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Preliminary Phytochemical Screening, Antioxidant and Antimicrobial Activities of the Aqueous, Methanol, Acetone, and Hexane Fractions of *Centaurea cyanoides* Wahlenb

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ABSTRACT

Centaurea cyanoides Wahlenb aqueous, methanol, acetone, and hexane fractions has been evaluated for their phytochemical constituents, antioxidant and antibacterial activities. The fractions of were studied in order to support its use in the traditional medicine. Antioxidant activity was assessed employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging method. Antimicrobial activity was tested using broth microdilution method and agar dilution method. All fractions were contained proteins, amino acids, carbohydrate, reducing sugars, volatile oil, phytosterols and alkaloids. Whereas Methanol fraction contained flavonoids (91.35±1.75 mg QUE/g), phenols (96.32±2.2 mg of GAE/g) and tannins (93.2±2.22 mg CaE/g) beside the earlier mentioned phytochemicals. Antioxidant activity for methanol fraction showed the highest activity (IC_{50}=3.16±1.70 µg/ml) followed by aqueous fraction (IC_{50}=16.59±1.89 µg/ml), hexane fraction (IC_{50}=23.98±1.94 µg/ml) and acetone (IC_{50}=42.65±2.13 µg/ml). All fractions exhibited remarkable potential antimicrobial activities. The methanolic fraction of *C. cyanoides* possesses high content of flavonoids, tannins and phenols, the plant demonstrated potential antioxidant and powerful antimicrobial activities.

Keywords: Centaurea Cyanoides, Antimicrobial, Antioxidant, Phytochemicals.

1. INTRODUCTION

Medicinal plants have been used in traditional medicine for centuries in the treatment or prevention of various diseases including infectious and noninfectious chronic disease, as they produce hundreds of various antioxidant and antimicrobial compounds for their self-protection[1-3]. This provides protection against many diseases such as oxidative stress related diseases like diabetes mellitus, cancer, neurological, ischemia/ reperfusion and cardiovascular diseases[4]. These diseases are among the leading cause of death worldwide. Lower respiratory infections remained the most deadly infectious disease, second leading cause of death after ischemia/repe fusion and cardiovascular diseases[5]. In addition, increase of antibiotic-resistant pathogens[6], due to inadequate use of them, increased the demand for antimicrobial agents. Therefore, to solve global health problems which associated with bacterial resistance and oxidative stress, recently antimicrobial and antioxidant screenings are being conducted for a large number of plants to investigate their potentials[7].

C. cyanoides Wahlenb. is commonly known as "Knapweed and Syrian cornflower-thistle" which belongs

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to the Asteraceae (Compositae) family. It is a showy annual, about 15-40 cm tall, appressed-cobwebbed or floccose, canescent. Its stems are almost erect or ascending, leafy, and sparingly branched. The plant Flowering heads are radiant and have 2.5-3 cm diameter, while its florets color is deep blue, and zygomorphic[8]. This species is widely and wildly spreading in Palestine, Lebanon, Syria and Cyprus[9]. In the folk medicine, the obtained decoction (hot aqueous extract) of this plant has been used for the treatment of conjunctivitis and inflammations and recently this plant is cultivated widely in Palestine to produce honey[10,11]. To the best of our knowledge, there are no previous studies on the antimicrobial and antioxidant therapeutic effects of C. cyanoides plant. The current study was carried out to first time determine, the phytochemicals present in aerial parts of C. cyanoides plant and evaluate its antimicrobial and antioxidant activities.

2. Materials and methods

Plant material used

The aerial parts C. cyanoides plant were collected from Nabuls region of Palestine during June 2016. The plant has been botanically identified by pharmacognosist Dr. Nidal Jaradat from the Pharmacy Department at An-Najah National University. A voucher specimen Pharm-PCT-543 has been retained in the herbarium of the Laboratory of Pharmacognosy. The plant was washed well several times with distilled water and then dried in the shade for four weeks at room temperature. After drying, the leaves they were grounded using blender into a fine powder and placed into airtight containers with proper labeling for future use.

Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), $AlCl_3$, catechin, vanillin, Chloroform, HCl, Folin-Ciocalteu's reagent, Quercetin and Potassium acetate were purchased from Sigma-Aldrich (USA). Methanol, millon's reagent, NaOH, Benedict's reagent and acetone were bought from Lobachemie (India). n-hexane, Ninhydrin solution, Molish's reagent, H_2SO_4 and Iodine solution were purchased from Alfa-Aesar (England). Magnesium ribbon, Acetic acid, Dimethyl sulfoxide (DMSO) and $FeCl_3$ were purchased from Riedeldehan (Germany). Nutrient broth and macConkey agar were purchased from Himedia (India), BBL Mueller Hinton II broth, Difco Sabouraud Dextrose Agar and Bacto tryptic soy broth were purchased from Dickinson and company (USA).

