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Phytochemical profiling of *Clerodendrum speciosum* leaves and evaluation of their antioxidant, antihyperglycemic and antiarthritic activities in vitro

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Abstract

Background *Clerodendrum speciosum* is a hybrid of *C. thomsonae* and *C. splendens*. Many biological and phytochemical studies have been performed on *C. thomsonae* and *C. splendens*, but few studies have been conducted on *C. speciosum*. Its methanol extract previously showed antioxidant activity in vivo via *Caenorhabditis elegans* model owing to its richness in flavonoids and phenylpropanoids.

Results Characterization of *C. speciosum* leaf volatile constituents (CSV) was performed via gas chromatography linked to mass spectrometry (GC–MS). This revealed the presence of 29 metabolites that belonged mainly to oxygenated monoterpenes, sesquiterpenes and their oxygenated compounds, fatty acids and their esters. The latter constituted the predominant metabolites, whereas linoleic acid (30.64%) is the major metabolite. Liquid chromatography linked to mass spectrometry (LC–MS) was performed on the ethyl acetate fraction of *C. speciosum* leaves (CSE). This resulted in a tentative assignment of twenty-four peaks, whereas six peaks were not identified. These identified metabolites mainly belonged to phenylpropanoids, whereas flavonoids, iridoid glycosides, phenolic acids and their derivatives were also detected. The leaf volatile constituents showed notable antiarthritic activity as evidenced by the significant inhibition of albumin denaturation with an IC_{50} of 32.50 $\mu\text{g/mL}$ comparable that of diclofenac sodium (15.12 $\mu\text{g/mL}$). The leaf ethyl acetate fraction revealed potent antioxidant effect estimated by 725.43 ± 5.95 mg TE/g, 333.82 ± 19.9 mg TE/g, 2.1 ± 0.08 mmol TE/g and 3.69 ± 0.3 mg EDTA/g. in cupric-reducing antioxidant capacity (CUPRAC), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), phosphomolybdenum (PHD) and metal-chelating activity (MCA) assays, respectively. It showed a reasonable α -glucosidase and α -amylase inhibition estimated by 1.88 ± 0.05 and 0.14 ± 0.01 mmol ACAE/g, respectively. ADME/TOPAKT assessment, processed on the prevalent identified components detected in CSV, displayed acceptable pharmacodynamic, toxicity and pharmacokinetic behaviors for most tested compounds except for *n*-nonadecane and *n*-heptacosane and *n*-octacosane.

Conclusion Thus, *C. speciosum* leaves could serve as a promising treasure for the treatment of many diseases such as arthritis and diabetes mellitus owing to its abundance with flavonoids and terpenoidal compounds.

Keywords *Clerodendrum speciosum*, Lamiaceae, GC/MS, LC/MS, Antioxidant, Antiarthritis, Antihyperglycemic

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Background

Oxidative stress is characterized by a pronounced alteration in the balance between naturally occurring antioxidants that exist within the living organisms and the quantity of the free radicals generated, which consequently results in cell destruction [1]. Despite the production of natural antioxidants, the massive generation of free radicals can be triggered by environmental pollution, unhealthy lifestyles and radiation, which further exacerbates oxidative stress [2, 3]. Moreover, inflammation could be triggered by the unregulated formation of these free radicals, which further worsens the situation via the formation of additional free radicals. This ultimately leads to a plethora of ailments such as diabetes mellitus and inflammatory abnormalities such as arthritis [4–6].

Diabetes mellitus results in uncontrolled blood glucose level that causes many micro- and macrovascular deterioration, such as retinopathy, cardiovascular, neuropathy and nephropathy disorders, if left without regular monitoring and strict treatment [7]. Currently, it is regarded as the third most lethal factor after cancer and heart disorders, affecting people worldwide. It is estimated to reach 592 million by 2035, as predicted by the International Diabetes Federation [8]. Arthritis is defined as swelling, inflammation and tenderness of the joints, accompanied by stiffness and pain in the joints and synovial inflammation resulting in tissue destruction. The main symptoms get worsen with the age progress. Rheumatoid arthritis and osteoarthritis represent the most common types of arthritis [9]. Additionally, arthritis imposes a significant economic and healthcare burden [10].

Despite tremendous developments in the discovery of potent drugs and the presence of many synthetic entities in the market for the alleviation of hyperglycemia and arthritis and their concomitant complications, adverse effects remain a great challenge compared to the benefits of these drugs [11]. Thus, new medicines obtained from natural sources that are safer and cheaper than synthetic drugs are required owing to their acceptance by people worldwide [12]. Many phytoconstituents have been previously reported to alleviate oxidative stress and its complications such as carotenoids, curcumin, flavonoids, which showed promising antioxidant and anti-inflammatory potential [13–17]. Besides, the scientific community has recently shed a light on the prominent functional potential of phenolic compounds and flavonoids such as quercetin, ellagic acid, catechin myricetin, delphinidin, cyaniding and caffeic acids in the alleviation of diabetes and its hazardous consequences. This could be interpreted by the virtue of their pronounced ability to inhibit reactive species, modify intestinal microbiota, scavenge free radicals, or even enhance oxidative enzymes. Additionally, they decrease blood glucose levels, prevent

protein glycation, dipeptidyl peptidase-IV activity and other enzymes implicated in the metabolism of carbohydrates. They also enhance the insulin secretion from the pancreatic β -cell [18]. Moreover, their anti-inflammatory potential manifested by the inhibition of IL-6 is mainly correlated to their arthritis improving behavior [19].

The genus *Clerodendrum* belonging to the family Lamiaceae, includes nearly 150 species that are native to warm temperate and tropical areas worldwide [1]. Members of this genus are popular because of their effectiveness in alleviating the inflammatory related diseases, rheumatism, skin diseases, malaria, coughing and asthma. This could be due to the prevalent existence of phenolics, flavonoids, phenylpropanoids and volatile oils within this genus [20]. *C. speciosum* is a hybrid of *C. thomsonae* and *C. splendens*. Despite the presence of many biological and phytochemical reports on *C. thomsonae* and *C. splendens*, few studies have been conducted on *C. speciosum*. It was previously studied by the authors and proven to possess antioxidant activity in vivo via *C. elegans* model owing to the richness of the methanol extract with flavonoids and phenylpropanoids [1, 21, 22].

Thus, this study aimed to chemically identify the volatile constituents of *C. speciosum* leaves using GC–MS, and the ethyl acetate fraction via LC–MS. In addition, the antioxidant and antihyperglycemic activities of the ethyl acetate fraction in vitro using CUPRAC, ABTS, phosphomolybdenum and metal-chelating assays for the former, and α -glucosidase and α -amylase inhibition experiments for the latter were carried out for the first time. The antiarthritis activity of the volatile constituents was also assessed here in vitro for the first time using the album denaturation test. ADME/TOXPAKT evaluation was performed on the prevalent identified secondary metabolites detected in the volatile constituents to evaluate their probable, pharmacodynamic, toxicity and pharmacokinetic behaviors.

Methods

Plant material and preparation of extracts

Plant material

Fresh leaves of *Clerodendrum speciosum*, Lamiaceae, were collected from the Mazhar Botanical Garden which is located in Egypt, on the Cairo-Alexandria Road in Egypt in April 2018. Authentication of the collected samples was performed by Dr. Terasse Labib, Plant Taxonomy Consultant at El-Orman Botanical Garden and the Ministry of Agriculture in Egypt through examination of its morphology. The sample was maintained at the Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University, with voucher code (PHG-P-CS 156).

Preparation of volatile constituents of *C. speciosum* leaves

Hydrodistillation of fresh leaves of *Clerodendrum speciosum* (500 g) was processed by a Clevenger apparatus for 6 h. Drying of the collected oil was achieved using anhydrous sodium sulfate that was collected in vials and stored at 30 °C for extensive phytochemical and biological analyses. The yield was computed as % v/w based on the initial weight of the plant.

