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Isolation of beta-sitosterol and evaluation of antioxidant Activity of Iraqi *Campsis grandiflora* flowers

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Abstract

Campsis grandiflora (Bignoniaceae) is a fast growing deciduous climber, the dried flowers have been used as a carminative, blood tonic, and febrifuge in Chinese traditional medicine. This plant has an anti-inflammatory, anti-oxidant, anti-depressant, and anti-bacterial effect; with a beneficial role in stagnant blood and endometriosis conditions. In this study, the detection of beta-sitosterol in the hexane extract of Iraqi *C.grandiflora* flowers was performed using thin layer chromatography (TLC) and high performance liquid chromatography(HPLC); while the isolation done by preparative layer chromatography then structure elucidation of isolated compound was done by FTIR and ^{1}HNMR. Furthermore, assessment of the anti-oxidant activity of the ethyl acetate extract of Iraqi *C.grandiflora* flowers using three different methods and total flavonoid content, then measuring the pearson's correlation coefficient between these methods. The results showed that the hexane extract of Iraqi *C.grandiflora* flowers contain beta-sitosterol compound and the ethyl acetate extract of this plant possesses an excellent anti-oxidant effect using the single-electron transfer (SET) pathway in scavenging the free radicals, and this activity attributed to the potent antioxidant i.e. polyphenols.

Keywords: Anti-oxidant activity, Betasitosterol, *Campsis grandiflora*, Total flavonoids content.

عزل مركب البيتا سيتوستيرول وتقييم الفعالية المضادة للأكسدة لأزهار نبات البوق الزاحف العراقي

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الخلاصة

نبات البوق الزاحف (عائلة البنونية) هو متسلق نفضي سريع النمو ، تم استخدام الزهور المجففة لهذا النبات كمادة طارد للريح ، منشط للدم ، ومضاد للحمى في الطب التقليدي الصيني. هذا النبات له تأثير مضاد للالتهابات , مضاد للأكسدة , مضاد للاكتئاب ومضاد للبكتيريا مع دور مفيد في حالات ركود الدم والانتباذ البطاني الرحمي. في هذه الدراسة تم الكشف عن مركب البيتا سيتوستيرول في مستخلص الهكسان لأزهار نبات البوق الزاحف العراقي باستخدام كروماتوجرافيا الطبقة الرقيقة (TLC) والكروماتوجرافيا السائلة عالية الأداء(HPLC) بينما تم العزل بواسطة كروماتوجرافيا الطبقة التحضيرية (PLC) ثم تم توضيح الستركجر الكيميائي للمركب المعزول بواسطة(FTIR و HNMR) علاوة على ذلك ، تم تقييم النشاط المضاد للأكسدة لمستخلص أسيتات الإيثيل لأزهار نبات البوق الزاحف العراقي بأستخدام ثلاث طرق مختلفة وقياس محتوى الفلافونويد الكلي, ثم حساب معامل الارتباط (بيرسون) بين هذه الطرق. أظهرت النتائج أن مستخلص الهكسان من أزهار نبات البوق الزاحف العراقي يحتوي على مركب البيتا سيتوستيرول وأن مستخلص أسيتات الإيثيل لهذا النبات يمتلك تأثيرًا ممتازًا مضادًا للأكسدة باستخدام مسار نقل الإلكترون الفردي(SET) في كسح الجذور الحرة ، و يُعزى هذا النشاط إلى مضادات الأكسدة القوية مثل البوليفينول.

الكلمات المفتاحية: الفعالية المضادة للاكسدة ، بيتاسيتوستيرول ، نبات البوق الزاحف ، محتوى الفلافونويد الكلي .

