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ANTI-INFLAMMATORY ACTIVITY OF THE FOOD PLANT *CALLIGONUM POLYGONOIDES* L. FLAVONOIDS TARGETING NF-κB

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Calligonum polygonoides L. subsp. comosum (Polygonaceae) is a wild shrub that grows on the sandy deserts of various regions in North Africa, Western Asia and Southern Europe. In some of Asian countries, the flower buds and young branches of the plant are used to prepare traditional food dishes. Also, different organs of C. polygonoides L. are used traditionally in treatment of stomach diseases. The isolation of anti-inflammatory lead compounds was performed by using different normal silica gel, reversed phase C-18, and sephadex LH-20 open columns. The identification of the isolated compounds was done using NMR spectral techniques. The isolated compounds are of different flavonoid classes; two flavonol glycosides; kaempferol-3-O-β-D-glucuronide and mequilianin, one flavan-3-ol; catechin, and one dihydro-flavonol; taxifolin. The methanol extract and ethyl acetate fraction of Calligonum polygonoides L. subsp. comosum exhibited anti-inflammatory activity against NF-κB translocation pathway on HEK293 cells. All of them were active against NF-κB translocation, and previously isolated from the plant under study. Kaempferol-3-O-β-D-glucuronide, a flavonoid glycoside demonstrated the most potent NF-κB inhibition in comparing with other flavonoids; taxifolin, catechin, and mequilianin. Therefore, the food plant aerial parts of C. polygonoides are rich in nutraceuticals (flavonoids and flavonoid glycosides) that can be utilized in the treatment of vascular inflammation.

INTRODUCTION

Calligonum polygonoides L. subsp. *comosum* is a wild shrub that belongs to family Polygonaceae; it grows on the sandy deserts of tropical regions in North Africa, Western Asia and Southern Europe[1]. The flower buds and young branches of the plant are used to prepare some traditional food dishes in some Asian countries[1]. The fresh flowers are edible as sugars and nitrogenous constituents rich bread[1]. The plant fruits also are used as fodder for cattles[2]. The plant aerial parts are used for treatment of stomach disorders and toothaches in folk medicine[3]. Additionally, the flowers are traditionally used for cough, cold, and asthma and shoot latex is applied topically to help in healing of dog bites, scorpion tingle and eczema[4].

Previously reported biological studies on the aerial parts of the plant extracts demonstrated protective activity in oxidative stress[5], antiosteoporotic[6], anti-ulcer[7], hypoglycaemic[8], cytotoxicity, and antioxidant activities[3]. The enzymes inhibition activities of different *C. polygonoides* extracts against amylase, tyrosinase, and acetylcholinesterase enzymes have been reported as well[4]. The preliminary phytochemical screening study in the seeds, buds, stems, and flowers extracts resulted in detection of flavonoids at all plant parts, in addition to alkaloids, terpenoids, steroids, carbohydrates, and phenols in seeds, buds and flowers[9]. High level of proteins has been detected in seeds and flowers only[9]. Several flavonoids such as kaempferol, quercetin, kaempferol-3-O-rhamnopyranoside, quercitrin, isoquercitrin, kaempferol-3-Oglucuronide, quercetin-3-O-glucuronide, pro-cyanidines, kaempferol-3-O-*β*-D-(6"-n-butyl glucuronide), taxifolin, (+)-catechin, dehydrodicatechin were previously isolated from the aerial parts of *C. polygonoides*[3].

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On the light of the valuable nutritional and biological importance of *C. polygonoides*, our study reports new biological assessment of the aerial parts extracts and isolated flavonoids in vascular inflammation through the inhibition of NF-κB pathway translocation, and hence inhibition of inflammatory mediator's release. Vascular inflammation is the common cause of many vascular diseases as atherosclerosis, myocardial infarction, and congestive heart failure[10]. The inhibition of NF-κB pathway has been established to display valuable impact in the treatment of various cardiovascular diseases as hypertension[11], myocardial infraction[12], and arteriosclerosi[13]. These findings support the idea of the inhibition of NF-κB as a promising strategy in minimizing cardiovascular diseases.