Preparation of the ethyl acetate fraction of *C. speciosum* leaves

Fresh leaves of *Clerodendrum speciosum* (1 kg) were dried in the shade and crushed to give 100 g of coarse powder, followed by maceration with (1.5 L×3) distilled methanol and filtration. Then, the collected filtrate was exposed to reduced pressure using rotary evaporator (Heidolph, Germany) at 45 °C, followed by lyophilization to give 8.95 g of completely lyophilized powder of total methanol extract. Subsequently, it was subjected to dissolution in methanol (70%) and fractionated successively using *n*-hexane, dichloromethane and finally ethyl acetate. It was dried at 45 °C under reduced pressure and lyophilized to give 2.18 g the dried powdered fraction.

Metabolite profiling using GC–MS and LC–MS**Metabolic profiling of volatile constituents obtained from *C. speciosum* leaves via GC/MS analysis**

GC/MS was carried out using a Shimadzu GCMS-QP 2010 (Shimadzu Corporation, Koyoto, Japan) supplied with a Rtx-5MS capillary column (30 m×0.25 mm i.d.×0.25 µm film thickness) (Restek, PA, USA), equipped with a Shimadzu mass spectrophotometer using a previously reported method [23]. The initial temperature was set at 50 °C for the column that lasted approximately three minutes and was then gradually increased from 50 to 300 °C at a rate of 5 °C/min. The isothermal pattern was kept for ten min. at 300 °C. The injector temperature was kept at 280 °C, whereas that of the interface was maintained at 220 and the ion source temperature was 280 °C. The carrier gas, helium, was adjusted to a flow rate of 1.37 mL/min. Quantitation of the detected compounds was achieved using the normalization method, taking into consideration the retention indices of the metabolites compared to *n*-alkanes (C8–C28) homologous series that were injected under the same conditions. Additionally, the mass spectra of the identified compounds were compared with those recorded in NIST and Wiley library databases and with data collected from the literature [24–27].

Phytochemical profiling of the ethyl acetate fraction of *C. speciosum* leaves using LC/MS technique

Phytochemical profiling of the ethyl acetate fraction of *C. speciosum* leaves was performed on (250×2 mm, ID) Agilent 1100 Series by Knauer that was pre-packed with a 100-5 C18 Eurospher, together with an integrated pre-column as previously described [7]. The mobile phase composed of acetonitrile/H₂O with 0.1% formic acid, gradient elution was adopted beginning with acetonitrile/H₂O (10:90) to 100% acetonitrile at a flow rate 1 mL/min. A Finnigan LCQ-DECA mass spectrometer was used, and the ESI interface was operated in the negative mode. Xcalibur TM 2.0.7 software (Thermo Scientific, Karlsruhe, Germany) was used for data and chromatogram integration.

Biological investigations of *C. speciosum* leaves***In vitro* antiarthritic activity of volatile constituents obtained from *C. speciosum* leaves**

The albumin denaturation assay was performed to assess the antiarthritic activity of volatile constituents obtained from *C. speciosum* leaves, as previously described, with slight modifications [28]. Preparation of twofold serial dilutions of the tested samples as well as diclofenac sodium, the standard drug used, diclofenac sodium, with a concentration ranging between of 7.81 and 1000 µg/mL was performed using a 96-well plate. Addition of 100 µL of 1% bovine albumin solution to 100 µL of tested sample or the standard drug was done for each concentration, to prepare the reaction mixture. To induce protein denaturation, the previously prepared reaction mixtures were incubated for 15 min at 28 °C. Subsequently, cooling was achieved, and the turbidity was determined at 660 nm via a microplate reader. Calculation of the inhibition percentage of protein denaturation was performed utilizing a control that contained no drug and applying the following equation: protein denaturation percentage of inhibition = $1 - [A_1/A_2] \times 100$, where A_1 = the control absorbance; and A_2 = the tested sample or the standard absorbance. The average was obtained after repeating each experiment three times. The IC₅₀ value for protein denaturation was also calculated.

***In vitro* antioxidant activity of the ethyl acetate fraction of *C. speciosum* leaves**

The antioxidant potency of *C. speciosum* leaves ethyl acetate fraction (0.2–2 mg/ml) was determined in vitro using different methods, comprising phosphomolybdenum (PHD), metal-chelating activity (MCA), free radical scavenging capacity 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), reducing-power cupric-reducing antioxidant capacity (CUPRAC) methods, as reported

before [29]. The results was expressed as Trolox equivalents (TE/g sample) for all the antioxidant assays except for metal-chelating activity (MCA) as EDTA equivalents (mg EDTA/g sample). Each sample was analyzed in triplicate.

In vitro antihyperglycemic activity of the ethyl acetate fraction of C. speciosum leaves

The antihyperglycemic activity of *C. speciosum* leaves ethyl acetate fraction (0.2–2 mg/ml) was determined in vitro using both α -glucosidase and α -amylase inhibitory assays. The former was performed using the *p*-nitrophenyl- β -D-glucopyranoside (pNPG) assay, whereas the latter was performed via Caraway–Somogyi iodine/potassium iodide (IKI) assay, as previously described [30]. The results were expressed as acarbose equivalents (mg ACAE/g) for both assays. Each sample was measured in triplicate.

In silico predictions

In silico ADME/TOPKAT prediction of C. speciosum leaves volatile constituents

The 2D structures for the identified volatile constituents obtained from *C. speciosum* leaves were designed via ChemDraw 13.0 and kept as pdb files that were followed by their preparation using the ligand preparation default protocol built in the software. Subsequently, they were subjected to ADME evaluation and toxicity postulation (TOPKAT) using Discovery Studio 2016 (Accelrys Inc., San Diego, CA, USA) to determine their pharmacokinetics, toxicity and pharmacodynamic behavior. The chosen ADME descriptors included penetration levels in the blood–brain barrier (BBB), prediction of plasma protein binding pattern (PPB), inhibition of cytochrome P450 2D6, hepatotoxicity level, aqueous solubility and human intestinal absorption (HIA). Meanwhile, for TOPKAT assessment, Ames mutagenicity, the impact of carcinogenic agents on FDA male and female rats, rat chronic lowest observed adverse effect level (LOAEL), rat oral LD₅₀ and skin and eye irritant behavior were the selected parameters [27, 31].

Statistical analyses

The results of the biological assessments were carried out in triplicate and are expressed as the mean $\pm$ S.D. Graphs were drawn using GraphPad Prism 5 (GraphPad Software Inc., CA, USA).

Results

GC/MS analysis of the volatile constituents obtained from C. speciosum leaves

The volatile constituents of *C. speciosum* leaves were quantitatively and qualitatively identified using GC/MS

analysis. The volatile constituents were pale yellow with a characteristic odor. Profiling of the volatile constituents resulted in the identification of twenty-nine components. They belong mainly to oxygenated monoterpenes, sesquiterpenes and their oxygenated derivatives, in addition to fatty acids, and fatty acid esters, which constitute the predominant metabolites, as illustrated in Table 1 and Fig. 1. Linoleic acid (30.64%) was the major metabolite, followed by linolenic acid (29.53%), 9-hexadecenoic acid (9.29%), palmitic acid (12.42%) and β -caryophyllene (2.34%). A schematic representation of the main volatile constituents in the leaves of *C. speciosum* is shown in Fig. 2.

