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Citharexylum spinosum promotes antioxidant, anti-inflammatory, and anti-acetylcholinesterase activities

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Abstract

Citharexylum spinosum L., a species of flowering plants, belongs to the family Verbenaceae and known with the common name Fiddlewood. The main aim of the present research was to evaluate the anti-acetylcholinesterase, antioxidant and anti-inflammatory activities of the plant. Six successive leaves extract of *C. spinosum* L.; *n*-hexane (*n*-Hex), *n*-hexane: benzene (*n*-Hex:Bz), chloroform ($CHCl_3$), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) and ethanol:H_2O 50% (EtOH:H_2O 50%) were evaluated for their anti-acetylcholinesterase, antioxidant and anti-inflammatory activities. The highest anti-acetylcholinesterase activity was obtained by *n*-BuOH extract followed by *n*-Hex: Bz and EtOH: H_2O 50% (IC_{50}; 0.60, 0.80 and 1.86 µg/ml, respectively). The antioxidant results showed that, *n*-BuOH exhibited the highest DPPH$^{\bullet}$ scavenging activity (85.91, 87.32, 56.33 and 39.43%) at different concentrations (1-100µg/µl), and 391.71±3.2µg/ml for H_2O_2 antioxidant method. While EtOH: H_2O 50% extract exhibited the highest antioxidant by reducing power method, FRAP and ABTS$^{\bullet}$ scavenging activity (87.94%, 1382.29µmol of Fe^{2+}/g sample and 20.01mmol of Trolox/g sample, respectively). The highest anti-inflammatory effect obtained by *n*-BuOH extract followed by EtOH: H_2O 50% (IC_{50} 74.50 and 109.80µg/ml, respectively). The screening of bimolecular components was carried out for *n*-BuOH and EtOH: H_2O 50% extracts by using LC-MS/MS. The LC-MS/MS profile showed the presence of phenolic acids (chlorogenic, gallic, caffeic, coumaric, ellagic, cinnamic, syringic, ferulic, and 3,4-dihydroxybenzoic), methyl gallate, and flavonoids (rutin, vanillin, naringenin, quercetin and luteolin). The current study evaluated for the first time the anti-acetylcholinesterase activity of *C. spinosum* L. leaves besides, the determination of the antioxidant and anti-inflammatory activities by different methods.

Keywords: *Citharexylum spinosum* L., successive extracts, acetylcholinesterase inhibitory activity, antioxidant, anti-inflammatory, LC-MS/MS profile

1. Introduction

There is an association between cholinergic enzymes and both oxidative stress and inflammatory pathways. The cholinergic system involves brain neurotransmitter; acetylcholine (ACh) and its hydrolyzing enzymes [butyryl cholinesterase (BChE) and acetylcholinesterase (AChE)] [1]. Anti-cholinesterases or its inhibitors inhibit the cholinesterase enzyme from breaking down the ACh, increasing both the duration and level of the neurotransmitter action [2]. The inactivation of enzyme induced by different inhibitors results in the accumulation of acetylcholine, the hyperstimulation of muscarinic and nicotinic receptors, as well as disrupted neurotransmission [2]. Antioxidants are the substances that delay or prevent the autoxidation processes of other compounds in addition they neutralize the free radicals. Antioxidants types include synthetic and natural antioxidants as the major types besides, others endogenous, exogenous, and dietary antioxidants … etc. [3]. Free radicals have gained growing importance in medicine and biology fields. They arise from many different sources of endogenous and exogenous [4]. Endogenous sources are cellular organs with high consumption of oxygen (mitochondria, endoplasmic reticulum and peroxisomes) while exogenous sources like pollution, smoking, alcohol, heavy or transition metals, pesticides, industrial solvents, etc. However,

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the overproduction of these radicals' damage macromolecules including; lipids, proteins and nucleic acids subsequently, they lead to the damage of tissue in various degenerative and chronic diseases [4]. These diseases include atherosclerosis, stroke cancer, myocardial infarction, inflammatory joint disease, degenerative eye disease asthma, senile dementia and diabetes [5, 6]. Synthetic antioxidants are products of artificial synthesis with side effects on health at the long term [3]. However; natural antioxidants are products of natural synthesis exist in plants, animals or bacteria with benefits of; promotion the eco-friendly food system/circular economy, maximizing the exploitation of natural antioxidants and repositioning food systems to reduce/prevent some chronic diseases [3]. Therefore, the replacement of synthetic antioxidants with natural ones is necessary.

Inflammation is the root cause of various chronic diseases such as Alzheimer's, obesity, hypertension, cancer and atherosclerosis disease [7]. In the presence of inflammatory signals, neutrophils (the main source of reactive oxygen species (ROS) during inflammation) first infiltrate the site of inflammation, injury or infection [8]. Thus, oxidative stress develops and further aggravates the inflammation [9]. In oxidative stress, the overproduction of ROS species will drive the inflammatory progression throughout activation the expression of the pro-inflammatory genes [10].

Different species of *Citharexylum* (Verbenaceae family) have anti-allergic, cytotoxic, nephroprotective, antimicrobial, immunomodulatory, hepatoprotective, anti-schistosomal, antihypertensive and antiulcer activities [11-15]. Thus, the current study aims to evaluate *in vitro* anti-acetylcholinesterase, antioxidant and anti-inflammatory properties of *Citharexylum spinosum* L. different extracts. In addition, identify the bioactive compounds of the two most promising extracts by LC-MS/MS method.

2. Materials and methods

2.1. Materials

2.1.1. Collection and identification of plant material

Fresh *C. spinosum* L. leaves were gathered from the farm of El-Orman Botanical Garden, Egypt during September 2021. The plant was authenticated by Prof. Dr. Mohamed El-Khateeb, Professor of Ornamental Plant Taxonomy, Faculty of Agriculture, Cairo University and the plant material was kept at the herbarium of Cairo University, Faculty of Agriculture, Ornamental Horticulture Department (Egypt). The given voucher specimen for *C. spinosum* L. was 001359CC-000326, 05-01-01-0031. The leaves were ground and homogenized into a coarse powder and allowed to air dry in the shade. The leaves powder was kept until use in opaque screw-tight jars.