Instrumentation

Rotary evaporator (Heidolph OB2000); freeze dryer (Mill rock technology model BT85, Danfoss); shaker device (Memmert shaking incubator); grinder (Moulinex model, Uno); balance (Radwag, AS 220/c/2); filter papers (Macherey-Nagel, MN 617 and Whatman no.1); micropipettes (Finnpipetted); automatic deionizer unit (Mime water inc); syringe filter 0.45 µm pore size (Microlab); micro broth plate (Greiner Bio-one); sterile syringe filter (0.45µm 25 mm); stir-mixer (Tuttnauer); incubator with Co_2 (Tuttnauer) and multichannel micro-pipet (Eppendorf).

Preparation of plant fractions

The air-dried, powdered aerial parts of C. cyanoides (25 g) were fractionated sequentially by adding solvents of increasing polarity: hexane (non-polar solvent), acetone (polar aprotic solvent), methanol (polar protic solvent) and water (polar protic solvent). Plant powder was soaked in 500 ml of hexane and placed in a shaker at 100 rounds per minute for 72 hours at room temperature. Then it was filtered through Whatman's No. 1 filter paper. The filtrate was reduced under vacuum pressure with rotatory evaporator. The remaining residue was soaked in acetone, and all the above mentioned steps were repeated for methanol and water fractions. However, aqueous fraction was dried using freeze dryer. Finally, all fractions were stored at 4 ºC in the refrigerator for further use.

Antioxidant activity

The free radical scavenging activity of C. cyanoides fractions and standard were measured according to the procedure described by Jaradat et al, 2016[12]. DPPH radical solution at a concentration of 0.002% w/v was mixed with methanol and the prepared concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/ml) from four plant fractions of 1mg/ml in methanol and standard Trolox in a ratio of 1:1:1 respectively[13]. The solutions were left in dark incubation for 30 min at room temperature. Absorbance readings were recorded at 517 nm. The antioxidant activity was reported as the percentage of inhibition. IC_{50} was calculated using the BioDataFit fitting program in which the sigmoidal fitting model was the fit model.

Qualitative phytochemical analysis

Preliminary qualitative phytochemical screening of primary and secondary metabolic compounds such as proteins, starch, phenols, glycosides, saponin glycosides, flavonoids, steroids, volatile oils, and tannins was carried out for the four plant fractions according to the standard phytochemical methods[14,15].

Determination of total flavonoid contents present in methanol fraction

Total flavonoid content of methanol fraction of C. cyanoides was determined by aluminum chloride colorimetric assay[16]. 10 mg of quercetin dissolved in 100 ml methanol, the solution was serially diluted to 10, 30, 50, 70, and 100 µg/ml. In each working test tube, 0.3 ml of 5 % sodium nitrite solution was added. After 5 minutes, 0.3 ml of 10 % aluminum chloride was added and at the 6th minute, 2 ml of 1 M sodium hydroxide was added. Finally, volume was made up to 10 ml with distilled water. The procedure was repeated for 1% concentration (100 mg/10ml) methanolic fraction. The absorbance was measured at 510 nm spectrophotometer by using UV-visible Jasco V-630 instrument. The samples were analysed in triplicate. The calibration curve was plotted using standard quercetin. The data of total flavonoids content of the methanol fraction was expressed as mg of quercetin equivalents/g of methanolic fraction.

Determination of total phenol contents present in methanol fraction

Total phenol content of methanol fraction of C. cyanoides was determined using a modified Folin-Ciocalteu's colorimetry assay[17]. Gallic acid was used as a standard. 0.5 ml of aqueous methanolic fraction was mixed with 2.5 ml 10% Folin-Ciocalteu's reagent, followed by 2.5 ml 7.5% sodium carbonate. After 45 minutes at 45 ºC incubation, absorbance was measured by using spectrophotometer apparatus at 765 nm wavelength. All samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The procedure was repeated for the standard solution of gallic acid and the calibration curve was construed. The data of total phenols content of the methanol fraction was calculated as mg gallic acid equivalent (mg of GAE/g of fraction).

Determination of total tannin contents present in methanol fraction

Total tannin content (Proanthocyanidin) was estimated according to procedure reported by Sun et al., 1998[18]. A volume of 0.5 ml from each diluted Catechin solution of concentrations (0.1, 0.4, 0.5, 0.7 and 1 mg/ml) was mixed with 3 ml of 4% vanillin/methanol solution and 1.5 ml of concentrated hydrochloric acid. The mixture was allowed to stand for 15 minutes, and absorption was measured at 500 nm and methanol was used as a blank. Total tannin content was expressed as mg (+)-catechin/g plant fraction. All samples were analyzed in triplicate.