Alzheimer's disease, a chronic neuro-inflammation in which the levels of pro-inflammatory mediators; cytokines, interleukins, interferons, chemokines, and tumor necrosis are elevated in the brains[14]. Hence, the possibility of inhibiting or delaying the onset of Alzheimer's disease can be accomplished through the inhibition of NF-κB activation[15].

However, the anti-inflammatory effects of some flavonoids such as quercetin, luteolin, kaempferol, fisetin, apigenin, isoliquiritigenin, chrysin, rutin, genistein, kaempferol, and silymarin through the modulation of NF-κB translocation were previously reported[16], our study reveals the activity of flavonoid glycosides; kaempferol-3-O-glucuronoide (**1**) and mequilianin (**4**) as new potent inhibitors of NF-κB activation pathway.

MATERIAL AND METHODS

Plant material collection and extraction

C. polligonoides is a wild shrub that grows in Al-Wadi Al-Ebrahimi, Assiut Governorate, Eastern desert of Egypt. The aerial parts of *C. polligonoides* were collected in April 2019, from its wild natural habitat. The plant is not considered as rare species, hence, no permission was mandatory from Assiut Governorate for collection the plant samples. The collected plant samples were then identified by Dr. Ahmed M. Fareed, Associate professor of plant taxonomy, Department of Botany, Faculty of Science, Assuit university, Assiut, Egypt. The dried sample of *C. polligonoides* was deposited at the Herbarium of Department of Botany, Faculty of Science, Assuit University with voucher number 1722015. The remaining fresh aerial parts of the plant were air-dried at room temperature, and then powdered and weighed to obtain 2.5 kg dried powders. The dried plant powder (2.5 kg) was extracted by maceration in 5 L methanol (70%) with gentle shaking using shaker (Selecta, Kiev, Ukraine), then filtered every 48 hr. The extraction process was repeated three times and the combined methanol extracts were concentrated by Rota vapor (Buchi RII, Essen, Germany). The dried methanol extract (250 g) was kept in refrigerator till isolation of bioactive compounds.

Isolation of compounds 1-4

The potent anti-inflammatory ethyl acetate fraction was conducted to the open glass column (75 mm × 600 mm) prepacked with 400 g silica gel (Silica gel 60, 0.015-0.040 mm). The mobile phase used in chromatography were consisted of solvent A (dichloromethane) and solvent B (methanol). The solvents were eluted in a gradient concentration (0% -100% methanol). The effluent was collected in a 200 ml flasks and monitored on TLC under UV lamp, and after spraying with sulphuric acid 10%. Four main sub-fractions (I, II, III, and IV) were obtained. Four flavonoids **1-4** were isolated from sub-fractions (I-IV) by using open columns packed with different adsorbents. Silica gel G60 (60-120 mesh, Merck, Darmstadt, Germany) was used as packing material in open column chromatography. Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan) was utilized as packing material for molecular sieving in column chromatography. Nuclear magnetic resonance (NMR) spectra including 1D- (^{1}H-NMR and ^{13}C-NMR) of the isolated compounds were recorded on Bruker Avance DRX spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C).

Characterization of isolated compounds 1-4

Kaempferol-3-O-glucuronide (**1**). Yellow amorphous powder. ^{1}H-NMR (400 MHz, CD_3OD): δ 8.10 (2 H, d, *J*= 8 Hz, H-2', H-6'); δ 6.90 (2 H, d, *J*= 8 Hz, H-3', H-5'); δ 6.39 (1 H, d, *J*= 2.4 Hz, H-8); δ 6.19 (1 H, d, *J*= 2.4 Hz, H-6); δ 5.31 (1 H, d, *J*= 8 Hz, H-1"); δ 3.61 (1 H, m, H-5"); δ 3.55 (1 H, m, H-4"); δ 3.51 (1 H,

