoid compounds as well as the investigated fraction in the negative ion mode using MS/MS product ion scans where the

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deprotonated molecules $[M–H]^-$ were observed for all the studied compounds; for flavonol and flavone glycosides, the spectra exhibit both the deprotonated molecule $[M–H]^-$ of the glycoside and the ion corresponding to the deprotonated aglycone $[A–H]^-$, the latter ion is formed by loss of the sugar residue from the glycosides; different fragmentation pattern was observed for flavone-C-glycosides which involved additional fragmentation in the sugar part while for methyl ether aglycones, formation of $[M–CH_3]^{-\bullet}$ was observed.

The gained results revealed the elution of eleven phenolic compounds from the ethyl acetate fraction, namely; isovanillin, gallic acid, caffeic acid, apigenin-8-*C*-glucoside (vitexin), quercetin 3-*O*-galactoside (hyperoside), luteolin-7-*O*-glucoside, quercetin, isorhamnetin, luteolin 6-methyl ether (neptein), kaempferol 3,7-dimethyl ether and 5,7,3',4'-dihydroquercetin tetramethyl ether (taxifolin 5,7,3',4'- tetramethyl ether) successively.

The methanol extract and different fractions of *Leucaena leucocephala* legumes were subjected to *in vitro* evaluation of their α-glucosidase inhibitory activity at different dose levels compared to the reference standard drug "Acarbose", the gained results revealed that methanol extract and ethyl acetate fraction exhibited significant percent inhibitory activity at dose level of 2.5 $mgml^{-1}$ (71.55±0.009%, IC_{50} 0.210±0.003 mg ml^{-1} & 65.44±0.005%, IC_{50} 0.270±0.042 mg ml^{-1}) respectively compared to Acarbose (55.45±0.051%, IC_{50} 0.650±0.051 mg ml^{-1}) which affords both as effective natural antihyperglycemic drugs.

Keywords: Phenolic; flavonoids; Leucaena leucocephala; HPLC/MS/MS; antihyperglycemic.

1. INTRODUCTION

Leucaena is a genus of flowering plants belonging to subfamily Mimosoideae, family Fabaceae, it contains about 22 species of trees and shrubs, which are commonly known as lead trees [1], they are native to the Americas [2] but are now naturalized throughout the tropics [3], among which the *Leucaena leucocephala* which is a one of the fastest growing invasive species of woody plants distributed throughout the tropics and subtropics, it was referred to as the "miracle tree" due to its medicinal efficacy and multipurpose nature [3,4].

Leucaena leucocephala whole aerial parts were reported to contain flavonoid constituents namely, caffeic acid, isorhamnetin, chrysoeriol, isorhamnetin 3-*O*-galactoside, kaempferol-3-*O*-rubinoside, quercetin-3-*O*-rhamnoside, luteolin-7-glucoside [5], quercetin-3-*O*-α-rhamnopyranosyl -(1'''→2'')–*β*-glucopyranoside, quercetin-7-*O*-*α*–rhamnopyranosyl-(1'''→2'')-*β*-gluco-pyranoside, quercetin-3-*O*-*α*-rhamnopyranoside, quercetin-3-*O*-*β*–glucopyranoside, isovitexin, vitexin, acylated flavanol glycoside "quercetin–3–*O*-(2''–trans–*p*–coumaryl)–α–rhamnopyranosyl-(1'''→6'') -β-glucopyranoside", quercetin [6], steroid constituents; 5α,8α-epi-dioxy-(24ζ) -ergosta-6,22-dien-3β-ol, β-sitosterol, β-sitostenone & stigmastenone; a terpenoid 'lupeol' ; one glyceride; 1,3-dipalmitoyl- 2-oleoylglycerol; one alkanoid: linoleic acid; two benzenoids; methylparabene and isovanillic acid and five chlorophylls; pheophytin-a, pheophorbide a methyl ester, methyl- 13^2 - hydroxy - (13^2-S) - pheophorbide-b, 13^2 - hydroxy - (13^2-S) - pheophytin-a and aristophyll-C [7].