2.1.2. Chemicals and standards

The standards and experimental materials were purchased from Sigma-Aldrich (India). All of the compounds came from Sigma Products, Merck, and Aldrich and were of analytical grade. The kits were made by Sigma Chemical Company (St. Louis, MO, USA), Biodiagnostic Company (Cairo, Egypt), and Biosystems (Alcobendas, Madrid, Spain). Sigma-Aldrich Chemical Company (USA) provided the standards for phenolic acids and flavonoids. Fisher Chemical Company (UK) supplied formic acid (LC grade). Supelco in the United States provided the methanol and acetonitrile (LC grade). The water was Milli-Q from Merck Millipore.

2.2. Methods

2.2.1. Preparation of successive extracts of C. spinosum L. leaves

Extraction was carried out according to the method of El-Baz et al. [16]. Powdered leaves of *C. spinosum* L. (1.5 kg) were extracted by soaking using successive six solvents with different polarities (in ascending order) as follow: *n*-hexane (*n*-Hex, with percentage yield 1.20%), *n*-hexane: benzene (*n*-Hex: Bz, 2.50%), chloroform ($CHCl_3$, 1.60%), ethyl acetate (EtOAc, 14.94%), *n* Butanol (*n*-BuOH, 0.70%) and Ethanol: H_2O 50% (EtOH: H_2O 50%, 12.19%) and put on shaker (Heidolph UNIMAX 2010) at 150 rpm for 24 hrs. The extract was filtered by Whatman No. 4 filter paper and a Buchner funnel. The plant residue was re-extracted for another two times by adding the same volume of *n*-Hex. The three filtrates were combined and concentrated by Rotary evaporator (Heidolph-Germany) under vacuum at 40°C. The plant residual was dried and soaked in *n*-Hex: Bz, $CHCl_3$, EtOAc, *n*-BuOH and EtOH: H_2O 50%, successively as described earlier.

2.2.2. Anti-inflammatory activity

2.2.2.1. Cell culture

The American Type Culture Collection (ATCC) provided the RAW 264.7 macrophage cell line. RAW 264.7 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media, which was supplemented with 10% heat-inactivated fetal bovine serum and 1% pen/strep. The cells were cultured in a humidified incubator at 37 °C with 5% CO_2 in the air. The cells underwent two subcultures prior to the experiment.

Using a Laminar Flow Cabinet biosafety Class II level (Baker, SG403INT, Sanford, ME, USA), all procedures were completed in a sterile environment.

After 24 hours of seeding 1 x 10^5 cells per ml (in 96 well plates), the RAW 264.7 cells were suspended in RPMI medium and the experiments were then incubated for 24 hours. The examined extracts (EtOAc, *n*-BuOH and EtOH: H_2O 50%) were applied to the cells at concentrations of 12.5, 25, 50, and 100 g/ml, followed by an hour of incubation. The cells were stimulated with 10 µg/ml of lipopolysaccharide (LPS) for an additional 24 hours. To measure nitric oxide (NO), the supernatant was transferred to brand-new 96-well plates. The remaining cells in the old plate, however, were examined for cell viability using the MTT assay. Stocked samples were dissolved in DMSO, while the working samples were prepared in the medium. The mitochondrial dependent reduction of yellow MTT to purple formazan was used to measure the viability of the cells [17]. The following formula was used to compute the viability change percentage:

$$\frac{\text{Reading of extract}}{\text{Reading of negative control}} - 1 \times 100$$

2.2.2.2. Nitric oxide assay

Nitrite measurements in the supernatants of cultivated RAW 264.7 cells were used to gauge NO production. The quantity of nitrite, a stable metabolite of NO, was utilized as a sign of NO generation. The experiment was carried out with a minor modification to the Yoon et al. [18] procedure. The amount of nitrite in the culture medium was calculated using Griess reagent following a 24-hour pre-incubation of RAW 264.7 cells (1 x 10^5 cells/ml) with 10 µg/ml LPS. Phosphoric acid (2.5%), naphthylethylenediamine dihydrochloride (0.1%) and sulfanilamide (1%) make up the Griess reagent. The cell culture media (50 µl) were combined with 50 µl of Griess reagent. This combination was then left to sit at room temperature for 15 minutes. At 540 nm, the absorbance was measured with a microplate reader. In all experiment, the medium fresh culture was used as a blank. The nitrite quantity was determined from the standard curve of sodium nitrite as shown in the following equation:

$$\text{Nitric oxide inhibition \%} = \frac{\text{Control- Extract}}{\text{Control}} \times 100$$

2.2.3. In vitro acetylcholinesterase activity

Acetylcholinesterase (AchE) activity was evaluated for the six extracts according to Ellman et al. [19] method. Suitable volumes of phosphate buffer (PBS), pH 8, AChE and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) stock solutions were mixed and temperature was regulated at 25 °C for 5 min. Under vigorous mixing, a suitable volume of acetylthiocholine iodide stock solution was added. This moment represented the zero time (t = 0) of hydrolysis. The total volume (25 ml) of the reaction mixture. Within the whole reaction, course the dependence of absorbance A (412 nm) vs. time was continually evaluated. The IC_{50} of AChE inhibition was calculated from the equation:

$$\text{\% Relative inhibition} = \frac{\text{Slope of enzyme control} - \text{Slope of extract}}{\text{Slope of enzyme control}} \times 100$$

2.2.4. Antioxidant assays

2.2.4.1. DPPH• assay

Quantitative measurement of free radical scavenging properties for *C. spinosum* L. extracts was evaluated according to McCue et al. [20] method. DPPH• solution (0.1 ml) was freshly prepared in absolute methanol (100 ml). DPPH• solution (1 ml) was added to 1 ml of *C. spinosum* L. tested extracts and ascorbic acid (the reference drug) at four concentrations; 1, 5, 10 and 100 µg/µl). After 30 min incubation, the discoloration was recorded at 517 nm. The measurements were taken in triplicate at least. The DPPH• scavenging activity of was calculated from the equation:

Scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$

A_0: DPPH• solution absorbance (without the extract) and A_1: The tested extracts absorbance with DPPH• solution.