LC/MS profiling of the ethyl acetate fraction of C. speciosum leaves

Profiling of the major phytoconstituents in the ethyl acetate fraction of *C. speciosum* leaves was performed using LC/MS–MS techniques, as shown in Table 2 and Fig. 3. Tentative assignments of twenty-four peaks were performed; however, six peaks remained unidentified. Identification was achieved via comparing the molecular ions [M–H] – in the negative mode together with the MS/MS fragments with those already published in the literature and online public databases. These identified metabolites were mainly to phenylpropanoids, whereas flavonoids, iridoid glycosides, phenolic acids and their derivatives were also detected. Phenyl propanoid glycosides represented the predominant class of metabolites existed in the ethyl acetate fraction represented by 13 compounds with molecular ions [M–H] – at *m/z* of 693.40, 451.01, 609.03, 623.32, 667.01, 753.28, 721.23, 723.14 and 887.19 corresponding to acetyl martynoside [32], fuhsioside [33], calceolarioside C [34], verbascoside [35], isoverbascoside [36], scroside B [37], rocymosin A [38], cinnamoyl verbascoside, diacetyl leucosceptoside and its isomer [39], diacetylplantamajoside and its isomer [40] and maruboside [41]. Many types of phenylpropanoid glycosides have been detected in the ethyl acetate fraction of *C. speciosum*, including verbascoside and isoverbascoside, which are characterized by the presence of dihydroxy phenylethanol and caffeoyl moieties connected to deoxyhexose and hexose sugars. Their fragmentation showed MS² fragment ions at *m/z* of 161 matching with the loss of the caffeoyl moiety and 461 corresponding to the remaining fragment [M – H – caffeoyl] –, in addition to the presence of cinnamoyl verbascoside with [M–H] – at *m/z* 753.28. Calceolarioside C represents another type of phenylpropanoid that contains pentose and hexose sugars in addition to the caffeoyl moiety and shows MS² fragment ions at *m/z* of 161 corresponding to the loss of the caffeoyl moiety. In scroside B, two hexose sugars were detected, in addition to the presence of a feruloyl moiety,

Table 1 Compositions of volatile constituents obtained from *C. speciosum* leaves utilizing Rtx-5MS column

No.	Compounds ^[a]	R_i		% compositions	References
		Measured ^[b]	Reported ^[c]		
1.	Isoamyl acetate	869	869	0.15	MS, RI
2.	<i>n</i> -Nonane	891	900	0.18	MS, RI
3.	1-Octen-3-ol	984	984	tr	MS, RI
4.	<i>n</i> -Decane	999	999	0.43	MS, RI
5.	<i>trans</i> -Decalin	1055	1055	tr	MS, RI
6.	<i>n</i> -Undecane	1100	1100	0.41	MS, RI
7.	Dodecane	1199	1200	0.26	MS, RI
8.	Edulan I, dihydro-	1301	1292	tr	MS, RI
9.	β -Damascenone	1390	1390	tr	MS, RI
10.	β -Caryophyllene	1428	1428	2.34	MS, RI
11.	α -Guaiene	1446	1446	tr	MS, RI
12.	<i>trans</i> -Geranylacetone	1457	1455	tr	MS, RI
13.	α -Humulene	1463	1463	0.92	MS, RI
14.	β -Ionone	1495	1495	tr	MS, RI
15.	β -Caryophyllene oxide	1595	1595	0.22	MS, RI
16.	Pentadecanal-	1718	1717	tr	MS, RI
17.	Myristic acid	1763	1763	0.24	MS, RI
18.	Hexahydrofarnesyl acetone	1848	1848	tr	MS, RI
19.	<i>n</i> -Nonadecane	1893	1900	1.4	MS, RI
20.	Palmitic acid, methyl ester	1928	1928	tr	MS, RI
21.	9-Hexadecenoic acid	1954	1957	9.29	MS, RI
22.	Palmitic acid	1973	1973	12.42	MS, RI
23.	Methyl linoleate	2103	2098	0.56	MS, RI
24.	Phytol	2122	2122	1.23	MS, RI
25.	Linoleic acid	2159	2157	30.64	MS, RI
26.	Linolenic acid	2167	2178	29.53	MS, RI
27.	Stearyl acetate	2218	2211	0.19	MS, RI
28.	<i>n</i> -Heptacosane	2703	2700	1.8	MS, RI
29.	<i>n</i> -Octacosane	2797	2800	1.46	MS, RI
	Oxygen containing monoterpene			tr	
	Sesquiterpene hydrocarbons			3.62	
	Oxygen containing sesquiterpene			0.22	
	Fatty acid and their esters			82.87	
	Others			6.96	
	Total identified components			93.67	

^a Arrangement of the compounds based on their elution on RTX-5MS column^b Kovats index determined experimentally on RTX-5MS column relative to a standard mixture of C8–C30 n-alkanes^c Published Kovats retention indices. Identification was based on comparison of the compounds mass spectral data (MS) and Kovats retention indices (RI) with those of NIST Mass Spectral Library (2011), Wiley Registry of Mass Spectral Data 8th edition and literature

which revealed a fragment ion at m/z 175. Moreover, diacetyl leucosceptoside was detected with two acetylated sugars (hexose and deoxyhexose), connected to dihydroxy phenylethanol and feruloyl moiety, respectively, showing a fragment at m/z 175 matching with the loss of caffeoyl ion. Meanwhile, diacetylplantamajoside showed two acetylated hexose moieties and a dihydroxy phenylethanol and caffeoyl moiety and showed MS² fragment

ions at m/z 161, matching with the loss of the caffeoyl moiety. In addition, two iridoid glycosides, serratoside A with $m/z=503.18$ [42, 43] and coumaroyl oxyugandoside with $m/z=519.08$ [44], were identified in the negative ionization mode. Moreover, six flavonoid glycosides were detected in negative ionization mode and represented by acacetin *O*-hexouronide methyl ester ($m/z=473.25$) [45], tiliroside ($m/z=593.35$) [46], rhamnazin-*O*-rutinoside

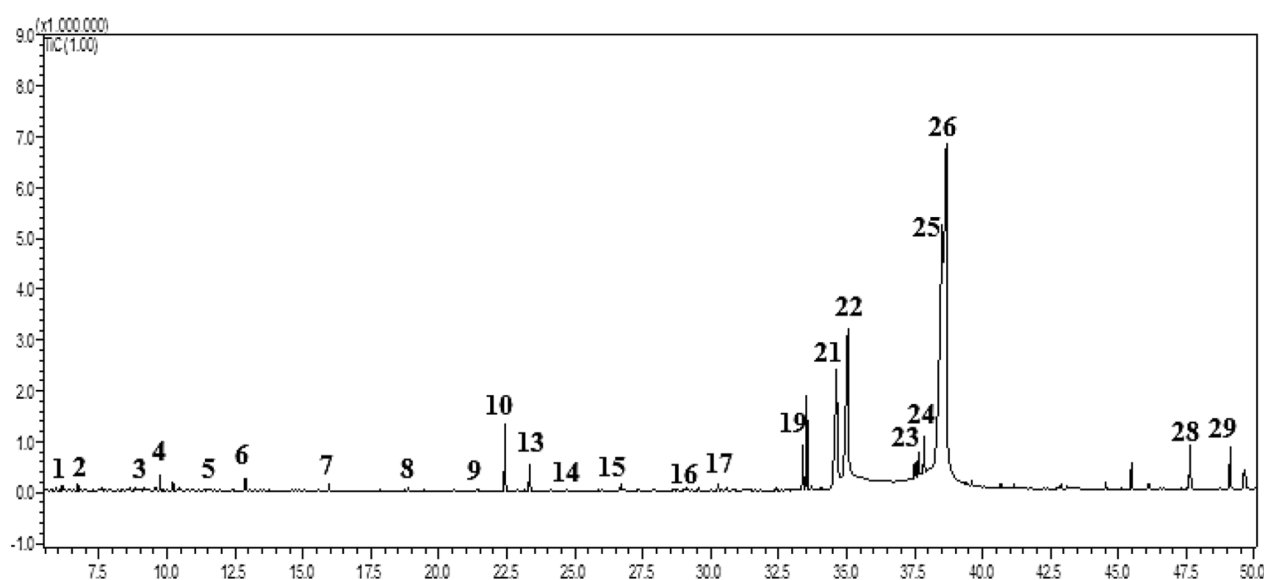


Fig. 1 GC-MS chromatogram of volatile constituents obtained from *C. speciosum* leaves using Rtx-5MS column

($m/z=637.38$) and its isomer [35], acacetin trioxide ($m/z=723.14$) [40] and neobudofficide ($m/z=737.14$). Additionally, rosmarinic acid, quinic acid and its derivatives were also identified at molecular ions $[M-H]^-$ at m/z of 359.00 [35], 191.19 [47] and 569.11 [48], respectively (Table 2).