Leucaena leucocephala seeds contain fixed oil , tannins, sulphated polysaccharides [8,9], proteins; the most significant is memosine [10], alkaloids, saponins, flavonoids, mimosin, leukanin, protein, fat, tannins, calcium, phosphorous, iron, vitamin A and vitamin B [11]; the whole legumes contain red, brown and black dyes which are tannin in nature and are used as natural dyes in leather and cotton industries [12] while, the roots are studied for tannin content as gallocatechin, epigallocatechin, catechin and epicatechin [13].

The reported medicinal uses of different parts of *Leucaena leucocephala* are versatile, they include central nervous system depressant, anthelmintic, anti-inflammatory, antioxidant, antidiabetic, contraceptive, abortive and nutritive activities [6,14-16] meanwhile, the seeds exhibited cancer chemo-preventive, anti-proliferative [10] and hair growth inhibitory activities [17], moreover, the seed gum has been reported to be useful as a tablet binder [18].

High pressure liquid chromatography coupled with mass spectrometry (HPLC/MS/MS) is one of the modern sensitive, accurate and precise techniques used for characterization of phenolic compounds [19], it is considered as one of the powerful tools for their investigation due to their soft ionization, in addition, it is one of the most

favored techniques for analysis of flavonoids as they are polar, non-volatile, and thermo-labile compounds [20-22].

In this current research work, HPLC/MS/MS technique with heated electrospray ionization (HESI) mode is used for characterization of phenolic and flavonoid compounds in the ethyl acetate fraction of *Leucaena leucocephala* legumes was carried out monitored with screening of their *in vitro* antihyperglycemic activity.

2. MATERIALS AND METHODS

2.1 Plant Material

Legumes of *Leucaena leucocephala* were collected during their flowering and fruiting stage from National Agricultural Center, Giza, Egypt in July 2015, their identities were established by Prof. Dr. Abdo Marey, Prof. of Botany, Faculty of Science, Al-Azhar University. Voucher specimens were deposited in a herbarium in department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, they were washed with distilled water, oven dried at 50°C, powdered and kept in tightly closed amber coloured glass containers protected from light at low temperature.

2.2 Extraction and Fractionation

500 g of powdered *Leucaena leucocephala* legumes were exhaustively extracted with 1500 ml of 80% (v/v) aqueous methanol for one hour to yield the methanol extract which was subsequently filtered under vacuum through Whatmann No. 1 filter paper, the residue was re-extracted following the same procedure two more times, the filtered extracts were pooled together and concentrated under vacuum at 40°C to dryness to yield the collective methanol extract (65 g).

50 g of the methanol extract were suspended in sufficient volume of distilled water and successively fractionated using n-hexane, methylene chloride and ethyl acetate where each fraction was concentrated under reduced pressure at 40°C; they yielded 11 g, 8 g and 15 g respectively.

10 g of the ethyl acetate fraction were chromatographed on 200 g Silica gel CC (C_{18}-reversed phase silica gel, 40-63 µm, 230-400 mesh, 90 A° pore size, Sigma-Aldrich) packed on glass column (5 cm, diameter, 1m length), elution with gradient mode was carried out starting water 100% to methanol 100% at flow rate 10 ml min^{-1} to give 38 fractions, 100 ml each. The collected fractions were monitored with paper chromatography Whatmann No.1 sheets for PC (Whatmann Ltd., Maidstone, Kent, and England) using S_1; n-butanol: acetic acid: water (4:1:5 v/v), upper layer, S_2; acetic acid: water (15:85 v/v) as solvent systems, the resultant spots were visualized with ferric chloride [23] and aluminum chloride [24] spray reagents for phenolic compounds and flavonoids respectively where similar fractions were pooled together to give three collective subfractions A, B and C.