2.2.4.2. Hydrogen peroxide scavenging assay

The ability of *C. spinosum* L. different extracts to scavenge H_2O_2 was determined as described in Ruch et al. [21] method. Aliquot of *C. spinosum* L. extracts (0.1 g at different concentrations; 25-400 µg/ml) was transferred into Eppendorf tubes. The extracts were mixed with 50 mM PBS (pH 7.4) and then H_2O_2 solution (0.6 ml; 2 mM) to reach the volume 0.4 ml. The mixture was vortexed for 10 min, and then the absorbance was recorded at 230 nm. The extracts ability to scavenge H_2O_2 was calculated from the equation:

$$H_2O_2 \text{ scavenging activity \% (µg/ml)} = \frac{A_0 - A1}{A_0} \times 100$$

A_0 = the control absorbance, A_1 = the extract absorbance.

2.2.4.3. Reducing power assay

With just minor modifications, the reducing power activity of *C. spinosum* L. extracts was determined using the Oyaizu et al. [22] technique. For each concentration (ranging from 25 to 400 g/ml), 0.1 g of *C. spinosum* L. extracts (*n*-Hex, *n*-Hex: Bz, $CHCl_3$, EtOAc, *n*-BuOH, and EtOH:H_2O 50% extracts) were combined with 0.5 ml of 1% potassium ferricyanide and 0.5 ml of PBS (0.2 M, pH 6.6). For 20 minutes at 50 °C, this mixture was incubated in a water bath. After adding 0.5 ml of 10% trichloroacetic acid at

room temperature, the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was collected, and 0.5 ml of it was combined with 0.5 ml of distilled water. The final mixture received 0.1 ml of ferric chloride (0.1%) and was allowed to sit at room temperature for 10 minutes. At 700 nm, the absorption was recorded. The positive control used was ascorbic acid. The absorbance of the reaction mixture at 700 nm against ethanol, in the absence (A_0) or presence (A_1) of the tested extracts was determined to calculate the inhibitory rate as:

$$IR\ (\%) = (1 - A_1/A_0) \times 100.$$

2.2.4.4. FRAP assay

The FRAP assay was carried out using the approach that Benzie and Strain [23] previously described. In order to match the linearity range, *C. spinosum* L. extracts were first diluted with deionized water. The 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) working reagent was made by combining 10 volumes of acetate buffer (300 mM; pH 3.6), one volume of TPTZ at a concentration of 10 mM in 40 mM HCl, and one volume of $FeCl_3$ (20 mM). The FRAP working reagent, 2.25 ml, was warmed to 37 °C. After that, 225ml of deionized water and 75 ml of *C. spinosum* L. extracts were introduced to the FRAP reagent. The absorbance was red at 593 nm against the reagent blank after 30 minutes of incubation. Calculated and represented as µmol of Fe^{2+} equivalent/g were the FRAP values.

2.2.4.5. ABTS$^{\bullet}$ assay

Using the Re et al. approach [24], the antioxidant capacity by Trolox equivalent was calculated. The stock solution of ABTS$^{\bullet}$ in water was combined with sodium persulphate (2.45 mM) to create the $ABTS^+$ solution. In order to achieve a stable oxidative state, the mixture was allowed to stand for 12-16 hours while being shaken in the dark at room temperature. The stock solution was diluted with methanol at 734 nm to the absorbance 0.720 for $ABTS^+$ assay. For the spectrophotometric experiment, 2 ml of the $ABTS^+$ reagent and 20 µl of the *C. spinosum* L. extract sample solution were combined. The 10 minute absorbance measurement was made at 734 nm and 37 °C. The Trolox standard solution was used to plot the calibration curve. The results were given in terms of mmol Trolox equivalent per gram of extract or per 100 g of sample.

2.2.5. LC-MS/MS of n-BuOH and EtOH:H_2O 50% extracts

Circumstances and parameters

Instrument

LC-MS/MS was performed using ExionLC™ AC system coupled with AB Sciex Triple Quadruple 5500+ mass spectrometer. The electron spray ionization (ESI) was used and the analysis was performed using the multiple reaction monitoring (MRM) modes.

Positive and negative MRM mode

ZORBAX Eclipse Plus C18 Column (4.6100 mm, 1.8m) performed the separation procedure. Furthermore, there were two eluents (A and B) in the mobile phases. Eluent B was LC grade acetonitrile, and eluent A contained 0.1% formic acid in water. The mobile phase's programming was as follows: from 0 to 1 minute: 2% B, from 1 to 21 minutes: 2 to 60% B, from 21 to 25 minutes: 60% B, and from 25.01 to 28 minutes: 2% B. The flow rate was 0.8 ml/min, and the injection volume was 3 µl. The selected polyphenols were subjected to MRM analysis using both positive and negative ionization modes using the following parameters: curtain gas: 25 psi; IonSpray voltage: 4500 and -4500 for the positive mode and negative mode, respectively; temperature source: 400°C; ion source gas 1 and 2: 55 psi with a declustering potential: 50; collision energy: 25; and collision energy spread: 10.

2.2.6. Statistical analysis

All values obtained in the present study were represented as mean ± SD. In between the obtained means, the statistical differences were determined by one-way analysis of variance (ANOVA) using the program; Statistical Package for the Social Sciences (SPSS), the version 11. The determination of AChE activity required Co-state computer program to show differences between letters whereas at $P \leq 0.05$, different letters were significant.