Introduction

Campsis grandiflora (Bignoniaceae) is a fast growing deciduous climber, native to central and southern China; its flowers are hermaphrodite, curled, and bright orange-red in color. This plant blooms for about eight months, between the last week of March and the second week of October[(1)].Flowering of *C.grandiflora* plant belongs to the cornucopia pattern, in which a large number of plant flowers are produced in each inflorescence for about several months[(2)]. The dried flowers of this plant, also known as "aborticide" in chinese folk, have been used as a carminative, blood tonic, febrifuge, and depurative diuretic in Chinese traditional medicine. Rheumatoid pains and menstrual problems exacerbated by blood stagnation, swelling breast after child birth, rubella, bleeding rectum, and diabetes have been treated with a decoction of this flowers[(3,4)]. *C.grandiflora* flowers have several pharmacologic activities e.g. cellular protection and anti -oxidant effect[(3)], anti-

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-depressant effect[3], with a beneficial role in stagnant blood, and endometriosis conditions[6].The objective of this study was detection and isolation of the beta-sitosterol present in the Iraqi *C.grandiflora* flowers with the evaluation of the anti-oxidant effect and total flavonoid content of the ethyl acetate extract of Iraqi *C.grandiflora* flowers and measuring the correlation between these methods.

Materials and Methods

Plant material collection

Flowers of *Campsis grandiflora* were collected from Baghdad – hay Aljamea`a in July 2020. The plant material was authenticated at the University of Baghdad / College of sciences by a professional taxonomist. Flowers were separated from the rest of the plant and washed thoroughly. It was then dried under shade conditions at room temperature for seven days. After that, the dried flowers were ground in a mechanical grinder and finally stored in a glass container at 4◦C.

Chemicals and reagents

Beta-sitosterol and quercetin standards were purchased from Changdu Biopurify; hexane, ethanol, acetone, DPPH, TPTZ, ABTS, sodium nitrite were obtained from Sigma Aldirch. Ethyl acetate, methanol, FeCl3 hexahydrate, Potassium persulfate, chloroform and toluene were supplied from Alpha Chemika, Ascorbic acid was purchased from Merk, Sulphuric acid and aluminum chloride were obtained from BDH.

Preparation of extract for the detection and isolation of compound 1

Powdered plant (100g) was extracted by soxhlet apparatus with *n*-hexane (1200ml) till exhaustion. The extract was dried using a rotary evaporator, weighted and labelled as hexane extract (HE)[7].

TLC analysis

An aliquot of hexane extract dissolved in about 2ml of hexane then applied to analytical TLC plate against standard beta-sitosterol, and developed in the following mobile phases(MP) [8–11]:

- MP1: aceton:hexane (1:3)
- MP2: hexane :ethyl acetate (7:2)
- MP3: toluene: chloroform :ethyl acetate (5:4:1)
- MP4: chloroform :acetone (9:1)

The plates were sprayed with anisaldehyde – sulphuric acid reagent followed by heating for the detection of distinct spots.

HPLC analysis

One milligram from hexane extract and standard beta-sitosterol was dissolved separately in HPLC grade methanol (1ml) using a mobile phase consisting of acetonitrile:methanol(70: 30, v/v). The flow rate was 1 ml/min, and the detector was monitored at 210 nm[12].

Isolation of compound 1

Hexane extract (1.5 gram) was dissolved in hexane and conducted on Preparative Layer Chromatography plates (PLC) against standard beta-sitosterol[13] and developed in MP2 mobile phase[10]. The detection was done by spraying the plates' side by anisaldehyde-sulphuric acid reagent followed by heating. The bands at R_f = 0.32 were scrapped off and the scrapped silica then eluted with warmed hexane, the purity of the C1 compound was confirmed by analytical TLC using the MP4 mobile phase.

Spectral analysis of the isolated compound 1

The isolated compound was subjected to different spectral analysis e.g. FTIR and ^{1}HNMR.

Preparation of extract for the anti-oxidant assay

Twenty-five gram of powdered plant material was defatted with 250 ml *n*-hexane by soxhlet apparatus. The marc was further extracted with ethanol 80% (250 ml) till exhaustion also by using the soxhlet apparatus. Firstly, the ethanolic extract was dried by rotary evaporator and weighted. Secondly, the dried extract was dissolved in 20 ml distilled water and partitioned with ethyl acetate (3×50 ml). The upper layer was collected, dried by rotary evaporator, weighted, labelled as ethyl acetate extract (EAE) and stored at 4◦C while the lower aqueous layer was discarded[14].