m, H-2"); δ 3.47 (1 H, m, H-3"). ^{13}C-NMR (100 MHz, CD_3OD): δ 159.15 (CH, C-2); δ 135.92 (C, C-3); δ 179.74 (C, C-4); δ 163.23 (C, C-5); δ 100.31 (CH, C-6); δ 166.91 (C, C-7); δ 95.06 (CH, C-8); δ 158.80 (C, C-9); δ 105.78 (C, C-10); δ 122.84 (C, C-1'); δ 132.63 (CH, C-2'); δ 116.34 (CH, C-3'); δ 161.78 (C, C-4'); δ 116.34 (C, C-5'); δ 132.63 (CH, C-6'); δ 104.33 (CH, C-1"); 75.66 (CH, C-2"); δ 77.94 (CH, C-3"); δ 73.55 (CH, C-4"); δ 77.23 (CH, C-5"); δ 176.37 (C, C-6").

(*2R, 3R*)-Taxifolin (**2**). Yellow crystals, m.p. 242°C. ^{1}H-NMR (400 MHz, CD_3OD): δ 4.94 (d, 1H, *J*= 12.0 Hz, H-2); δ 4.50 (d, 1H, *J*= 12.0 Hz, H-3); δ 5.93 (brs, 1H, H-6); δ 5.90 (brs, 1H, H-8); δ 6.87 (d, 1H, *J*= 8.0 Hz, H-6'); δ 6.82 (d, 1H, *J*= 8.0 Hz, H-5'); δ 6.97 (brs, 1H, H-2'). ^{13}C-NMR (100 MHz, CD_3OD): δ 85.16 (CH, C-2); δ 73.88 (CH, C-3); δ 198.67 (C, C-4); δ 164.72 (C, C-5); δ 97.44 (CH, C-6); δ 168.96 (C, C-7); δ 96.48 (CH, C-8); δ 165.53 (C, C-9); δ 102.09 (C, C-10); δ 130.07 (C, C-1'); δ 116.08 (CH, C-2'); δ 116.28 (CH, C-5'); δ 147.35 (C, C-4'); δ 146.53 (C, C-3'); δ 121.10 (CH, C-6').

(+) Catechin (**3**). White needles, m.p. 175-176°C. ^{1}H-NMR (400 MHz, CD_3OD): δ 2.53 (1 H, dd, *J*= 16.1 & 8.0 Hz, H-4b); δ 2.85 (1 H, dd, *J*= 16.1 & 8.0 Hz, H-4a); δ 3.98 (1 H, m, H-3); δ 4.57 (1 H, d, *J*= 8.0 Hz, H-2); δ 5.93 (1 H, d, *J*= 2.4 Hz, H-6); δ 5.86 (1 H, d, *J*= 2.4 Hz, H-8); δ 6.72 (1 H, dd, *J*= 8.0 & 1.8 Hz, H-6'); δ 6.76 (1 H, d, *J*= 8.0 Hz, H-5'); δ 6.83 (1 H, d, *J*= 1.8 Hz, H-2'). ^{13}C-NMR (100 MHz, CD_3OD): δ 83.03 (CH, C-2); δ 68.89 (CH, C-3); δ 28.70 (CH, C-4); δ 157.77 (C, C-5); δ 96.50 (CH, C-6); δ 157.11 (C, C-7); δ 95.71 (CH, C-8); δ 158.02 (C, C-9); δ 101.03 (C, C-10); δ 132.41 (C, C-1'); δ 115.46 (CH, C-2'); δ 116.29 (CH, C-5'); δ 146.44 (C, C-4'); δ 146.42 (C, C-3'); δ 120.25 (CH, C-6').