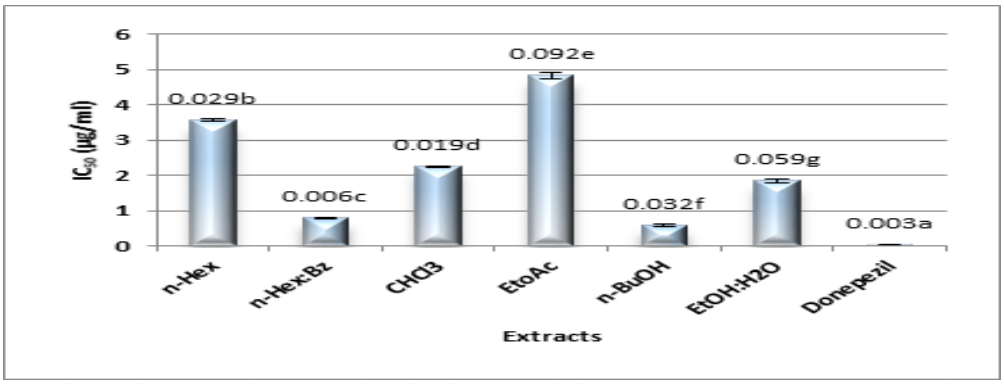
3. Results and discussion

3.1. Anti-acetylcholinesterase activity of C. spinosum L. different extracts

The anti-acetylcholinesterase activity was determined for *C. spinosum* L. six extracts to identify the extract with the highest activity to use it in *in vivo* anti-aging experiment study (unpublished data). In addition, the determination of cytotoxicity for the six extracts (unpublished data) to identify the safe effect of extracts on experimental animals. Depending on the results of anti-acetylcholinesterase activity and cytotoxicity, the three extracts (EtOAc extract, *n*-BuOH and EtOH: H_2O 50%) were chosen for the anti-aging experiment. In alignment with our previous (under publication), Alzheimer's disease (AD), a neurological condition that slowly worsens with age and is conceptualized as a continuous process, is linked to aging and includes mild cognitive impairment (MCI) as well as mild,

moderate, and severe clinical stages of AD dementia. AD is seen as a complicated, multifaceted illness. Recently, the treatment for AD patients has been the use of cholinesterase inhibitors (ChEI), such as tacrine, donepezil, rivastigmine, and galantamine. Interestingly, there is proof that ChEIs also support neuroprotective effects and provide some benefit to Alzheimer's disease patients.

According to Figure (1), *n*-BuOH extract exhibited the highest anti-acetylcholinesterase activity with IC_{50} value 0.60±0.032 µg/ml followed by *n*-Hex:Bz (IC_{50} 0.80±0.006 µg/ml), EtOH:H_2O 50% (IC_{50} 1.86±0.059 µg/ml), $CHCl_3$ (IC_{50} 2.26±0.019 µg/ml), *n*-Hex (IC_{50} 3.57±0.029 µg/ml) and finally EtOAc extract (IC_{50} 4.80±0.092 µg/ml) as compared to standard drug donepezil (IC_{50} 0.05±0.003 µg/ml).



Data are mean of three replicate in each group. Statistical analysis is carried out using SPSS computer program (version 11), One Way Analysis of Variance (ANOVA), coupled with Co-state computer program. Different letters are significant at $P \leq 0.05$. IC_{50}: the half-maximal inhibitory concentration, *n*-Hex: *n*-hexane, *n*-Hex: Bz: *n*-hexane: benzene, $CHCl_3$: chloroform, EtOAc: ethyl acetate, *n*-BuOH: *n*-butanol, EtOH: H_2O 50%: ethanol: H_2O 50%.

Figure 1. Anti-acetylcholinesterase activity of IC_{50} of *C. spinosum* L. different extracts

It is the first time to evaluate the anti-acetylcholinesterase activity of *C. spinosum* L. leaves. Cholinesterase inhibitors possess a strategy for the cure of neurodegenerative diseases *viz* Parkinson's and Alzheimer's [25]. This strategy presented in the degradation of choline (an important neurotransmitter associated with memory) throughout blocking the enzyme of cholinesterase [26]. Flavonoids with more hydroxyl groups exhibited a greater inhibition on AChE [27]. Luteolin isolated was reported to have AChE inhibition potential of 17.26 ± 0.23 µM [28]. Luteolin protects against AD by lowering neuroinflammatory reactions [29]. It inhibited macrophage/monocytes, mast cells and T cells, as well as decreased the release of inflammatory mediators [29]. Hence, the anti-acetylcholinesterase of *C. spinosum* L. extracts may be due to the presence of such bioactive constituents.

3.2. Anti-inflammatory effect of C. spinosum L. different extracts

The three extracts (EtOAc, *n*-BuOH and EtOH:H_2O 50%) were chosen (based on our data under publication in *in vivo* anti-aging experiment), for the anti-inflammatory effect determination. Table (1) revealed the effect of *C. spinosum* L. extracts on the inhibition percentage of NO. *n*-BuOH extract exhibited the highest anti-inflammatory effect followed by EtOH:H_2O 50% with IC_{50} 74.50±4.20 and 109.80±6.98 µg/ml, respectively. While, all extracts of *C. spinosum* L. at concentration of 100 µg/ml showed significant cellular cytotoxicity against RAW cells with cell viability percentage 92.80±5.10, 76.80±4.33 and 82.00±6.22 % for EtOAc, *n*-BuOH and EtOH: H_2O 50% extracts, respectively (Table 2).

Table 1. The effect of *C. spinosum* L. extracts on the inhibition percentage of NO

Extracts	NO inhibition %		IC_{50} µg/ml		
	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	
EtOAc	0.00	0.00	0.00	18.70±0.44^{a}	0.00
***n*-BuOH**	25.00±1.65^{b}	37.50±2.00^{c}	43.70±3.10^{d}	56.20±3.54^{e}	74.50 ±4.20^{f}
EtOH:H_2O 50%	8.70±0.23^{g}	15.30±1.00^{h}	21.60±1.02^{i}	37.50±3.00^{c}	109.80 ±6.98^{j}
Indomethacin	61.00±4.10^{k}	68.00±5.00^{l}	72.00±6.10^{f}	76.00±5.55^{f}	18.23±1.72^{m}

Data are mean of three replicate in each group and expressed as mean ± SD. Statistical analysis is carried out using SPSS computer program (One Way Analysis of Variance (ANOVA)) coupled with Co-state computer program. Different letters are significant at ≤0.05. EtOAc: ethyl

acetate, *n*-BuOH:*n*-butanol, EtOH:H_2O 50%: ethanol:H_2O 50%. LPS: Lipopolysaccharides. IC_{50}: The concentration of extract that is needed to inhibit nitric oxide to half of maximum.