2.3 Phenolic Standards

Quercetin from Sigma (St. Louis, MO, USA), quercetin 3-O-galactoside (hyperoside), isorhamnetin, luteolin-7-*O*-glucoside, isovanillin, apigenin-8-*C*-glucoside (vitexin), luteolin 6-methyl ether (Neptein) were obtained from Pharmacognosy Department, Al- Azhar University Cairo, Egypt while gallic and caffeic acids (Fluka, Buchs, Switzerland) were obtained from Faculty of Pharmacy, Deraya University, El-Minya, Egypt.

2.4 HPLC/MS/MS Analysis

HPLC apparatus used consisted of Accela 1200 LC-10AD pump, auto sampler Accela and a Hypersill gold (2.1 µm) (Phenomenex) 50X2.0 mm preceded by a C_{18} security guard cartridge Gemini 5 µm C_{18} (Phenomenex) 4 x 3 mm. Gradient elution was carried out with water: 0.1% formic acid (solvent A) and acetonitrile: 0.1% formic acid (solvent B) at a constant flow rate of 300 µl min^{-1} where acetonitrile (HPLC grade) from SDS (Peypin, France), formic acid from Probus (Badalona, Spain) and Ultrapure water (Milli-Q).

Mass spectrometric analysis was carried out using a TSQ Quantum Access MAXtriple quadrupole system. Data acquisition for quantification and confirmation are performed in Full scan mode. Samples are individually tuned for each target analyte by direct injection of individual solutions (1 mg ml^{-1}), data acquisition and processing is performed using Thermo Scientific Xcalibur 2.1 software.

The conditions used were as follows; ionization mode: Heated Electrospray (HESI), Polarity: Negative ion mode; Spray voltage: 3000 Volt; Scan type: Full scan; Ion sweep gas pressure: 0 arb; Vaporizer temperature: 400°C; Sheath gas

pressure: 25 arb; Aux gas pressure: 5 arb; Capillary temperature: 370°C; Collision gas pressure: 0 mTorr; Cycle time: 0.6 seconds & Peak width was full width of a peak at half its maximum height (FWHM) of 0.70 Da. HPLC/ MS/MS analysis procedure was carried out in the Central Laboratory, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

2.5 Antihyperglycemic Activity

α-glucosidase inhibition can be used as reflective parameter for evaluating antihyperglycemic activity [25] i.e. drugs capable of being competitive inhibitors of intestinal α-glucosidase enzyme can delay the digestion and subsequent absorption of glucose resulting in deceasing the elevated blood glucose levels [26].

In-vitro assessment of α-glucosidase inhibition capacity was carried out as per standard procedure [27] where 200 µl of α-glucosidase enzyme solution, Sigma (St. Louis, MO, USA) was pre-incubated with methanol extract, different fractions and Acarbose (Bayar) as standard antihyperglycemic drug at dose levels of 2.5, 1.250, 0.625 & 0.313 mg ml^{-1} solutions for 5 min, the reaction was initiated by adding 200 µl of 37 mM sucrose as substrate to all the test tubes, then all tubes were incubated for 30 min at 37°C to allow enzymatic action as well as drug action, then the enzymatic action was terminated by heating at 100°C for 10 min. The liberated glucose was determined spectrophotometrically using Genesys Spectrophotometer (Milton Roy, INC., Rochester, NY) by glucose oxidase-peroxidase (GOD-POD) method at 546 nm and by calculating with relative blank controls, the α-glucosidase inhibitory activity of the test drug was calculated as follows;

> % α-glucosidase inhibition =
> [{Absorbance (blank) - Absorbance (test/ standard)} / Absorbance (blank)] X 100

The results were expressed also in the form of IC_{50} values (mg ml^{-1}) where IC_{50} is the concentration of extract required to inhibit 50% of the enzyme activity at standard conditions.

2.6 Statistical Analysis

The percent activity was plotted against the sample concentration and a linear regression test was done using Graph Pad INSTAT and IC_{50} value was interpolated from standard curve, all tests were done in triplicates and results were expressed in mean ± standard error of mean (SEM). The data was analyzed statistically using Tukey-Kramer test.