Mequilianin (quercetin-3-O-glucuronide) (**4**). Yellow amorphous powder. ^{1}H-NMR (400 MHz, CD_3OD): δ 7.99 (1 H, brs, H-2'); δ 7.51 (1 H, d, *J*= 8.0 Hz, H-6'); δ 6.88 (1 H, d, *J*= 8.0 Hz, H-5'); δ 6.40 (1 H, brs, H-8); 6.21 (1 H, brs, H-6); δ 5.36 (1 H, d, *J*= 8.0 Hz, H-1"); δ 3.61 (1 H, m, H-5"); δ 3.55 (1 H, m, H-4"); δ 3.51 (1 H, m, H-2"); δ 3.47 (1 H, m, H-3"). ^{13}C-NMR (100 MHz, CD_3OD): δ 159.16 (CH, C-2); δ 135.72 (C, C-3); δ 179.36 (C, C-4); δ 163.03 (C, C-5); δ 99.99 (CH, C-6); δ 166.27 (C, C-7); δ 94.68 (CH, C-8); δ 158.45 (C, C-9); δ 105.62 (C, C-10); δ 122.67 (C, C-1'); δ 116.11 (CH, C-2'); δ 117.99 (CH, C-5'); δ 149.05 (C, C-4'); δ 145.81 (C, C-3'); δ 122.67 (CH, C-6'); δ 104.32 (CH, C-1"); δ 75.58 (CH, C-2"); δ 77.62 (CH, C-3"); δ 73.39 (CH, C-4"); δ 78.10 (CH, C-5"); δ 176.35 (C, C-6").

Anti-inflammatory activity assay

Chemicals

The materials used for anti-inflammatory assay were HEK293 cells (Human embryonic kidney cells) (Thermo Fisher Scientific, Waltham, USA), Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, USA), Opti-MEM medium free from antibiotic (Thermo Fisher Scientific, Waltham, USA), Tumor Necrosis Factor-α human (TNFα) (Sigma Aldrich, Louis, MO, USA), Thaw medium for assay (Sigma Aldrich, Louis, MO, USA) consisted of MEM medium (Hyclone, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FBS, 1% non-essential amino acids (Hyclone, Thermo Fisher Scientific, Waltham, USA), 1 mM Na pyruvate (Hyclone, Thermo Fisher Scientific, Waltham, USA), and 1% Penicillin/Streptomycin (Hyclone, VWR, Radnor, PA, USA), Growth medium consisted of Thaw medium, and 50 µg/ml of hygromycin B (Hyclone, VWR, Radnor, PA, USA). Also, 500 µl (60 ng DNA/ µl) of reporter (Component A) consisted of NF-κB luciferase reporter vector (BPS Biosciences, San Diego, CA, USA) and constitutively expressing *Renilla* luciferase vector (BPS Biosciences, San Diego, CA, USA), and 500 µl (60 ng DNA/ µl) of negative control reporter (Component B) consisted of non-inducible luciferase vector (BPS Biosciences, San Diego, CA, USA), and constitutively expressing *Renilla* luciferase vector (BPS Biosciences, San Diego, CA, USA) were used. Luciferase assay reagent (Azure biosystem, Dublin, CA, USA) was also used in the protocol.

Determination of the dose response of HEK293 cells transfected with NF-κB reporter to TNFα

The procedure in generalized transfection and assay protocols were previously described[17]. Briefly, the HEK293 cells were suspended in a 96 well plate containing 100 µl growth medium in each well at a density of 30,000 cells per well, and incubated for 24 hrs. The DNA mixture (Reporter component A)

was diluted by Opti-MEM medium (antibiotic-free) (1:15) with gentle shaking. Also, the dilution of 0.35 µl lipofectamine 2000 with 15 µl of Opti-MEM medium (antibiotic-free) was performed with the incubation for 5 min. at room temperature. After that, the diluted DNA mixture was gently mixed with the diluted lipofectamine 2000, and the whole mixture was incubated for 25 min. at room temperature. The DNA-Lipofectamine 2000 mixture (30 µl) was added to each well. The 96 well plate was incubated at 37°C in a CO_2 incubator for 24 hrs, to carry out the dual luciferase assay. Both firefly luminescence and *Renilla* luminescence were measured by using a luminometer.