Biological investigations of *C. speciosum* leaves

Evaluation of the antiarthritic activity of volatile constituents of *C. speciosum* leaves in vitro

The volatile constituents of *C. speciosum* leaves (CSL) showed a significant inhibition of albumin denaturation, with percentage inhibition ranging between 21.35 ± 1.20 and $82.35 \pm 1.60\%$ in the concentration range of 7.81–1000 $\mu\text{g/mL}$. It approaches that diclofenac sodium, the standard drug, showed percentage inhibition ranging between 31.12 ± 1.20 and $89.35 \pm 0.58\%$ within the same concentration range previously mentioned for *C. speciosum* tested sample. It is worthy to highlight that CSL showed IC_{50} of 32.50 $\mu\text{g/mL}$ that is comparable to diclofenac sodium (15.12 $\mu\text{g/mL}$) reflecting its potent antiarthritic activity. The results are presented in Table 3.

Evaluation of the antioxidant activity of the ethyl acetate fraction of *C. speciosum* leaves in vitro

The antioxidant potential was assessed in vitro using various assays, including the free radicle scavenging capacity (ABTS), metal-chelating activity (MCA), reducing-power cupric-reducing antioxidant capacity (CUPRAC) and phosphomolybdenum (PHD) assays. The results revealed the potent antioxidant effect of the tested plant, as it showed a high free radicle scavenging capacity in ABTS

estimated to be 333.82 ± 19.9 mg TE/g. Additionally, it showed a remarkable reduction and electron-donation capability, as evidenced by the conversion of Cu^{2+} to Cu^+ , which is equal to 725.43 ± 5.95 mg TE/g. Meanwhile, the PHD assessment reflects the ability of secondary metabolites to reduce Mo (VI) into Mo (V) by employing acidic pH, which is considered as the total antioxidant capacity measure. *C. speciosum* leaves ethyl acetate fraction showed a promising effect in this assay with antioxidant effect estimated by 2.1 ± 0.08 mmol TE/g. Besides, the tested sample showed a notable MCA that is performed by the ferrozine method that is estimated by 3.69 ± 0.3 mg EDTA/g.

Evaluation of the antihyperglycemic activity of *C. speciosum* leaves ethyl acetate fraction in vitro

C. speciosum leaves ethyl acetate fraction revealed a reasonable α -amylase inhibitory potential showing 0.14 ± 0.01 mmol ACAE/g in the amylase inhibition assay. Meanwhile, it showed a promising α -glucosidase inhibitory activity estimated by 1.88 ± 0.05 mmol ACAE/g in the glucosidase inhibition assay.

In silico ADME/TOPKAT prediction of *C. speciosum* leaves volatile constituents

The main volatile constituents of *C. speciosum* leaves extract were predicted using ADME/TOPKAT in silico. The results shown in Table 4 reflect the pharmacokinetics, toxicity and pharmacodynamic behaviors of the detected compounds. By careful inspection of the table, it was clearly obvious that β -caryophyllene displayed good absorption within the intestine, whereas α -humulene,

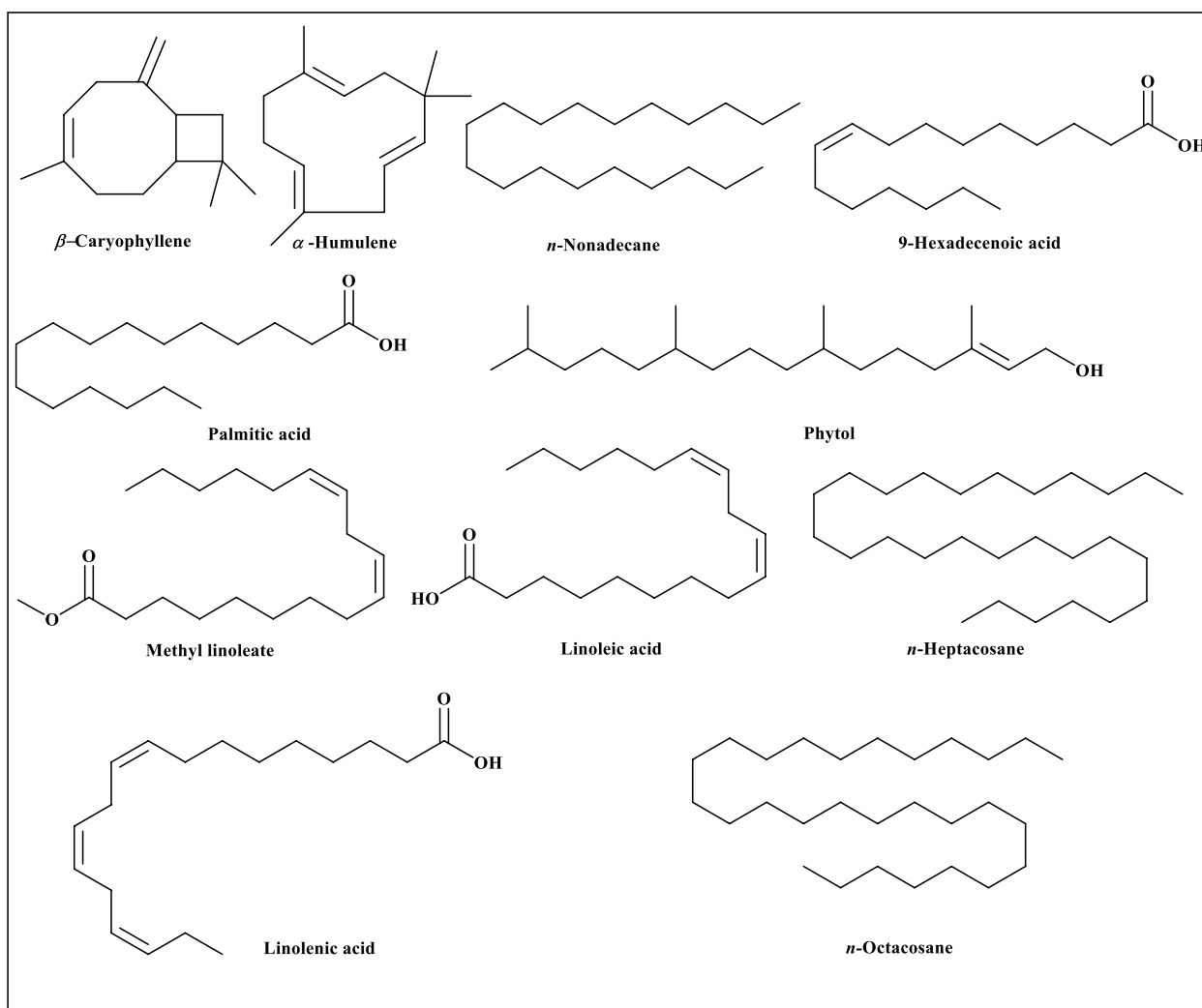


Fig. 2 Scheme of the major secondary metabolites identified in the volatile constituents of *C. speciosum* leaves

9-hexadecanoic acid, palmitic acid, methyl linoleate, linoleic acid and linolenic acid showed moderate absorption, which lies between the 95 and 99% absorption ellipses, as displayed ADMET plot (Fig. 4). In addition, all the tested components revealed very low but possible solubility, except for *n*-nonadecane, which displayed very low solubility, and *n*-heptacosane and *n*-octacosane, which showed extremely low solubility. Moreover, all of the tested components showed very high penetration within BBB, which was positioned within the 99% confidence eclipse of BBB (Fig. 4), except phytol *n*-nonadecane *n*-heptacosane and *n*-octacosane, which displayed undefined BBB and existed outside the confidence eclipse (Fig. 4). However, all the tested components revealed more than 90% binding to plasma protein with no observed hepatic toxicity; meanwhile, most of them exerted no inhibition on CPY2D6, except *n*-nonadecane,

n-heptacosane, *n*-octacosane and methyl linoleate, which inhibited CPY2D6.