Table 2. Cell viability percentage against RAW cells

Extracts	Cell viability % against RAW cells at 100 µg/ml
EtOAc	92.80±5.10[a]
***n*-BuOH**	76.80±4.33[b]
EtOH:H_2O 50%	82.00±6.22[c]
LPS at 10 µg/ml	100

Data are mean of three replicate in each group and expressed as mean ±SD. Statistical analysis is carried out using SPSS computer program (version 11), One Way Analysis of Variance (ANOVA), combined with Co-state computer program. Different letters are significant at $p \leq 0.05$. EtOAc: ethyl acetate, *n*-BuOH: *n*-butanol, EtOH:H_2O: ethanol:H_2O 50%. LPS: Lipopolysaccharides.

By successfully suppressing the nuclear transcription factor-ß pathway in LPS-activated RAW 264.7 macrophages and lowering inflammatory substances such NO, interleukin 6, and TNF-, phenolic acids demonstrates excellent anti-inflammatory capabilities [30]. Because they can eliminate ROS, polyphenols and flavonoids have strong anti-oxidant and anti-inflammatory capabilities [31]. By altering the Nrf2-HO-1-NF-B signaling pathway, gallic acid reduced inflammation and oxidative stress [32].

Hydroxylated polyphenols or flavonoids are richly present in fruits, vegetables, cereals, herbs, nuts, stems, seeds and flowers of numerous plants [33]. They exhibit numerous medicinal properties such as antioxidant, anti-microbial, anti-cancer, neuroprotective and anti-inflammation [33]. Flavonoids possessing anti-inflammatory properties which can interact with many molecules (Nuclear factor-κB, Phosphoinositide 3-kinases and nuclear factor erythroid 2-related factor 2) included in inflammatory pathways and decrease the activity of chemokines, cytokines and inflammatory enzymes [33]. This unconventional substance inhibits the activation of the MAPK and NF-B/AP-1 signalling pathways, which decreases the expression of inflammatory proteins and associated enzymes. [33].

The current results of LC-MS/MS showed the presence of bioactive compounds including phenolic compounds and flavonoids. Thus, the anti-inflammatory properties of *C. spinosum* L. extracts may be related to the occurrence of such bioactive constituents.

3.3. Antioxidant activity

3.3.1. DPPH• scavenging activity of C. spinosum L. different extracts

Table (3) indicated the DPPH• scavenging activity percentage of *C. spinosum* L. different extracts. The extract *n*-BuOH exhibited the highest DPPH• inhibition activity with a percentage 85.91±7.32, 87.32±4.90, 56.33 ±3.11 and 39.43±2.87% for the concentrations of 1, 5, 10 and 100µg/µl, respectively in a dose independent manner. The DPPH• scavenging activity of *n*-BuOH followed by EtOAc extract with percentages 78.87±4.90, 81.69±7.58, 29.57±2.00 and 0.00 %, EtOH: H_2O 50 % with values of 23.94±2.10, 45.07±3.00, 14.08±1.00 and 0.00%, *n*-Hex: Bz with values of 14.08±1.00, 15.491.36, 0.00 and 0.00%, $CHCl_3$ with values of 2.81±0.01, 12.67±0.98, 0.00 and 0.00%, respectively for the same concentrations. While *n*-Hex extract showed the lowest percentage with values of 1.40±0.02, 14.08±1.21, 9.85±0.06 and 3.80±0.04% for the same concentrations. The extracts *n*-Hex: Bz, $CHCl_3$ and EtOAc at high concentrations showed no results due to the developing color is out of the range of absorbance (517nm).

Table 3. DPPH• scavenging activity percentage of *C. spinosum* L. extracts

Extracts	DPPH• scavenging activity %			
	1 µg/µl	**5 µg/µl**	**10 µg/µl**	**100 µg/µl**
***n*-Hex**	1.40±0.02[a]	14.08±1.21[b]	9.85±0.06[c]	3.80±0.04[d]
***n*-Hex: Bz**	14.08±1.00[b]	15.49±1.36[b]	-	-
$CHCl_3$	2.81±0.01[d]	12.67±0.98[b]	-	-
EtOAc	78.87±4.90[e]	81.69±7.58[e]	29.57±2.00[f]	-
***n*-BuOH**	85.91±7.32[g]	87.32±4.90[g]	56.33±3.11[h]	39.43±2.87[i]
EtOH: H_2O 50 %	23.94±2.10[j]	45.07±3.00[k]	14.08±1.00[b]	-

Data are mean of three replicate in each group and expressed as mean ±SD. Statistical analysis is carried out using SPSS computer program (version 11), One Way Analysis of Variance (ANOVA) combined with Co-state computer program. Different letters are significant at $p \leq 0.05$. *n*-Hex: *n*-Hexane, *n*-Hex:Bz: *n*-hexane: benzene, $CHCl_3$: chloroform, EtOAc: ethyl acetate, *n*-BuOH: *n*-butanol, EtOH:H_2O 50%: ethanol:H_2O 50%.