RESULTS AND DISCUSSION

Structure elucidation of compounds 1-4 isolated from ethyl acetate fraction of *C. polygonoides*

The structural elucidation of the isolated compounds (**1-4**) (Fig. 1) was based on spectroscopic analyses (^{1}H & ^{13}C-NMR) compared with the data published in the literatures. The compounds were identified as kaempferol-3-O- glucuronide (**1**)[18], (*2R,3R*)-taxifolin (**2**)[19&20], (+) catechin (**3**)[21&22] and mequilianin (**4**)[18]. Compounds (**1-3**) were previously reported to be isolated from the leaves of the plant under investigation[3].

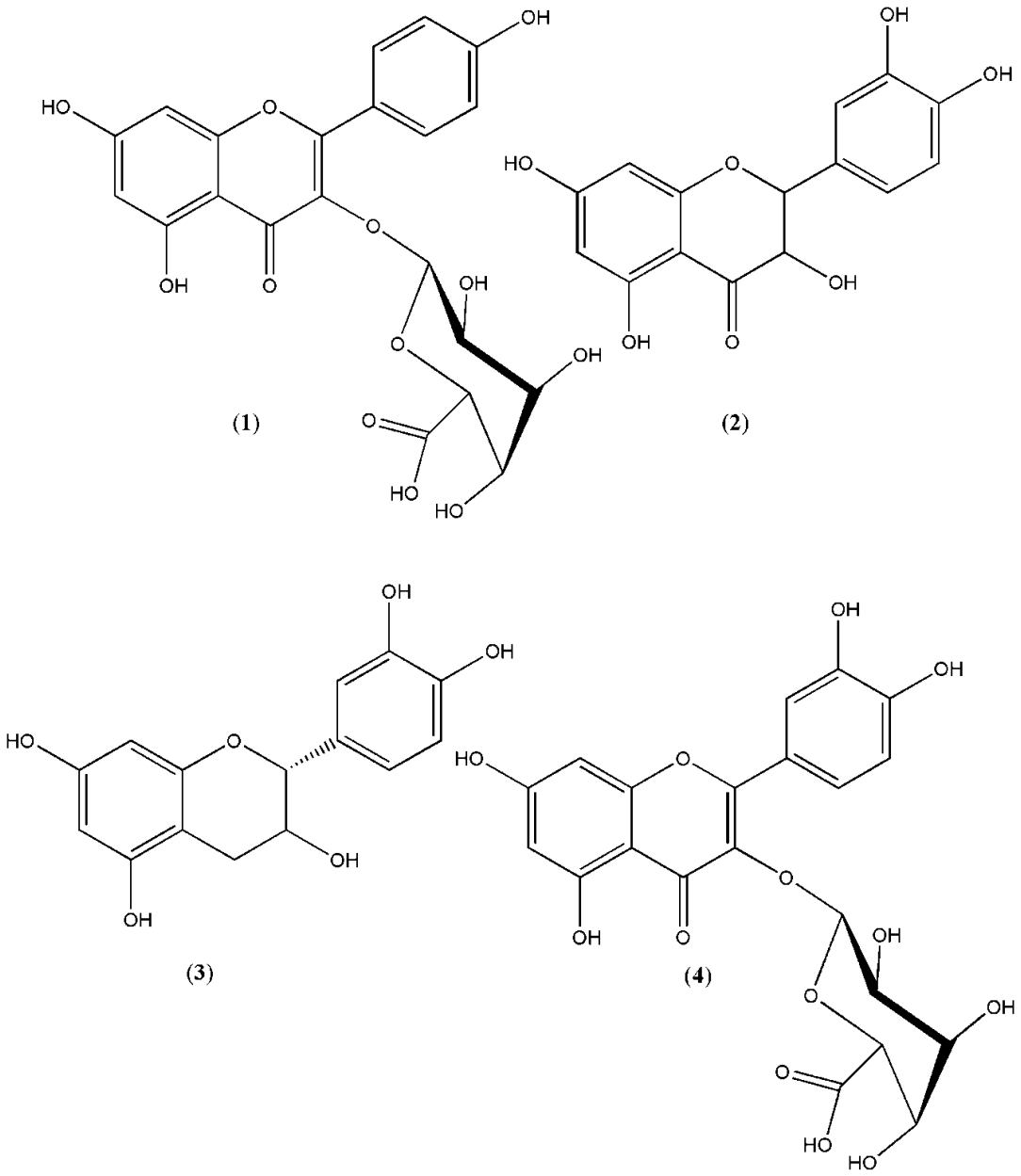

Fig. 1: List of isolated compounds (**1-4**).

Anti-inflammatory activity of the isolated compounds through the inhibition of NF-κB pathway

Previous reported biological studies of our plant under investigation revealed the anti-inflammatory activity of methanolic extract of the leaves and its ability to exert reduction in the oxidative stress induced by haloperidol[5]. This scientific basic corresponding was augmented in our study through the assessment of the anti-inflammatory activity of the plant methanolic and ethyl acetate extract, together with the isolated compounds (**1-4**) through the inhibition of the NF-κB pathway induced by α-TNF. Both the methanolic and ethyl acetate extracts, together with