Concerning TOPKAT evaluation, all the tested metabolites revealed no mutagenicity in Ames prediction, with no carcinogenic potential in the male rat FDA, except β -caryophyllene and α -humulene. In contrast, they exhibited carcinogenic potential in the female rat FDA, except for 9-hexadecanoic acid, palmitic acid, ethyl linoleate and linoleic acid. Furthermore, they caused no eye irritation, but moderate to severe skin irritation (Table 5). The examined metabolites displayed rat chronic lowest observed adverse effect level (LOAEL) ranging from 0.01–0.93 g/kg body wt, whereas β -caryophyllene showed the lowest LOAEL and palmitic acid exerted the highest. Furthermore, the tested metabolites revealed LD50 ranging between 0.78 g/kg body wt that is for β -caryophyllene to 38.06 g/kg body wt. for *n*-octacosane.

Table 2 Chemical components present in the ethyl acetate fraction of *C. speciosum* leaves via LC–ESI–MS/MS in the negative ion mode

Peak no.	t_R (min)	Name	$[M-H]^-$ (m/z)	MS^2 (m/z)	Mol. formula	Class	References
1	2.2	Quinic acid	191.19	111, 173	$C_7H_{12}O_6$	Organic acid	[47]
2	5.83	Quinic acid derivative	569.11	173, 191, 389	–	Organic acid derivative	[48]
3	13.21	Serratoside A	503.18	179, 341	$C_{25}H_{28}O_{11}$	Iridoid glycoside	[42, 43]
4	15.82	Acacetin O-hexouronide methyl ester	473.25	283	$C_{23}H_{22}O_{11}$	Flavonoid glycoside	[45]
5	16.89	Acetylmartynoside	693.40	–	$C_{33}H_{42}O_{16}$	Phenyl propanoid	[32]
6	20.25	Fuhsioside	451.01	179, 345, 405	$C_{21}H_{24}O_{11}$	Phenyl propanoid	[33]
7	20.79	Calceolarioside C	609.03	179, 161	$C_{28}H_{34}O_{15}$	Phenyl propanoid	[34]
8	21.83	Tiliroside	593.35	285	$C_{30}H_{26}O_{13}$	Flavonoid glycoside	[46]
9	23.56	Verbascoside	623.32	161, 461	$C_{29}H_{36}O_{15}$	Phenyl propanoid	[35]
10	25.51	Isoverbascoside	623.29	161, 461	$C_{29}H_{36}O_{15}$	Phenyl propanoid	[36]
11	32.74	Rhamnazin-O-rutinoside	637.38	475, 329, 297	$C_{29}H_{34}O_{16}$	Flavonoid glycoside	[35]
12	36.40	Coumaroyl oxyugandoside	519.08	163, 145	$C_{25}H_{28}O_{12}$	Iridoid glycoside	[44]
13	36.99	Rhamnazin-O-rutinoside isomer	637.38	475, 329, 297	$C_{29}H_{34}O_{16}$	Flavonoid glycoside	[35]
14	39.81	Rosmarinic acid	359.00	197, 179, 161	$C_{18}H_{16}O_8$	Phenolic acid	[35]
15	40.78	Unidentified	719.00	245, 341	–	–	–
16	41.61	Scroside B	667.01	623, 461, 193, 175	$C_{31}H_{40}O_{16}$	Phenyl propanoid	[37]
17	44.35	Rocymosin A	467.14	–	$C_{21}H_{24}O_{12}$	Phenyl propanoid	[38]
18	55.80	Unidentified	727.00	325, 401	–	–	–
19	57.14	Acacetin trioside	723.14	283	$C_{33}H_{40}O_{18}$	Flavonoid glycoside	[40]
20	63.40	Unidentified	595	355, 551	–	–	–
21	67.66	Unidentified	759.16	433, 601	–	–	–
22	70.26	Neobudofficide	737.14	283	$C_{34}H_{42}O_{18}$	Flavonoid glycoside	[69]
23	77.88	Unidentified	883.00	–	–	–	–
24	79.89	Cinnamoyl verbascoside	753.28	161	$C_{38}H_{42}O_{16}$	Phenyl propanoid	[70]
25	79.97	Diacetyl leucosceptoside	721.23	175, 193	$C_{34}H_{42}O_{17}$	Phenyl propanoid	[39]
26	80.69	Diacetyl leucosceptoside isomer	721.23	175, 193	$C_{34}H_{42}O_{17}$	Phenyl propanoid	[39]
27	81.55	Unidentified	697.07	175, 193	–	–	–
28	82.79	Diacetylplantamajoside	723.14	161, 179	$C_{33}H_{40}O_{18}$	Phenyl propanoid	[40]
29	83.98	Diacetylplantamajoside isomer	723.14	161, 179	$C_{33}H_{40}O_{18}$	Phenyl propanoid	[40]
30	84.41	Marruboside	887.19	–	$C_{39}H_{52}O_{23}$	Phenyl propanoid	[41]

Discussion

Clerodendrum, which belongs to the Lamiaceae family, varies greatly from 150 to nearly 500 species that are widely distributed worldwide. Many of these species have been used in folk medicine to alleviate fatal disorders. The biological potential and phytoconstituents of these 11 species have been thoroughly studied. Approximately 283 compounds belong to flavonoids and their glycosides, including mono-, di-, tri- and sesquiterpenes, steroids and their glycosides, phenylpropanoids, cyanogenic glycosides and anthraquinones. These secondary metabolites are undoubtedly responsible for the observed biological pattern, including anti-inflammatory, neuro-protective, antihyperglycemic, antihypertensive, antioxidant, antimicrobial, hypolipidemic and anticancer potential [43].

C. speciosum constitutes a hybrid between *C. thomsonae*, the bleeding heart glory bower, and *C. splendens*, the scarlet glory bower. *C. thomsonae* showed anticonvulsant and antiepilepsy and was utilized for relieving headaches and parasitic diseases [22], whereas *C. splendens* showed immunomodulatory, sedative and analgesic activities and was used to treat inflammation, spasm and cardiac distress [21]. Although many biological capabilities and phytochemical analyses have been reported for *C. thomsonae* and *C. splendens*, few studies were conducted on *C. speciosum*. It was previously studied by the authors and proven to possess antioxidant activity in vivo in *C. elegans* model owing to the richness of the methanol extract with flavonoids and phenylpropanoids [1, 21, 22].