The DPPH [1,1–Diphenyl – 2 picryl–hydrazyl] assay

The DPPH assay was performed using a colourimetric method described by Anna Floegel and coworkers[15] in which a methanolic dilution of DPPH was used. (2.95) ml of DPPH solution (1 Mm DPPH in 80% methanol) was mixed with (0.05) ml of EAE or ascorbic acid (concentrations ranging from 12.5 to 200 mcg per 1 ml methanol). The assay was performed in triplicate, and the developing mixture was incubated in the dark for 30 minutes with aluminium foil over it. At 517 nm, a decrease in the absorbance was observed. The control was prepared by replacing the amount of EAE or standard by 80% methanol. The findings were reported as a percentage of radical scavenging and estimated using the formula below[16]:

$$\%\ of\ inhibition = \frac{(A_0 - A_1)}{A_0} \times 100$$

A_0 is the absorbance of the control, A_1 is the absorbance of plant extract (EAE) or standard ascorbic acid.

The ABTS [2,2'–azino – bis (3–ethylbenzothiazoline–6–sulphonic acid) diammonium salt] assay

A colourimetric method[17] has been used to measure the antioxidant activity of EAE against $ABTS^{\bullet+}$ radical in which aqueous solutions (1:1) of ABTS (7 mM) and potassium persulphate (140 mM) were mixed. Then, this mixture was incubated in a

dark place for 12 hours at room temperature enabling the radical $ABTS^{\bullet+}$ to formed. After that, (1.96) ml from the previous solution was mixed with (0.04) ml of EAE or ascorbic acid (concentrations ranging from 12.5 to 200 mcg per 1 ml ethanol) and let to sit at room temperature. The assay was performed in triplicate, and the decrease in the absorbance was measured at 734 nm. The control was prepared by replacing the amount of EAE or standard by ethanol, and the findings were reported as a percentage of radical scavenging using the previously mentioned formula.

The FRAP (ferric reducing antioxidant power) assay

The FRAP assay was carried out using a colourimetric method[18] in which 3.8 ml of FRAP reagent was mixed with 0.2 ml of EAE or ascorbic acid (concentrations ranging from 12.5 to 200 mcg per 1 ml methanol). The FRAP reagent (10:1:1) was made by incorporating 10 parts of sodium acetate buffer solution (300 mM, pH=3.6) with 1 part from each of ferric chloride hexahydrate (20 mM) and TPTZ (10 mM). The assay was performed in triplicate, and the mixture was kept at 37°C for 30 minutes. The absorbance was determined at 593 nm, and the control was made by replacing the diluted sample with the same volume of methanol. The findings were expressed in microgram of ascorbic acid equivalents (AAEs) per milligram of dried EAE[19] and calculated using the formula from the ascorbic acid standard curve:

$y = 0.0025x + 0.1802$

y is the sample's absorbance at 593nm, x is the concentration of ascorbic acid equivalent measured in mcg/ml.

The Total Flavonoids Content (TFC)

The total flavonoids content of Iraqi *C.grandiflora* was estimated by using the aluminium chloride colourimetric method[20] in which 1ml of plant EAE extract (100 mcg/ml) or standard quercetin solution (5,10,20,40,60,80 and 100 mcg/ml) was mixed with 4ml of distilled water and then adding 0.3 ml of sodium nitrite solution (5%) to all test tubes. After five minutes, 0.3 ml of aluminium chloride solution (10%) was applied, followed subsequently by 2 ml of sodium hydroxide solution (1%). The assay was performed in triplicate, and the mixture was mixed well, then, the absorbance was measured at 510 nm against blank. Total flavonoid content (TFC) has been determined using an equation derived from the quercetin standard curve[20]

$y = 0.0009x + 0.0283$

y is the sample's absorbance at 510 nm, x is the concentration of the flavonoids in the sample measured in mcg/ml.