the isolated flavonoids (**1-4**) exhibited potent anti-inflammatory activity through the inhibition of NF-κB translocation pathway (Table 1 and Fig. 2). *Calligonum polygonoides* L. subsp. *comosum* aerial part methanol extract showed significant anti-inflammatory activity against NF-κB translocation in comparison to bortezomib which was used as a positive control. The phytochemical study of the bioactive ethyl acetate fraction led to isolation of four NF-κB inhibitors. Kaempferol-3-O- glucuronide (**1**) and mequilianin (**4**) are first reported as NF-κB inhibitors. Taxifolin (**2**), and (+) catechin (**3**) have been reported to display anti-inflammatory activity through inhibition of NF-κB mechanism[16,23-26].

Table 1: The inhibitory effect of the isolated compounds (**1-4**) on NF-κB release.

Compound	NF-κB / IC_{50} (µg/mL)
Ethyl Acetate fr.	18.37 ± 0.40
Methanol fr.	10.14 ± 0.22
Kaempferol-3-O- glucuronide (**1**)	2.19 ± 0.04
Taxifolin (**2**)	6.41 ± 0.14
Catechin (**3**)	3.08 ± 0.06
Mequilianin (**4**)	3.91 ± 0.08
Bortezomib*	2.79 ± 0.06

*Reference compound.
Values are displayed as means ± S.D., *n*= 3.
1Y: Kaempferol-3-*O*-*β*-D-glucuronide (**1**), 1R: Taxifolin (**2**), 2R: (+) Catechin (**3**), 2Y: Mequilianin (**4**), ET: Ethyl acetate fraction, ME: Methanol extract, Bortezomib reference compound.