3. RESULTS AND DISCUSSION

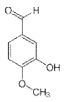
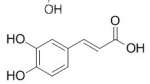
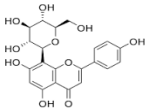
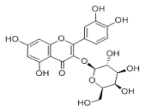
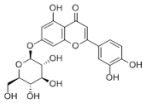
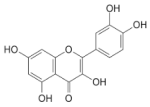
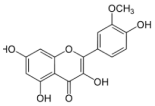
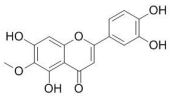
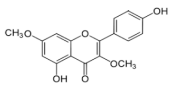
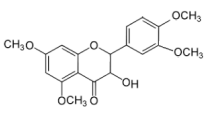
3.1 HPLC/MS/MS Identification of Phenolic Compounds in Ethyl Acetate Fraction

The three gained collective subfractions A, B and C were subjected to HPLC/MS/MS analysis in the fullscan mode where the results gained for subfraction A were as follows; the spectra generated revealed the existence of three phenolic compounds namely; isovanillin, gallic acid and caffeic acid (t_R 3.25, 4.40 and 6.15 min), subsequently they were fully identified by comparison with the retention time of their standards, their fragmentation patterns showing their *m*/*z* 151, 169 and 179 corresponding to the deprotonated molecules respectively, moreover, some other fragments appeared demonstrating the loss of CO_2 molecules for all of them giving the $[M–H–44]^-$ as characteristic ions, the gained data is matched with the previously published data concerning caffeic acid [6] while this is the first report for the existence of isovanillin and gallic acid in *L. leucocephala* legumes, Table 1.

Concerning the subfraction B, three flavonoid glycosides were eluted, the first two are flavonol and flavone *O*-glycosides namely; quercetin 3-*O*-galactoside (hyperoside) and luteolin-7-*O*-glucoside (t_R 10.85 & 11.25 min) were existing in the fullscan mode respectively, when traced, their mass spectra were characterized by the existence of both deprotonated molecule $[M–H]^-$ of the glycosides and the ion corresponding to the deprotonated aglycone $[A–H]^-$, the latter ion is formed by loss of the galactose and glucose moieties from the glycoside, this behavior was confirmed by the existence of their corresponding fragments (*m/z* 462 & 301 and 447 & 285) respectively [5,20], the third peak observed with t_R 10.45 min has product ion mass spectra showed ions at *m*/*z* 311 (loss of 120 u) and *m*/*z* 341 (loss of 90 u) which are characteristic of flavone *C*-glucosides those exhibit fragmentation pattern different from those of the *O*-glucosides involving cleavage of the sugar moieties as per the data published is several researches [28-30], moreover, they reported also that the characteristic fragment ions of $[M–H]^-$ (*m/z* 431) which allowed the differentiation between *C*-glycosylation at the 6- & 8-positions, hence the flavone vitexin (apigenin-8-*C*-glucoside) was preliminary identified as it showed these ions as

characteristic ions in the MS/MS mode; the deprotonated aglycone (*m*/*z* 269) showed a relative abundance of less than 5% suggesting that the glycoside is vitexin while there are no ions characteristic of any of the sugar parts of the three identified glycosides were observed in the negative ion mode, eventually, structural confirmation was carried out through comparison of the gained retention times with those of the reference standards Table 1.