3.3.2. Hydrogen peroxide scavenging and reducing power activity of C. spinosum L. different extracts

The hydrogen peroxide scavenging activity of *C. spinosum* L. extracts was determined. The results in Table (4) indicated that, *n*-BuOH extract revealed the highest hydrogen peroxide scavenging activity followed by EtOH:H_2O 50%, EtOAc, $CHCl_3$, *n*-Hex:Bz and finally *n*-Hex extract with inhibition values of; 391.71±3.2, 296.27±7.1, 247.18±4.5, 222.51±4.3, 210.96±3.9 and 81.43±2.6 µg/ml, respectively. Table (4) also showed the reducing power activity of successive extracts of *C. spinosum* L. The highest activity was obtained by EtOH: H_2O 50% extract followed by EtOAc, *n*-BuOH, $CHCl_3$, *n*-Hex: Bz and *n*-Hex extracts with percentages of 87.94±1.60, 80.88±2.10, 68.52±2.40, 61.55±2.60, 39.58±3.80 and 38.93±3.80%, respectively.

Table 4. Hydrogen peroxide scavenging and reducing power activity of *C. spinosum* L. extracts

Extracts	Hydrogen peroxide scavenging activity (µg/ml)	Reducing power activity %
***n*-Hex**	81.43±2.60[a]	38.93±3.80[a]
***n*-Hex: Bz**	210.96±3.90[b]	39.58±3.80[a]
$CHCl_3$	222.51±4.30[b]	61.55±2.60[b]
EtOAc	247.18±4.5 0[c]	68.52±2.40[c]
***n*-BuOH**	391.71±3.20[d]	80.88±2.10[d]
EtOH: H_2O 50%	296.27±7.10[e]	87.94±1.60[d]

Data are mean of three replicate in each group and expressed as mean ±SD. Statistical analysis is carried out using SPSS computer program (version 11), One Way Analysis of Variance (ANOVA), combined with Co-state computer program. Different letters are significant at $p \leq 0.05$. *n*-Hex: *n*-hexane, *n*-Hex:Bz: *n*-hexane:benzene, $CHCl_3$: chloroform, EtOAc: ethyl acetate, *n*-BuOH: *n*-butanol, EtOH:H_2O 50%: ethanol:H_2O 50%.

3.3.3. Ferric reducing power and ABTS$^{\bullet}$ scavenging activity of C. spinosum L. different extracts

According to the results in Table (5), EtOH:H_2O 50% exhibited the highest ferric reducing power activity with value of 1382.29±39.00 µmol of Fe^{2+}/ g sample followed by EtOAc, *n*-BuOH and $CHCl_3$ extracts with values of 925.88±22.00, 710.72±18.90 and 313.15±11.90 µmol of Fe^{2+}/ g sample, respectively. ABTS$^{\bullet}$ scavenging activity of *C. spinosum* L. extracts was indicated in Table (5). The highest scavenging effect was obtained by EtOH:H_2O 50% extract followed by EtOAc, *n*-BuOH which showed insignificant change, then finally $CHCl_3$ extracts with values of 20.01±1.10, 9.54±0.45, 8.31±0.56 and 2.54±0.10 mmol Trolox/g sample, respectively.

Table 5. Ferric reducing power and ABTS$^{\bullet}$ scavenging activity of *C. spinosum* L. extracts

Extracts	Ferric reducing power activity (µmol of Fe^{2+}/ g sample)	ABTS$^{\bullet}$ scavenging activity (Trolox mmol / g sample)
***n*-Hex**	87.16±5.33[a]	0.70±0.07[a]
***n*-Hex: Bz**	210.23±10.54[b]	1.80±0.28[b]
$CHCl_3$	313.15±11.90[c]	2.54±0.10[c]
EtOAc	925.88±22.00[d]	9.54±0.45[d]
***n*-BuOH**	710.72±18.90[e]	8.31±0.56[d]
EtOH:H_2O 50%	1382.29±39.00[f]	20.01±1.10[e]

Data are mean of three replicate in each group and expressed as mean ±SD. Statistical analysis is carried out using SPSS computer program (version 11), One Way Analysis of Variance (ANOVA), combined with Co-state computer program. Different letters are significant at $p \leq 0.05$. *n*-Hex: *n*-hexane, *n*-Hex: Bz: *n*-hexane: benzene, $CHCl_3$: chloroform, EtOAc: ethyl acetate, *n*-BuOH: *n*-butanol, EtOH:H_2O 50%: ethanol:H_2O 50%.

Khan

and Siddique [13] studied the antioxidant properties (catalase, superoxide dismutase, glutathione-S-transferase, glutathione reductase, glutathione peroxidase, reduced glutathione and lipid peroxidation) of the chloroform extract of *C. spinosum* leaves (CSCE) in Sprague–Dawley male rats. The authors found that, CSCE exhibited potent nephroprotective and antioxidant properties. They attributed these effects to the flavonoids content of CSCE (127 ± 14.6 as rutin equivalent mg/g of the extract) and other constituents (terpenoids, alkaloids and saponins) identified in the extract. Allam [14] tested two isolated polyphenolic compounds from the aerial parts of *C. quadrangulare* (synonym *C. spinosum*); 1,3,6-tri-*O*-galloyl-*β*-D-glucopyranose (TGG) and methyl gallate for the antioxidant activity by DPPH$^{\bullet}$ method. The two isolated compounds showed higher radical scavenging effect as compared to ascorbic acid with IC_{50} of 1.5 and 10.8 µM, respectively [14]. Moreover, the antioxidant effect by DPPH$^{\bullet}$ method for *C. spinosum* flowers (the essential oil and various solvent extracts) was estimated by

Mar and Pripdeevech [34]. The authors revealed that the essential oil had the highest antioxidant activity and the highest total phenol content with IC_{50} of 62.7 and 107.3 µg/ml, respectively.