Statistical analysis

The pearson's correlation coefficient (r) was computed using the SPSS-16.0 (Statistical Packages for Social Sciences- version 16), the correlation is significant at ($p \leq 0.05$).

Results and Discussion

TLC results

Phytosterol, such as beta-sitosterol, was reported in *C.grandiflora* flower extract[21]. The results in (Table1) showed a matching in the R_f values of both beta-sitosterol and compound 1(C1) spots in four mobile phases, and this finding suggested the presence of beta-sitosterol in hexane extract of Iraqi *C.grandiflora* flower, as shown Figure.1.

Table 1. The R_f values of compound 1(C1) as compared with the R_f values of standard beta-sitosterol.

Mobile phase	The R_f value of C1	The R_f value of standard beta-sitosterol
MP1	0.51	0.50
MP2	0.32	0.33
MP3	0.42	0.43
MP4	0.69	0.67

and beta-sitosterol using MP1 solvent system:(A) C1 spot, (B) standard beta-sitosterol spot.

HPLC results

The peak at (6.973) minutes in the HPLC analysis of hexane extract of Iraqi *C.grandiflora* flowers was matched the peak of standard beta-sitosterol with a retention time (6.913) minutes, as shown in Figure 2.

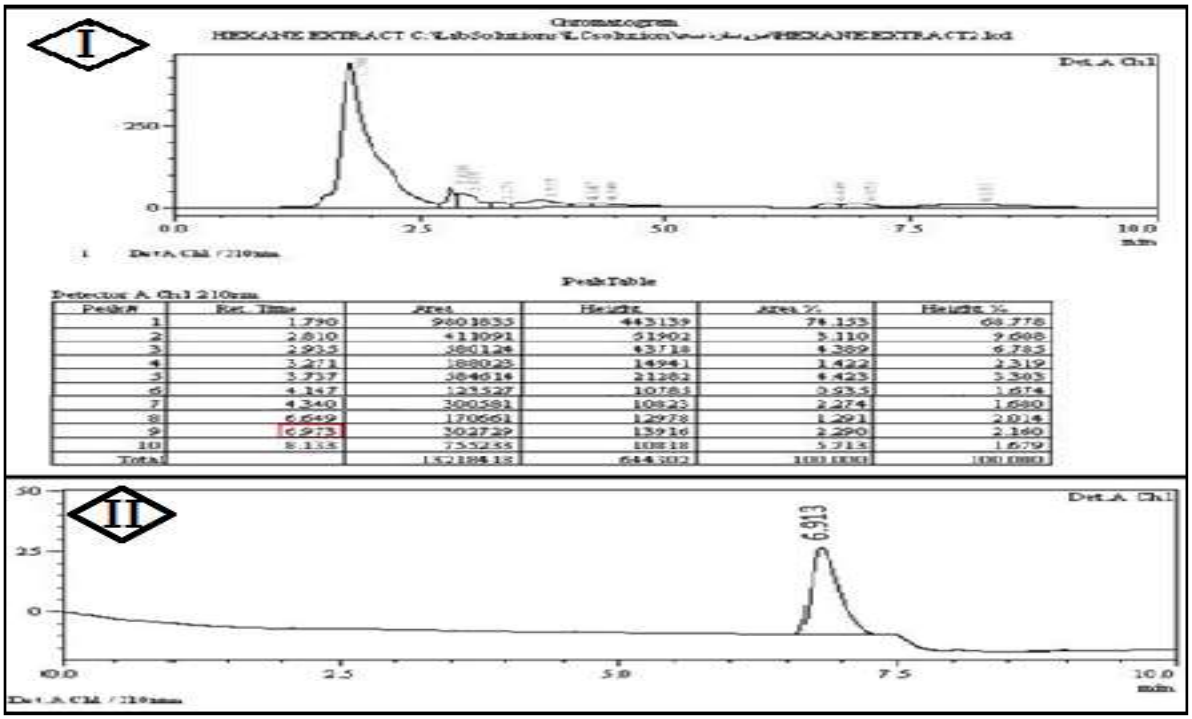


Figure2.The HPLC analysis: (I) hexane extract of Iraqi *C.grandiflora* flowers, (II) standard beta-sitosterol.