Table 1. Phenolic compounds investigated in the ethyl acetate fraction of *Leucaena leucocephala* legumes

Sub fraction	Peak No.	t_R (min)	MS-MS		Structure	Name
			M^+	Fragments		
A	1	3.25	152	151[100 %, M^+ - H], 152 [95%, M^+], 136 [30 %, M^+-H- $^{\bullet}CH_3$] & 107 [30%, M^+-H- CO_2].		Isovanillin **($C_8H_8O_3$)**
	2	4.40	170	169[100%, M^+- H], 152 [80%, M^+-OH-H] & 125[20%, M^+-H- CO_2].		Gallic acid **($C_7H_6O_5$)**
	3	6.15	180	179 [25% M^+ - H] & 135[100%, M^+-H-CO_2].		Caffeic acid **($C_9H_8O_4$)**
B	1	10.45	432	431[35%, M^+-H], 311[100% M^+-H-120] & 341 [30%, M^+-H-90].		Apigenin-8-*C*-glucoside (vitexin) **($C_{21}H_{20}O_{10}$)**
	2	10.85	464	463[5%, M^+] & 301 100% M^+-H-162].		Quercetin 3-*O*-galactoside (hyperoside) **($C_{21}H_{20}O_{12}$)**
	3	11.25	448	447 [100%, M^+-H] & 285 [100%, M^+-H 161].		Luteolin-7-*O*-glucoside **($C_{21}H_{20}O_{11}$)**
C	1	22.90	302	301[60%, M^+-H] & 151[100%, M^+-H-150].		Quercetin **($C_{15}H_{10}O_7$)**
	2	23.50	316	315 [60%, M^+-H], 300 [100%, M^+-H- $^{\bullet}CH_3$] & 151 [10%, M^+-H- 164].		Isorhamnetin **($C_{16}H_{12}O_7$)**
	3	24.85	316	316[100%, M^+] & 301[80%, M^+-H- $^{\bullet}CH_3$]		Luteolin 6-methyl ether (Neptein) **($C_{16}H_{12}O_7$)**
	4	28.50	314	314 [100%, M^+], 313 [85%, M^+-H] & 283 [45%, M^+-H-2$^{\bullet}CH_3$].		Kaempferol 3, 7-Dimethyl ether **($C_{17}H_{14}O_6$)**
	5	31.05	360	331 [65%, M^+-H-2$^{\bullet}CH_3$] & 180 [100%, M^+-H-179].		Dihydroquercetin 5,7, 3',4' tetramethyl ether (Taxifolin 5, 7, 3',4' tetramethyl ether) **($C_{19}H_{20}O_7$)**

Concerning subfraction C, five aglycones were eluted which gave retro-Diels-Alder fragmentation [31], for instance, the *m*/*z* 151 ion is common for the aglycones studied; the first aglycone is quercetin which has been described in the literature [7] while the other four aglycones were methyl ethers, exhibiting specific fragmentation pattern included the loss of $^{\bullet}CH_3$ radicals from the deprotonated aglycone molecule giving the characteristic fragments as follows; *m*/*z* 300 & 301 for isorhamnetin, luteolin 6-methyl ether (Neptein) $[M–CH_3]^{\bullet -}$ and 383 and 331 for kaempferol 3, 7-dimethyl ether and dihydroquercetin 3',4', 5,7 tetramethyl ether $[M–2CH_3]^{\bullet -}$ respectively [32-36], Table 1.

The first three aglycones were fully identified by comparing their retention times with those of the reference standards while the last two ones were tentatively identified according to their fragmentation patterns where this is the first report for their existence in *Leucaena leucocephala* plant.

3.2 Antihyperglycemic Studies

Diabetes mellitus is a chronic metabolic disorder characterized by elevated blood glucose levels, disturbances in the carbohydrate, fat and protein metabolism [37], its management is an important criterion as it has become a major health problem worldwide; it represents one of the greatest threats to the modern global health because its incidence rates are rising rapidly, and if the current rates of growth continue, the total number will exceed 435 million in 2030 [38,39].

Drug treatment for type II diabetes has been improved during the last decade but unfortunately, still drug resistance is a big concern that needs to be dealt with effective approaches, one of the strategies to control the elevated blood glucose levels is to inhibit the production of glucose from the small intestine through α-glucosidase inhibitors those interfere with carbohydrate digestion [38]. Synthetic α-glucosidase inhibitors have undesirable side effects, such as flatulence, diarrhea and abdominal cramping while, more recently several researches confirmed some natural products as effective and safe α-glucosidase inhibitors making them good sources for safe antihyperglycemic drugs [39].