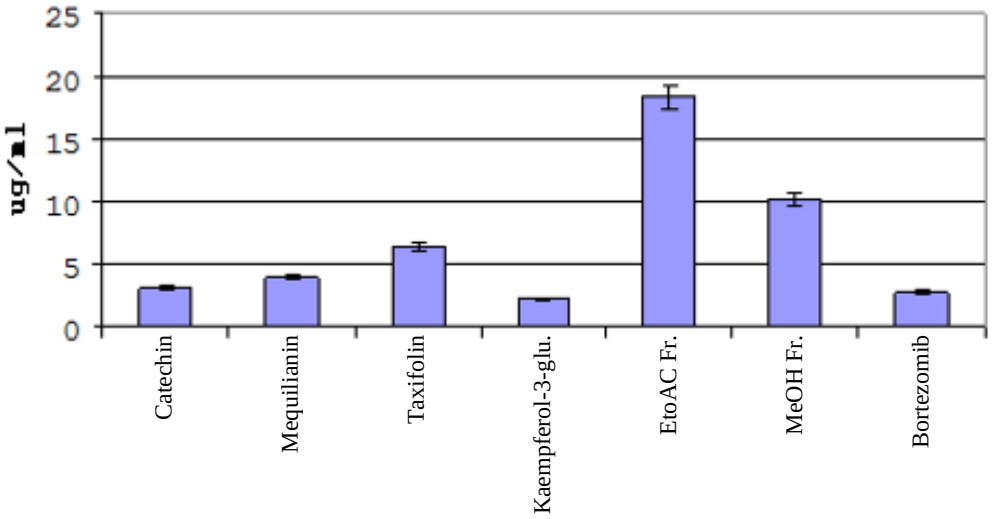

Fig. 2: The inhibitory effect of the isolated compounds (**1-4**) on NF-κB release.

Conclusion

The aerial parts methanol extract and ethyl acetate fraction of *C. polygonoides* showed for the first time inhibition of NF-κB translocation. From the ethyl acetate fraction, four potent anti-inflammatory flavonoids (**1-4**) were isolated. The isolated flavonoids were identified by NMR spectral techniques and confirmed by comparing with previously reported data. Flavonoid glycoside, kaempferol-3-*O*-*β*-D-glucuronide (**1**) exhibited the most potent activity similar to bortezomib (positive control) with IC_{50}= 2.78±0.06 µg/mL. Based on our findings, *C. polygonoides* extract is recommended as a valuable food supplement for treatment of vascular inflammation conditions; cardiovascular diseases, and Alzheimer.

List of abbreviations

NF-κB	Nuclear Factor-Kappa
HEK293 cells	Human embryonic kidney cells
IC_{50}	The half maximal inhibitory concentration
L	Litre
Kg	Kilogram
µM	Micro-molar
µg	Microgram
ml	Milliliter
µl	Microliter

Declarations

The authors have no conflict of interest. The author(s) declared that no grants were involved in supporting this work.

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تقيم المستخلصات والفلافونوديات المعزولة من نبات كاليجونم بلوجنويدي كمضادات للألتهابات

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كاليجونم بلوجنويدي هو نبات يتبع فصيلة الأرطاه التابعة للعائلة البطاطية ، وهي شجيرة برية تنمو على الصحاري الرملية في مناطق مختلفة في شمال إفريقيا وغرب آسيا وجنوب أوروبا. تُستخدم براعم الزهور وفروع النبات الصغيرة في بعض البلدان الآسيوية لإعداد أطباق الطعام. كما تستخدم أعضاء النبات المختلفة ايضا في علاج أمراض المعدة. في هذه الدراسة تم عزل مركبات ذات فاعلية كبيرة كمضادات للالتهابات من مستخلص خلالت الإيثيل باستخدام كروماتوجرافيا العموديه واطوار ثابته مختلفه مثل هلام السيليكا والسيفادكس. تم التعرف على المركبات المعزولة باستخدام تقنيات الطيف بالرنين المغناطيسي النووي والتي وجد أن جميعها تنتمي لفئات مختلفة من الفلافونويدات. وأحتوت هذه الدراسة ايضا على تقيم مستخلص الميثانول الكحولي ومستخلص خلالت الإيثيل والمركبات المعزولة من حيث قدرتها كمضادات للالتهابات عن طريق تثبيط مسار NF-κB. وأثبتت الدراسة فعالية كلاً من المستخلصات والمركبات المعزولة كمضادات للالتهابات مما يرجح ادراج هذا النبات تحت المغذيات الواعدة لعلاج اضطربات الاوعية الدموية.