Isolation of compound1

Hexane extract of *C.grandiflora* flowers was applied to PLC plates to give 52 mg (3.46 % w/w) of C1, and then the purity was confirmed using analytical TLC plate, as shown in Figure 3.

***Fourier Transform Infrared Spectrometry (FTIR)* of compound1**

The wavenumbers of the isolated compound (C1) showed a characteristic broad peak at 3242-3439 cm^{-1} that confirmed a hydroxyl group with peaks at 3007 and 1641 cm^{-1} that established a double bond. These results were confirmed by comparing the wavenumbers with the reported literature[22],as in Figure 4

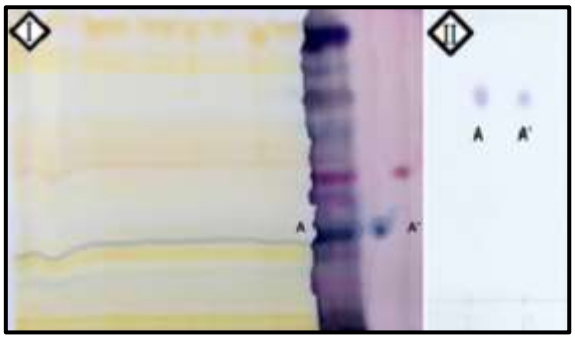


Figure 3. Isolation and purity confirmation of C1 from hexane extract: (I) PLC plate for isolation using MP2, (II) TLC plate for purity confirmation using MP4, (A)C1 spot, (A') standard beta-sitosterol spot.

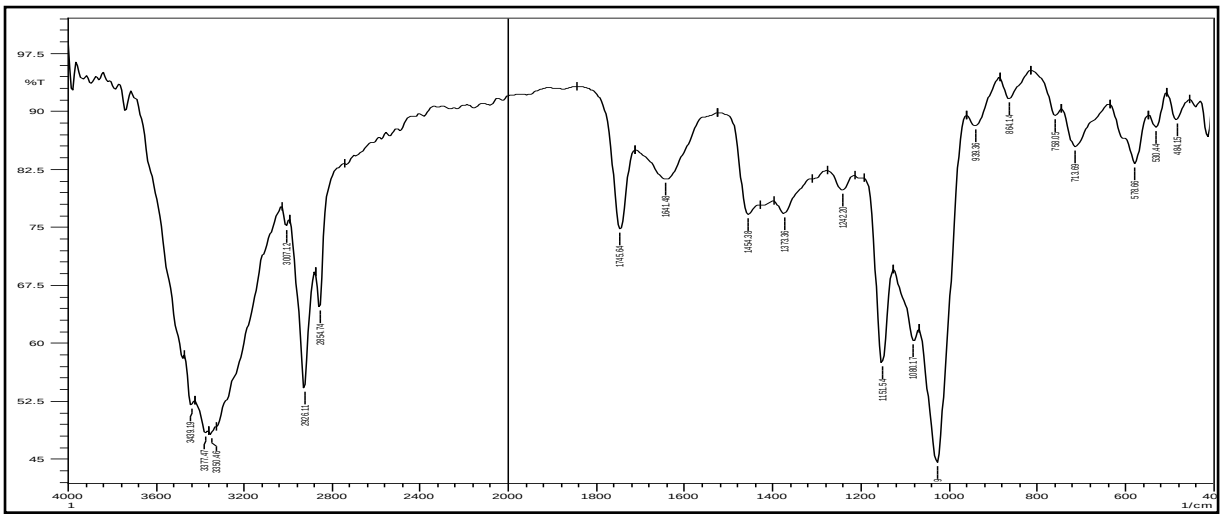


Figure 4. The IR spectrum of the isolated compound

Nuclear Magnatic Resonance (NMR) of compound1

The isolated compound 1 (C1) exhibited four distinct chemical shifts in the ^{1}HNMR spectrum (Figure5), which reflected the active functional groups. The chemical shifts began at 0.65 parts per million (ppm), which correspond to the hydrogens of saturated hydrocarbons, followed by the allylic protons, which have a chemical shift of 2.1 ppm. At 4.62 ppm, a proton next to a carbon adjacent to heteroatom can be observed. Finally, at 5.32 ppm, the vinylic proton can be seen.