Different researches attributed the antihyperglycemic activity of several plants to their flavonoid content which explained to be due to their antagonistic action to the intestinal glucose transporters preventing the absorption of glucose through the intestinal barrier, the dissipation of the electrochemical gradient of Na^+ on either sides of the membrane of the enterocytes [39,40].

Leucaena leucocephala legumes and seeds had been traditionally used to treat diabetes especially by Indonesians and Mexicans [11,15], their extract was shown to contain several bioactive compounds to which attributed their insulin-like property as several phenolic compounds, flavonoids, tannins, steroids, saponins, vitamins E & C and mimosine [40].

Leucaena leucocephala legumes exhibited significant antihyperglycemic activity this can be attributed to their flavonoid content, their aqueous extract exhibited insulin-like action, they enhance glucose uptake into rat adipocytes efficiently which is in line with its adipogenesis effect, nevertheless, they also exhibited lipolytic activity [41], hence, they can serve as insulin-like agents with less weight gain effects similar to metformin and is recommended as therapy for the management of hyperglycemia in overweight type 2 diabetic patients [42].

Table 2. Percent α-glucosidase inhibitory activity of acarbose, methanol extract and different fractions of *Leucaena leucocephala* legumes

Groups / Dose (mg ml^{-1})	Acarbose	Methanol extract	Methylene chloride fraction	*n*-hexane fraction	Ethyl acetate fraction
2.5	55.45±0.006	71.55±0.009	38.18±0.027^a	17.90±0.040^b	65.44± 0.005
1.250	48.35±0.003	66.50±0.006	30.15±0.023^a	13.44±0.030^b	61.88±0.007
0.625	45.90±0.004	51.35±0.008	24.75±0.020^a	10.85±0.024^c	49.36±0.009
0.313	29.80±0.005	34.60±0.005	18.50±0.031^a	8.45±0.041^c	31.25±0.005
IC_{50}	0.650±0.051	0.210± 0.003	n.d	n.d	0.270±0.042

n.d not determined; Values are expressed as mean ± SEM; Means with different superscripts vary significantly (p<0.001) with each other within rows

The present study included *in vitro* screening of the antihyperglycemic activity of *Leucaena leucocephala* legumes' different extracts and fractions revealed that both the methanol extract and ethyl acetate fraction exhibited strong inhibitory effect on α-glucosidase levels (71.55±0.009 & 65.44±0.005%) and their IC_{50} (0.210±0.003 & 0.270±0.042 mg ml^{-1}) demonstrating significant higher efficacy and potency than those of methylene chloride and *n*-hexane fractions (38.18±0.027 & 17.90 ±0.040%) compared to Acarbose, the reference standard drug (55.45±0.006 % with IC_{50} 0.650±0.051 mg ml^{-1}) Table 2. The gained results are confirming those reported previously in literature [39,40,43] as the flavonoid rich total methanol extract as well as the ethyl acetate fraction "flavonoid rich fraction" demonstrated significant antihyperglycemic activity.

4. CONCLUSION

HPLC/MS/MS investigation of the ethyl acetate fraction of *Leucaena leucocephala* legumes allowed the identification of eleven phenolic compounds (phenolic acids, flavonols and flavones), where extraction and fractionation resulted in partial purification and concentration of phenolic compounds in a few fractions, those when eluted using optimum conditions resulted their identification. The study also speculated that methanol extract and ethyl acetate fraction of *Leucaena leucocephala* legumes has significant α-glucosidase inhibitory effect thus, provided insight into their traditional uses as antihyperglycemic agents which can be helpful for developing medicinal preparations or nutroceuticals and functional foods for control of type 2 diabetes mellitus and related symptoms.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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DOI: 10.1007/s13596-016-0252-8

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/17867