The composition of the volatile components obtained from *C. speciosum* leaves was quantitatively and qualitatively identified using GC/MS analysis. Characterization

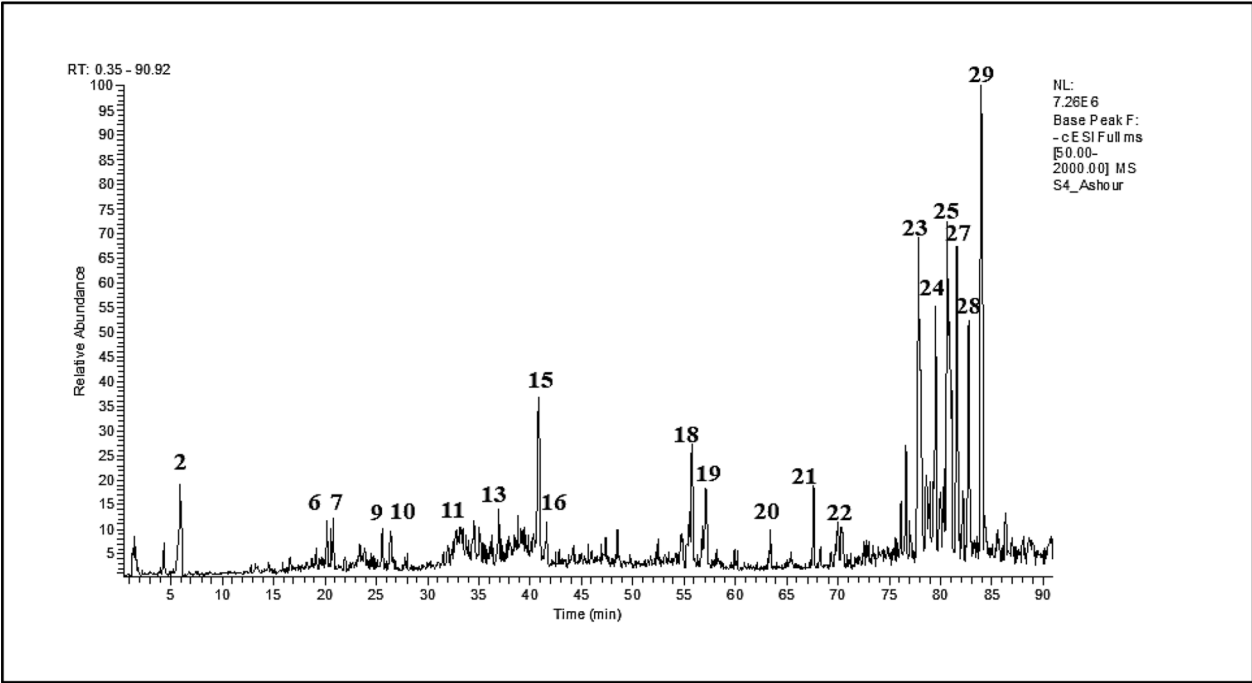


Fig. 3 LC-ESI-MS/MS chromatogram of the ethyl acetate fraction of *C. speciosum* leaves in the negative ionization mode

Table 3 Antiarthritic activity of volatile constituents of *C. speciosum* leaves (CSL) using albumin denaturation assay represented as mean of inhibition of protein denaturation *versus* diclofenac sodium as a standard

Conc. (µg/mL)	CSL	Diclofenac sodium
7.81	21.35 ± 1.20	31.12 ± 1.20
15.63	29.38 ± 2.50	51.21 ± 0.58
31.25	49.81 ± 2.10	59.14 ± 1.20
62.50	54.31 ± 1.30	68.28 ± 0.63
125.00	61.34 ± 0.63	70.14 ± 0.58
250.00	71.51 ± 1.20	76.52 ± 0.63
500.00	78.89 ± 0.58	84.12 ± 1.20
1000.00	82.35 ± 1.60	89.35 ± 0.58
IC ₅₀	32.50	15.12

The assessments were carried out in triplicate and are expressed as the mean ± S.D. All the data are significantly different from the standard at (*p* < 0.001)

of the volatile constituents led to the identification of twenty-nine secondary metabolites. They were mainly oxygenated monoterpenes, sesquiterpenes and their oxygenated derivatives, in addition to fatty acids and their esters, which were the predominant metabolites. The non-polar metabolites present in the volatile constituents isolated from the leaves revealed promising antiarthritic activity by inhibiting albumin denaturation. It was previously reported that changes and denaturation of the

proteins of the body tissues, together with the alteration of the hydrophobic electrostatic, disulfide and hydrogen bonding of the body, result in the generation of large quantities of auto-antigens. This, in turn, results in the occurrence of inflammatory arthritic diseases. Notably, many anti-inflammatory agents such as diclofenac sodium and indomethacin inhibit the denaturation of bovine serum albumin [49]. *C. speciosum* leaves showed promising inhibition of albumin denaturation with an IC₅₀ value of 32.50 µg/mL that is comparable to that of diclofenac sodium (15.12 µg/mL), reflecting its potent antiarthritic activity. This activity is mainly attributed to the synergistic effect of its metabolites, where linoleic acid (30.64%) is the major metabolite and was previously reported to possess anti-inflammatory activity. A recent large-scale study observing the consumed diet and its impact on the progression of rheumatoid arthritis has revealed an astonishing link between linoleic acid that exhibited notable anti-inflammatory potential and the inhibition of being attacked by rheumatoid arthritis [50]. Moreover, linolenic acid was previously reported to directly suppress proinflammatory cytokines, which could be attributed to its anti-inflammatory activity and consequent related disorders such as arthritis [51]. Moreover, hexadecenoic acid positional isomers are fatty acids with potential anti-inflammatory behavior [52]. Meanwhile, various reports have documented the antiarthritic behavior of β-caryophyllene, which

Table 4 ADMET of the main secondary metabolites detected in the volatile constituents of the leaves of *C. speciosum*

Compounds	Absorption level	Solubility level	BBB level	PPB level	CPY2D6	Hepato-toxic	Alog p98
β -Caryophyllene	0	2	0	True	NI	NT	4.297
α -Humulene	1	2	0	True	NI	NT	5.04
<i>n</i> -Nonadecane	3	1	4	True	Inh	NT	9.04
9-Hexadecanoic acid	1	2	0	True	NI	NT	5.95
Palmitic acid	1	2	0	True	NI	NT	6.39
Methyl linoleate	1	2	0	True	Inh	NT	6.64
Phytol	3	2	4	True	NI	NT	7.34
Linoleic acid	1	2	0	True	NI	NT	6.42
Linolenic acid	1	2	0	True	NI	NT	5.97
<i>n</i> -Heptacosane	3	0	4	True	Inh	NT	12.69
<i>n</i> -Octacosane	3	0	4	True	Inh	NT	13.15

3, 2, 1 and 0 indicate very low absorption, low, moderate and good, respectively;

5, 4, 3, 2, 1 and 0 indicate too soluble, optimal good, low, very low but possible, extremely low, respectively;

4, 3, 2, 1 and 0 represent undefined, low, medium, high and very high penetration via the BBB, respectively

PPB, plasma protein binding; True = more > 90%; false = less than 90%;

Inh, inhibitor; NI, non-inhibitor; NT, non-toxic

revealed anti-inflammatory and antiarthritic potency both in arthritis induced by Freund's complete adjuvant in rats and methotrexate-induced arthritic side effects in rats [53, 54]. Additionally, it was reported that β -caryophyllene relieved arthritis through cross-talk between PPAR- γ and CB2 receptors, which decreased the stimulation of NF- κ B and caused an increase in peroxisome proliferator-activated receptor-gamma (PPAR- γ) expression and its co-activator (PGC-1 α) in human articular chondrocytes triggered by lipopolysaccharides. These effects were reversed by treatment with AM630, which is a CB2 receptor antagonist. These results suggest that BCP relieves arthritis through crosstalk between CB2 and PPAR- γ [55]. Thus, all the present metabolites act synergistically to produce this prominent effect. However, the potential variability in bioactivity owing to factors such as extraction methods, solvent systems and regional and climatic variations may constitute a critical limitation for the consumption of *C. speciosum* leaves as an antiarthritic agent.

Moreover, ADME/TOPAKT prediction performed *in silico* on the prevalent identified components detected in the volatile constituents of leaves showed reasonable pharmacokinetic and pharmacodynamic behavior for most tested compounds and thus could be incorporated within various dosage forms to alleviate arthritis except for *n*-nonadecane, *n*-heptacosane and *n*-octacosane, which were found outside the confidence eclipse of both BBB and HIA. Regarding toxicity prediction, all the tested metabolites revealed no mutagenicity in Ames prediction with no carcinogenic potential in male rat FDA, except β -caryophyllene and α -humulene. β -caryophyllene was

previously examined for its cytotoxic potential, which was categorized as toxic at a dose greater than 2000 mg/kg body weight) of the Globally Harmonized Classification System of OECD Guideline 423. Meanwhile, virtual studies performed with ProTox-II and AdmetSAR 2.0) reported only its immunocytotoxic activity [56]. Regarding α -humulene, our results were in accordance with those previously reported, where α -humulene and linalool were among the most toxic terpenes. Their IC₅₀/LC₅₀ or LD₅₀ values vary with time and are dose dependent. Their cytotoxic effects mainly rely on their ability to disrupt the plasma membrane, with concomitant lipid peroxidation and reactive oxygen species production. This in turn affects additional organs such as the kidney, liver, brain and lungs [57]. However, the tested compounds caused no eye irritation but moderate to severe skin irritation, which in turn accounted for the utilization of some terpenes as skin penetration enhancers in various topical dosage forms [58].