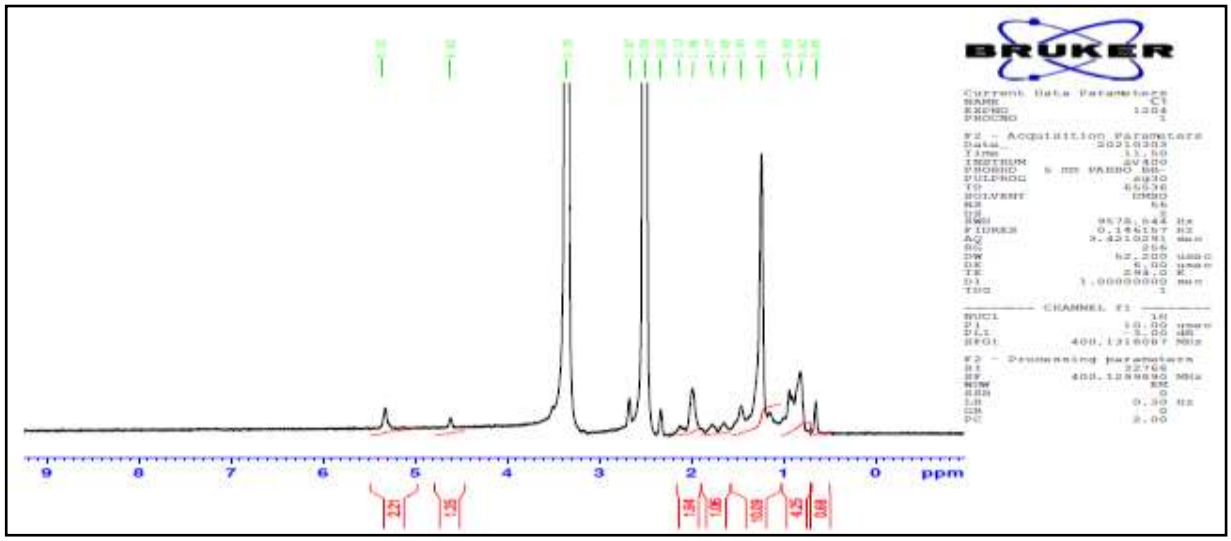


Figure 5. The ^{1}HNMR spectrum of C1

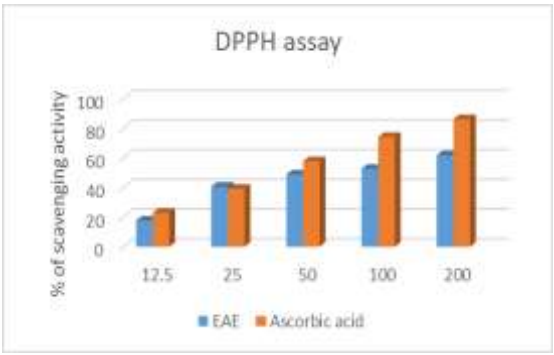
The results of the different chromatographic techniques and various spectral analysis proved that the compound 1 was beta-sitosterol.

The DPPH assay results

The DPPH radical is a popular substrate for quickly determining the antioxidant activity of biological samples because of the simplicity of the assay and the stability of radical. As the DPPH radical is quenched by the antioxidant using the single electron transfer (SET) and hydrogen atom transfer (HAT) reaction mechanisms, the color of the DPPH solution changes from purple to yellow and the absorbance was read at 517 nm[23]. The results of DPPH assay were illustrated in Table (2) and more visually expressed in Figure. 6.