Farrag et al. [35] studied the anti-schistosomal effect of chloroform extract of *C. quadrangular*e leaves. The authors mentioned that, the combination of the supplements (vitamin E, selenium, and chloroform extract of *C. quadrangulare* as well as their mixture) and Praziquantel (PZQ) drug improved the efficiency of PZQ. The authors showed also, the combination of supplement was effective in attenuating the oxidative insult as well as other parameters which included; lipid peroxidation (MDA), and total immunoglobin E (total IgE), interleukin- 10 (IL-10), tumor necrosis factor-alpha (TNFα), gamma-glutamyl transferase (γ-GT), alanine aminotransferase (ALT), hepatic hydroxyproline content, NO, reduced glutathione (GSH), catalase (CAT), thioredoxin reductase (TrxR), glutathione reductase (GR), associated with *Schistosoma mansoni* infection. The possible mechanism that may explain the anti-schistosomal effect of *C. quadrangular*e chloroform extract may be due to its content of active constituents (flavonoids and triterpenes) which affect the vitality of schistosome different stages and the fecundity of the remaining female adult worms [36]. Thus, the possible interpretation for the present antioxidant effect of *C. spinosum* L. extracts is that, they contain biological constituents such as phenolic compounds and flavonoids which known for their potential antioxidant activity.

3.4. LC-MS/MS

Both extracts of *n*-BuOH and EtOH: H_2O 50% exhibited the highest antioxidant and anti-inflammatory effect. Therefore, the LC-MS/MS was carried out for the two extracts to identify the bioactive constituents. Figure (2) indicated the phenolic compounds and flavonoids of *n*-BuOH extract compared to those in the standard one. It showed the presence of the organic acids (chlorogenic, gallic, caffeic, coumaric, ellagic, cinnamic, ferulic, syringic, and 3,4-dihydroxybenzoic), methyl gallate in addition, the presence of the five flavonoids (rutin, vanillin, naringenin, quercetin and luteolin). Caffeic acid revealed the highest content among phenolic compounds with value 366.00 mg/g however; methyl gallate showed the lowest content (0.09 mg/g). Vanillin gained the highest content among flavonoids (19.84 mg/g) however; rutin showed the lowest content (0.04 mg/g) (Table 6).

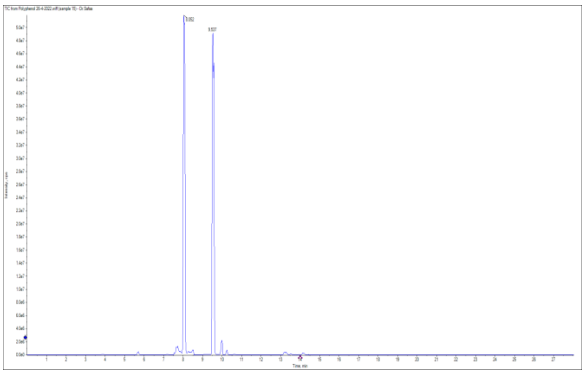


Figure 2. LC-MS/MS chromatogram of *n*-BuOH extract

Table 6. Content of polyphenols determined in *n*-BuOH extract by the proposed LC-MS/MS method

Ser.	Compound	Q1 (m/z)	Q3 (m/z)	RT	ng/g	mg/g
1	**Chlorogenic acid**	355.1	163	7.37	427.85	0.43
2	**Daidzein**	ND	ND	ND	ND	ND
3	**Gallic acid**	168.9	124.9	3.89	2996.25	3.00
4	**Caffeic acid**	178	135	8.07	366001.21	366.00
5	**Rutin**	609	299.9	9.74	36.73	0.04
6	**Coumaric acid**	162.9	119	9.55	218445.52	218.45
7	**Vanillin**	151	136	9.55	19837.98	19.84
8	**Naringenin**	271	119	14.99	5377.08	5.38
9	**Quercetin**	301	151	13.60	48.90	0.05
10	**Ellagic acid**	301	145	9.97	319.72	0.32
11	**3.4-Di hydroxyl benzoic acid**	152.9	109	5.74	8239.01	8.24
12	**Hesperetin**	ND	ND	ND	ND	ND
13	**Myricetin**	ND	ND	ND	ND	ND
14	**Cinnamic acid**	146.9	102.6	14.17	185423.90	185.42
15	**Methyl gallate**	183	124	7.47	85.90	0.09
16	**Kaempferol**	ND	ND	ND	ND	ND
17	**Ferulic acid**	192.8	133.9	10.26	24939.92	24.94
18	**Syringic acid**	196.8	181.9	8.41	8724.99	8.72
19	**Apigenin**	ND	ND	ND	ND	ND
20	**Catechin**	ND	ND	ND	ND	ND

21	**Luteolin**	284.7	132.9	13.54	1565.18	1.57

Q1: Quadrupoles Q1, Q3: Quadrupoles Q3, RT: Retention time, ND: Not detected.

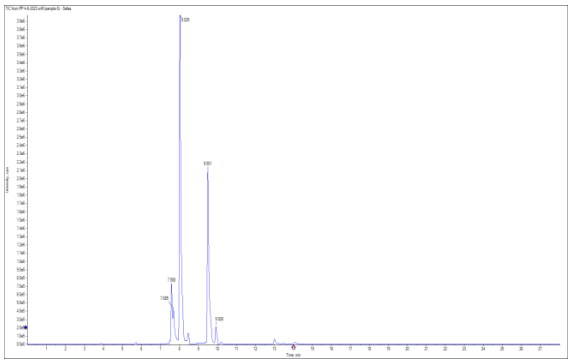


Figure 3. LC-MS/MS chromatogram of EtOH: H_2O 50% extract

Figure (3) indicated the phenolic compounds and flavonoids of EtOH: H_2O 50% extract compared to those in the standard one. Figure 3 showed the presence of the phenolic compounds; chlorogenic, gallic, caffeic, coumaric, ellagic, 3,4-dihydroxybenzoic, cinnamic, ferulic and syringic in addition to methyl gallate and the presence of the two flavonoids (rutin and luteolin). Caffeic acid showed the highest content with value 34.70 mg/g in spite, methyl gallate showed the lowest content (0.05 mg/g). The flavonoid, luteolin presented the highest content with value 0.21 mg/g. However, rutin showed the lowest content (0.03 mg/g) (Table 7).