Regarding the polar metabolites of the leaves that exist in the ethyl acetate fraction, as identified by LC/MS, resulted in the tentative assignments of 24 peaks, whereas six peaks remained unidentified. These identified metabolites mainly belonged to phenylpropanoids, whereas flavonoids, iridoid glycosides, phenolic acids and their derivatives were also detected and experienced notable antioxidant and antihyperglycemic activities in various *in vitro* assays. This could be attributed to their pronounced ability to inhibit reactive species, modify intestinal microbiota, scavenge free radicals, or even enhance oxidative enzymes. Additionally, they decrease blood glucose levels and prohibit protein glycation,

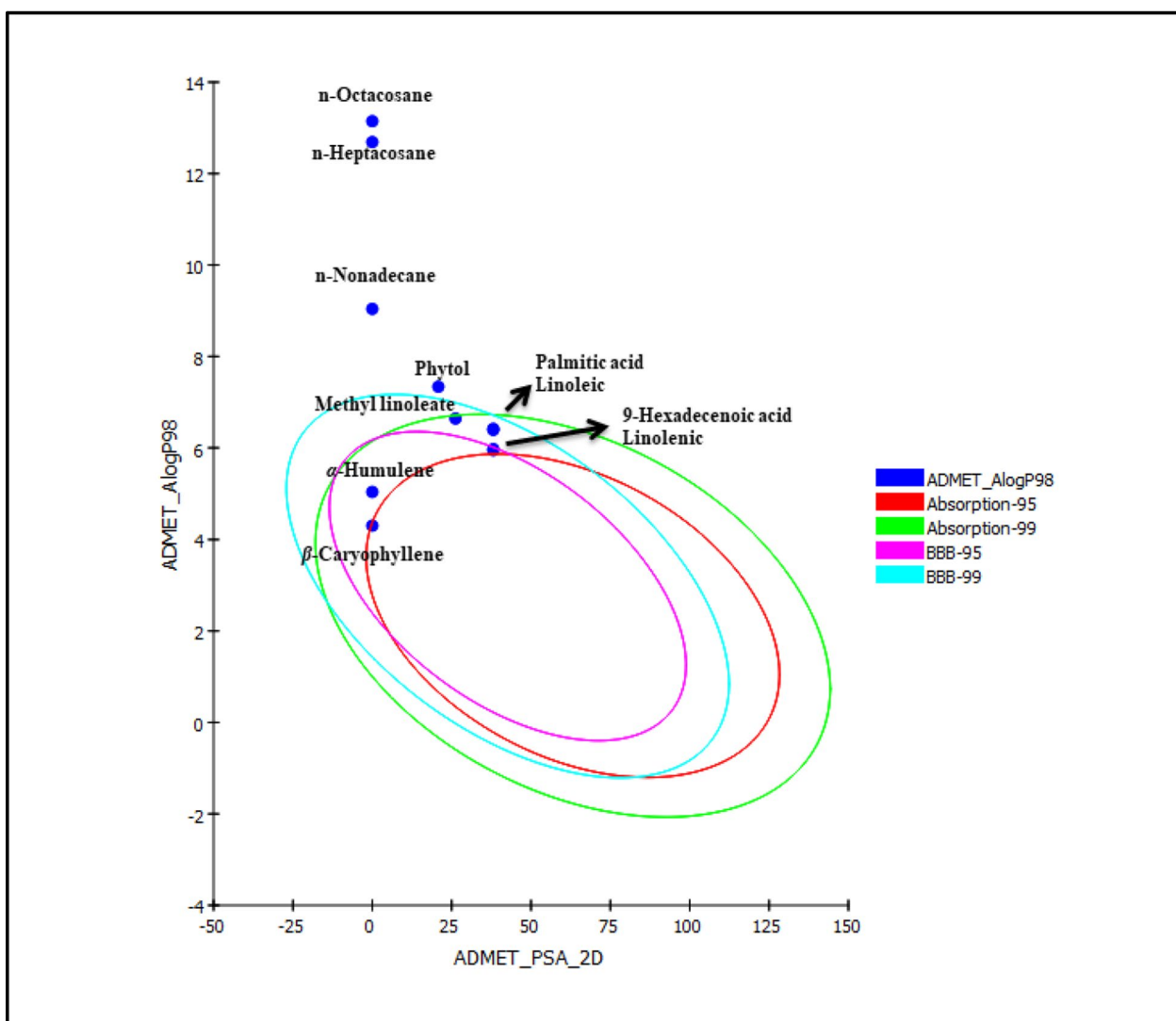


Fig. 4 ADME plot of the major components identified in the volatile constituents of *C. speciosum* leaves displaying 95% and 99% in confidence limit ellipses with respect to BBB and HIA models

dipeptidyl peptidase-IV activity and other enzymes implicated in the metabolism of carbohydrates. They also enhance insulin secretion from pancreatic β -cell [18]. Verbascoside, a predominant component in the genus, previously showed potent antihyperglycemic activity, with potent inhibition of α -amylase and α -glucosidase, as estimated by IC_{50} :337.4 and 42.28 μ g/mL, respectively, compared to acarbose (IC_{50} :363.29 and 122.07 μ g/mL in both assays, respectively). It also exhibited a pronounced aldose reductase activity with IC_{50} values of 0.73 μ g/mL. Similarly, it prohibited the generation of advanced glycation end products (AGEs), yielding IC_{50} values 68.31 μ g/mL, whereas aminoguanidine, the standard, revealed IC_{50} of 53.04 μ g/mL [59]. Additionally, rosmarinic acid

exhibited a notable effect on HFD-STZ-induced diabetes. It effectively reduces the levels of serum free fatty acids, triglycerides and cholesterol. Additionally, it decreased the plasma and pancreatic levels of protein carbonyls and lipid peroxides in diseased animals. Thus, it ameliorated the oxidative stress triggered by HFD-STZ in pancreatic β -cells. This obviously caused an antioxidant potential manifested by elevation in pancreatic catalase and glutathione-S-transferase superoxide dismutase glutathione peroxidase levels and nourishing plasma levels of vitamin E ceruloplasmin, reduced glutathione and vitamin C, which was confirmed by Nrf-2 and hemeoxygenase levels. Furthermore, it elevated the insulin sensitivity index and

Table 5 TOPKAT evaluation of the main secondary metabolites identified in the volatile constituents of *C. speciosum* leaves

Compounds	Ames prediction	Rat oral LD50	Rat chronic LOAEL	Skin irritancy	Ocular irritancy	Rat male FDA	Rat female FDA
β -Caryophyllene	Non-mutagen	0.78	0.01	Moderate	Non-irritant	Carcinogen	Carcinogen
α -Humulene	Non-mutagen	1.11	0.02	Moderate	Non-irritant	Carcinogen	Carcinogen
<i>n</i> -Nonadecane	Non-mutagen	14.80	0.90	Severe	Non-irritant	Non-carcinogen	Carcinogen
9-Hexadecanoic acid	Non-mutagen	5.35	0.35	Moderate	Non-irritant	Non-carcinogen	Non-carcinogen
Palmitic acid	Non-mutagen	7.60	0.93	Moderate	Non-irritant	Non-carcinogen	Non-carcinogen
Methyl linoleate	Non-mutagen	5.48	0.18	Moderate	Non-irritant	Non-carcinogen	Non-carcinogen
Phytol	Non-mutagen	9.43	0.27	Moderate	Non-Irritant	Non-carcinogen	Carcinogen
Linoleic acid	Non-mutagen	3.75	0.28	Severe	Non-irritant	Non-carcinogen	Non-carcinogen
Linolenic acid	Non-mutagen	4.56	0.21	Moderate	Non-irritant	Non-carcinogen	Carcinogen
<i>n</i> -Heptacosane	Non-mutagen	34.49	0.83	Severe	Non-irritant	Non-carcinogen	Carcinogen
<i>n</i> -Octacosane	Non-mutagen	38.06	0.82	Severe	Non-irritant	Non-carcinogen	Carcinogen

Rat Chronic LOAEL Rat oral LD50 is represented in g/kg body weight

plasma insulin levels, while decreasing blood glucose and advanced glycation end products [60].