Table 2.The DPPH-radical scavenging activity of *C.grandiflora* EAE

DPPH assay		
Conc. of vit C or extract (mcg/ml)	**% scavenging activity of vit C**	**% scavenging activity of EAE extract**
12.5	22.90	17.63
25	39.00	40.43
50	57.60	48.68
100	74.06	52.50
200	86.03	71.69



DPPH :1,1-Diphenyl-2-picryl-hydrazyl, EAE: ethyl acetate extract of Iraqi *C.grandiflora*.

Figure 6.The antioxidant activity of EAE measured by DPPH method and compared with ascorbic acid standard: (EAE) ethyl acetate extract of Iraqi *C.grandiflora*,(DPPHassay)1,1-Diphenyl-2-picryl-hydrazyl assay.

The ABTS assay results

The ABTS can react with potassium persulfate in an oxidation reaction in which the colored-ABTS radical is formed. The degree of decolorization indicates that the antioxidants had the ability to transfer electrons or hydrogen atoms to inactivate this radical[24]. The results of ABTS assay were illustrated in Table (3) and more visually expressed in Figure 7.

Table 3.The ABTS-radical scavenging activity of *C.grandiflora* EAE.

ABTS assay		
Conc. of vit C or extract (mcg/ml)	**% scavenging activity of vit C**	**% scavenging activity of EAE extract**
12.5	23.43	15.97
25	37.93	15.35
50	52.43	28.89
100	64.7	37.92
200	71.96	45.10

ABTS: [2,2'–azino – bis (3– ethylbenzothiazoline – 6– sulphonic acid) diammonium salt], EAE: ethyl acetate extract of Iraqi *C.grandiflora*.

The FRAP assay results

In FRAP assay, the antioxidant activity was assessed by measuring the capacity of the antioxidants in the EAE to convert ferric (Fe^{+3}) to ferrous (Fe^{+2}) in a redox-linked colorimetric assay using single electron transfer (SET) as a reaction mechanism[25]. The results were expressed as ascorbic acid equivalents (AAEs), measured in microgram ascorbic acid per milligram dried EAE, depending on the equation from ascorbic acid standard curve (table 4).

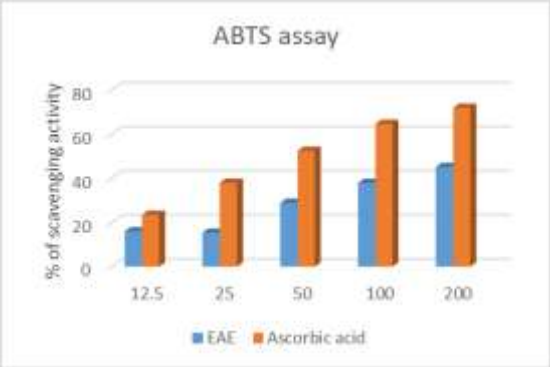


Figure 7. The antioxidant activity of EAE measured by ABTS method and compared with ascorbic acid standard: (EAE) ethyl acetate extract of Iraqi *C.grandiflora*, (ABTS assay) 2,2'azino–bis(3–ethylbenzothiazoline–6–sulphonic acid) diammonium salt.

Table 4. The antioxidant activity measured by the FRAP method.

EAE concentration (mcg/ml)	EAE absorbance (nm)	Mcg Ascorbic acid equivalent (mcg/ml)	AAEs (mcg/mg dried extract)
12.5	0.190±0.005	3.92±2.309	313.6±184.7
25	0.216±0.017	14.58±7.055	583.46±282.2
50	0.265±0.011	34.18±4.42	683.73±88.4
100	0.362±0.025	72.72±10.283	727.2±102.8
200	0.590±0.005	163.92±2.309	819.6±11.5

Each value represents the mean±SEM of 3 samples; FRAP: ferric reducing antioxidant power, EAE:ethyl acetate extract of Iraqi *C.grandiflora*, AAEs: ascorbic acid equivalents.