Table 7. Content of polyphenols determined in EtOH: H_2O 50% extract by the proposed LC-MS/MS method

Ser.	Compound	Q1 (m/z)	Q3 (m/z)	RT	ng/g	mg/g
1	**Chlorogenic acid**	355.1	163	7.34	340.79	0.34
2	**Daidzein**	ND	ND	ND	ND	ND
3	**Gallic acid**	168.9	124.9	3.89	970.93	0.97
4	**Caffeic acid**	178	135	8.04	34702.01	34.70
5	**Rutin**	609	299.9	9.71	25.71	0.03
6	**Coumaric acid**	162.9	119	9.52	11564.16	11.56
7	**Vanillin**	ND	ND	ND	ND	ND
8	**Naringenin**	ND	ND	ND	ND	ND
9	**Quercetin**	ND	ND	ND	ND	ND
10	**Ellagic acid**	301	145	9.94	1551.76	1.55
11	**3.4-Dihydroxy benzoic acid**	152.9	109	5.72	1655.10	1.66
12	**Hesperetin**	ND	ND	ND	ND	ND
13	**Cinnamic acid**	146.9	102.6	14.09	37056.93	37.17
14	**Methyl gallate**	183	124	7.42	46.51	0.05
15	**Kaempferol**	ND	ND	ND	ND	ND
16	**Ferulic acid**	192.8	133.9	10.2	2312.46	2.31
17	**Syringic acid**	196.8	181.9	8.37	1049.60	1.05
18	**Apigenin**	ND	ND	ND	ND	ND
19	**Catechin**	ND	ND	ND	ND	ND
20	**Luteolin**	284.7	132.9	13.48	213.41	0.21

Q1: Quadrupoles Q1, **Q3:** Quadrupoles Q3, **RT:** Retention time, **ND:** Not detected.

Oxidative stress, the imbalance between oxidants and antioxidants, leads to disruption of redox signaling and/or molecular damage [37]. If any disturbance occurs in the redox balance toward oxidation by excessive production of reactive oxygen species (ROS), hydrogen peroxide $(H_2O_2)^-$, superoxide anion radical ($O_2^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen (1 O_2), nitrogen monoxide ($NO^{\bullet}$), peroxynitrite (ONOO), hypochlorous acid (HOCl), oxidative stress $(OS)^-$will be induced [38].

Polyphenols are the most prevalent antioxidants that gaining large popularity [39]. Phenolic acids, a particular class of polyphenol, contain one carboxylic acid group [40]. Benzoic acid and cinnamic acid derivatives are examples of phenolic acids. Benzoic acid derivatives including; *p*-hydroxy benzoic, salicylic, gallic, and ellagic. Moreover, cinnamic acid derivatives imply *p*-coumaric, caffeic, and ferulic [41]. Numerous biological actions involving chlorogenic, ellagic and gallic acid have been documented [42, 43]. They exhibit good antioxidant activities due to their polyphenolic structure [44]. Any antioxidant's mode of action is determined by its chemical structure, therefore variations in chlorogenic acid and its derivatives' chemical structures result in variations in their antioxidant properties [45]. Gallic acid, a phenolic compound, also provides significant health advantages because of its antioxidant capabilities [46]. The main groups of secondary metabolites that were isolated are simple phenolic compounds.

Polyphenols with flavonoid structure are the most widely studied category of polyphenols. The properties of flavonoids in disease prevention are owing to their antioxidant, anti-inflammatory and anticancer activities [47]. For example, rutin can alleviate oxidative stress, suppresses the malondialdehyde formation and glutathione disulfide [48]. Luteolin, a flavonoid widely present in the plant kingdom, has shown a range of pharmacological properties such as antioxidant, anti-inflammatory, analgesic and neuroprotective in several research studies [49]. The antioxidant properties of luteolin may be contributed to the inhibition of chronic inflammation [49]. Naringenin is a bioflavonoid, which possesses intrinsic antioxidant effects. It contributes to revealing inflammation and oxidative stress process (an imbalance resulted from large amount of free radicals) [50]. Quercetin is a flavonol compound that has a variety of positive biological activities. It is widely exist in nature and human diet with potent oxidative properties and biological activities [51].

In vitro and *in vivo* studies demonstrated various biological activities of caffeic acid such as anti-inflammatory, hepatoprotection, antitumor, antimicrobial antioxidant and anticancer properties through several mechanisms of action in fighting towards this diseases including ROS prevention, prooxidant action, angiogenesis [52-54], suppression of matrix metalloproteinase-2 (MMP-2) and matrix metallopeptidase 9 (MMP-9) [54] justifying the differences in the results found. The cell and animal experiments revealed that methyl gallate has good therapeutic effects such as anti-inflammatory [55], anti-tumour [56], and anti-oxidation [57]. Cinnamic acid and its derivatives showed a range of cellular protection and a reduction in the metabolic syndrome's risk factors, as well as anti-inflammatory and anti-proliferative capabilities [58]. Another derivative of cinnamic acid, ferulic acid showed similar activities jointly with direct antioxidant activity, since it contains a phenolic moiety [59, 60].

The current results of LC-MS/MS profile of *n*-BuOH and EtOH: H_2O 50% extracts revealed the presence of the bioactive substances phenolic compounds and flavonoids. All these compounds are known with their various biological activities. Thus, the anti-acetylcholinesterase, antioxidant and anti-inflammatory properties of *C. spinosum* L. different extracts are attributed to the occurrence of such bioactive constituents.

4. Conclusion

It can be concluded from the current results, different extracts of *C. spinosum* L. leaves possessed anti-acetylcholinesterase, antioxidant and anti-inflammatory activities especially *n*-BOH and EtOH: H_2O 50% extracts. The results LC-MS/MS analysis of *n*-BuOH and EtOH: H_2O 50% extracts showed that these extracts were wealthy in caffeic acid followed by coumaric acid. However, further *in vitro* and *in vivo* researches are needed to isolate these bioactive constituents and evaluate their other biological activities.

Conflict of interest

There are no conflicts to declare.

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