LC/MS results are in accordance with those previously identified from various *Clerodendrum* species with phenylpropanoids representing a noticeable class of phytoconstituents. Isolation of phenylpropanoid glycosides from various species of the Lamiaceae family was observed in significant quantities, and they represent an important taxonomic marker among the species of the family, as reported by Erdtman [61]. Additionally, previous studies performed on *C. splendens* and *C. inerme* have revealed their richness in phenylpropanoids, iridoid glycosides, phenolic acids, flavonoids, fatty acid derivatives and diterpenoids. Verbascoside, magnolioside A or D and markhamioside B were previously detected in *C. splendens* and *C. inerme* and constituted their major phenylpropanoids. In addition, rhamnazin-3-*O*-rutinoside and rosmarinic acid were previously identified in *C. splendens* leaves and detected in the ethyl acetate fraction of *C. speciosum* and thus ascertained the relationship between both species. Moreover, acacetin 7-*O*-hexuronide, scutellarein, scutellarein-7-*O*-hexuronide and naringenin were detected in *C. inerme* leaves [35]. Moreover, from *C. infortunatum*, incanoside C, leucosceptoside A, jionoside C and D, verbascoside, isoacteoside, martynoside and its acetylated derivatives acacetin 7-*O*-glucuronide together with apigenin 7-*O*-glucuronide were identified, which further confirmed our findings [62]. Undoubtedly, the presence of these metabolites was reflected in the observed antioxidant and antihyperglycemic potentialities of the ethyl acetate fraction. It is noteworthy to mention that verbascoside predominately exists in *Clerodendrum* species and plays a crucial role in traditional medicine. It was also found in *C. glandulosum*

leaves, which are traditionally consumed as a vegetable or soup in Northeast India for the amelioration of diabetes and hypertension and further showed antioxidant, α -amylase and α -glucosidase inhibitory potential [63]. In addition, rosmarinic acid previously exhibited a potent antioxidant activity as evidenced by different assays, as ABTS and DPPH with TEAC equals 3.7 mM Trolox in the former and IC₅₀ of 72.3 μ M in the latter, where it serves as a free radical scavenger [64, 65]. Moreover, many studies have documented the effectiveness of phenylpropanoids as antioxidants and antihyperglycemic agents, where the introduction of hydroxyl groups increases the activities in contrast to additional glycosylation and methoxylation, which reduce the activity [66]. Additionally, pantamajoside acts as a protective agent versus ultraviolet light, exhibiting prominent antioxidant potential but with very low toxicity [67]. Additionally, leucosceptosides that were identified in many *Clerodendrum* species such as *C. bungei* and *C. phlomidis* roots, *C. infortunatum* leaves, and *C. chinense* showed antioxidant activity similar to that of ascorbic acid. It also showed potent enzyme inhibitory potential against different enzymes, such as α -glucosidase activity, with an IC₅₀ value of 273.0 μ M, which is slightly higher [68].

Conclusion

From the results illustrated in the following research, it is clear that *C. speciosum* leaves could serve as a source of components with various biological potentialities. The non-polar metabolites present in the volatile constituents isolated from the leaves and identified by GC/MS revealed promising antiarthritic ability. Its metabolites belong mainly to oxygenated monoterpenes, sesquiterpenes, their oxygenated derivatives, fatty acids and their

esters, which constituted the predominant metabolites. This was evidenced by the strong inhibition of albumin denaturation, with IC_{50} value approaching that of diclofenac sodium, the standard drug used. Meanwhile, the polar metabolites of the leaves that existed in the ethyl acetate fraction, as identified by LC/MS experienced notable antioxidant and antihyperglycemic activities. Its metabolites mainly represented by phenylpropanoids in addition to flavonoids, iridoid glycosides, phenolic acids and their derivatives. In addition, ADME/TOPAKT prediction, based on the prevalent identified secondary metabolites detected in leaf volatile constituents, showed reasonable pharmacokinetic, toxicity and pharmacodynamic pattern for most tested compounds, except for *n*-nonadecane, and *n*-heptacosane and *n*-octacosane, and hence could be incorporated within various dosage forms to alleviate arthritis. However, further *in vivo* studies exploring dose–response relationships, mechanism of actions, as well as the potential synergistic effects of the identified metabolites are highly recommended. Furthermore, clinical trials including safety pharmacology and bioavailability studies should be conducted to support the biological activities of plants.

Abbreviations

ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ACAE	Acarbose equivalent
ADME/TOPAKT	Absorption, distribution, metabolism, excretion and toxicity prediction
AM630	Iodopravadoline, a novel aminoalkylindole
BBB	Blood–brain barrier
BCP	β -Caryophyllene
CB2	Cannabinoid receptor 2
CPY2D6	Cytochrome P450 2D6
CSE	Ethyl acetate fraction of <i>C. speciosum</i> leaves
CSV	<i>C. speciosum</i> Leaf volatile constituents
CUPRAC	Cupric-reducing antioxidant capacity
2D	Two dimensional
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
GC-MS	Gas chromatography linked to mass spectrometry
HFD-STZ	High-fat-diet streptozotocin-induced diabetes
HIA	Human intestinal absorption
IC_{50}	Half-maximal inhibitory concentration
IKI	Iodine/potassium iodide
LC–MS	Liquid chromatography linked coupled with mass spectrometry
LD_{50}	Lethal dose, 50%
LOAEL	Rat chronic lowest observed adverse effect level
MCA	Metal-chelating activity
<i>m/z</i>	Mass-to-charge ratio
NF- κ B	Nuclear factor kappa B
NIST	National Institute of Standards and Technology
Nrf-2	Nuclear factor erythroid 2-related factor 2
PGC-1 α	Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α
PHD	Phosphomolybdenum
<i>p</i> NPG	<i>p</i> -Nitrophenyl- β -D-glucopyranoside
PPAR- γ	Peroxisome proliferator-activated receptor-gamma
PPB	Plasma protein binding pattern
S.D.	Standard deviation
TE	Trolox equivalent

TEAC

Trolox equivalent antioxidant capacity

Acknowledgements

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia, under Grant Number: (GPIP-885-249-2024). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Author contributions

SSE contributed to investigation, methodology, supervision and writing—review and editing. RFA contributed to conceptualization, data curation, formal analysis, methodology and writing—original draft. GZ contributed to investigation, methodology, formal analysis and writing—review and editing. RTM was involved in data curation, funding acquisition, supervision and writing—review and editing. MLA contributed to conceptualization, funding acquisition, supervision and writing—review and editing. FSF contributed to conceptualization, data curation, formal analysis, investigation, methodology and writing—original draft. All authors have read and agreed to the published version of the manuscript.

Funding

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University (KAU), Jeddah, Saudi Arabia, under grant number: (GPIP-885-249-2024). The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Availability of data and materials

All the data are available in the manuscript and further data could be given by the authors upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 31 December 2024 Accepted: 13 March 2025

Published online: 07 April 2025

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