The total flavonoids content result

The main antioxidant compounds in plants are phenols, which have an aromatic ring that enables the unpaired electrons in their arrangement to be stabilized and relocated, allowing electrons and hydrogen atoms to be donated from their hydroxyl groups[18]. Since phenolic compounds are one of the main classes of compounds known to serve as primary antioxidants, and the litreture have shown a good correlation between the total flavonoids content (TFC) and the antioxidant activity[23], it's essential to calculate the amount of these compounds that present in the EAE. The results of the total flavonoids content illustrated in table (5).

Table 5. The total flavonoid content of EAE of Iraqi *C.grandiflora*.

EAE concentration (mcg/ml)	EAE absorbance (nm)	Mcg Quercetin equivalent (mcg/ml)	Mcg Quercetin equivalent /mg EAE
100	0.0903±0.0008	68.925±0.979	689.25±9.799

Each value represents the mean±SEM of 3 samples; EAE: ethyl acetate extract of Iraqi *C.grandiflora*.

The pearson's correlation coefficient results

The data of pearson's correlation coefficient between the three different antioxidant methods and between these methods and TFC were summerized in Table (6).

Table 6.The pearson's correlation coefficient.

Method	DPPH assay	ABTS assay	FRAP assay	TFC
DPPH assay	—	0.886*	0.976*	-0.848
ABTS assay	0.886*	—	0.848	-0.992
FRAP assay	0.976*	0.848	—	-0.923
TFC	-0.848	-0.992	-0.923	—

DPPH assay :1,1-Diphenyl-2-picryl-hydrazyl assay ; ABTS assay : 2,2'–azino – bis (3– ethylbenzothiazoline – 6– sulphonic acid) diammonium salt assay ; FRAP assay : ferric reducing antioxidant power assay ; TFC : total flavonoid content ; * : means correlation is significant at ($p \leq 0.05$).

The results demonstrated that Iraqi *C.grandiflora* ethyl acetate extract possesses strong antioxidant capacity measured with DPPH, ABTS, and FRAP assays compared with ascorbic acid. These findings were consistent with another study in which *C.grandiflora* ethyl acetate extract exhibit the highest radical scavenging potency against DPPH and ABTS radicals with IC50=1.625mcg/ml; this result was excellent when compared to IC50 of ascorbic acid (8.6 mcg/ml), also the IC50 of other fractions such as ethanol, petroleum, butanol, and water were (8.75, 32.25, 10, 7.6 mcg/ml), respectively[3].

There was a positive correlation between the DPPH, ABTS, and FRAP assays, and this may be explained by the fact that these assays were suitable and accurate for determining the plant extracts' overall antioxidant capacities; these results were agreed with another research[26] in which a high correlation between these methods have been showed. A significantly high correlation coefficient was obtained from the DPPH and FRAPS assays (0.976) with a p-value ≤0.05. These findings suggested that the antioxidants in EAE of Iraqi *C.grandiflora* were used the single-electron transfer (SET) pathway in scavenging the free radicals; since the DPPH and FRAP assays were based on the SET pathway as a reaction mechanism.

There was a negative correlation between the antioxidant assays and the TFC, and these findings pointed that, although there are a relatively high amount of flavonoids in the extract but there are various factors affecting the anti-oxidant activity of a substance and results in negative correlation, these include substance solubility, pH of the medium, oxidation state, etc[23]. These results suggested that flavonoids not only the major contributor to the antioxidant effect of this plant , but there are other phytochemicals i.e. the non-flavonoidal compounds such as acetoside, caffeic acid, gallic acid, etc. that reported in *C.grandiflora* flower extract may contribute to this excellent activity since these compounds also exhibited a pronounced antioxidant effects[27,28].

Conclusion

Beta-sitosterol was isolated form the hexane extract of Iraqi *C.grandiflora* flowers, and this compound was confirmed with different chromatographic and spectroscopic techniques. Moreover, the ethyl acetate extract of the Iraqi *C. grandiflora* flowers has an excellent anti-oxidant effect using the single-electron transfer (SET) pathway in scavenging the free radicals, and this activity attributed to the potent antioxidant i.e. polyphenols.

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