Bacterial and fungal strains

Antibacterial activities of the aqueous and organic fractions of C. cyanoides were examined against 6 reference bacterial strains obtained from the American Type Culture Collection (ATCC). These were Staphylococcus aureus (ATCC 25923), Escherichia coli

(ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Enterococcus faecium (ATCC 700221) and Shegilla sonnie (ATCC 25931) and clinical isolate Staphylococcus aureus (MRSA). In addition, the antifungal activity was against Candida albicans (ATCC 90028) and Epidermatophyton floccosum (ATCC 52066).

Antimicrobial activities

Preparation of plants fractions for antimicrobial activity assessment

Aqueous fraction of plant powder was dissolved in sterile distilled water, while other organic fractions were dissolved in 10% DMSO to achieve a concentration of 50 mg/ml. These solutions were sterilized by syringe filter with 0.45 µm pore size.

Broth microdilution method

Detection of anti-bacterial activity of both organic and aqueous plant fractions was carried out using microdilution method. The applied procedure was similar to that of CLSI[19,20]. Briefly, plant fractions were serially diluted (2-fold) 11 times with Mueller-Hinton broth (MHB). Negative control of bacterial growth (no bacterial inoculation) assigned for well number 11, while positive control was MHB with bacterial inoculation only. For detection of any possible antibacterial activity of DMSO in broth microdilution method conditions, a serial 2-fold dilution of DMSO with nutrient broth was prepared with concentration 0.098% to 50%. The final bacterial concentration in each well (except negative control) was adjusted to 5×10^5 CFU/ml. After inoculation of bacteria, the plates were incubated at 35°C for 24 hours. Each plant fraction was examined in duplicate. The lowest concentration of plant fraction that did not allow any visible bacterial growth in the test broth was considered minimal inhibitory concentration (MIC).

MIC of plant fractions against Candida albicans was determined by broth microdilution method as mentioned above, but with slight modification[19,21]. RPMI1640 was used instead of MHB. A weight of 1.04 g of RPMI powder was dissolved in 90 ml sterile distilled water. MOP (3.456 g) was added to the solution and pH of solution was adjusted to 7. Sterile distilled water was added up to 100 ml. The solution was sterilized by filtration using 0.45 µm syringe filter.

Determination of anti-mold activity

Epidermatophyton floccosum mold inhibition by plant fraction was evaluated by agar dilution method[19,22]. Sabouraud dextrose agar (SDA) was placed in tubes and kept at 40°C water bath after sterilization by autoclave. Plant fractions were serially diluted with SDA. The prepared concentrations were from 0.78 to 25 mg/ml for plant fractions. Then, the prepared tubes were allowed to solidify in slanted position at room temperature. Meanwhile, a suspension with turbidity similar to 0.5 McFarland standard was prepared from fresh culture of Epidermatophyton floccosum. Then 20 µl of the suspension of was added to all tubes. Tubes with SDA and E. floccosum inoculation only were used as positive control of the mold. Results were taken after 10 days of incubation at 25°C. Minimum inhibitory concentration was the lowest concentration that completely inhibits the growth of Epidermatophyton floccosum.

3. Results

Qualitative phytochemical analysis

Qualitative preliminary phytochemical screening tests for aqueous, methanol, acetone, and n-hexane fractions of C. cyanoides showed the presence of the following phytochemical classes during the separation process; proteins, amino acids, carbohydrate, reducing sugars, glycosides, flavonoids, phytosterols, phenols, volatile oil, alkaloids and tannin in each collected fraction (Table 1). However, phenols, flavonoids and tannin were present in methanol fraction, but not in the other fractions.

Quantitative phytochemical analysis for methanol fraction

As shown above, methanol fraction contained phenols, flavonoids and tannin. Therefore, we quantified total contents of flavonoids, phenols and tannins present

in methanol fraction. Total flavonoids content was determined by aluminum chloride colorimetric method and quercetin was used as a standard. As shown in Figure-1, total content of flavonoids was 91.35±1.75 mg QUE/ g of methanol plant fraction. Total phenols content of methanol fraction of C. cyanoides was determined employing Folin-Ciocalteu's assay and gallic acid as a standard (Figure-2). Total content of phenols in the methanolic fraction was 96.32±2.2 mg of GAE/ g of methanolic plant fraction (Figure-2). Total tannin content of methanolic fraction of C. cyanoides plant was calculated from the standard calibration curve of catechin as presented in Figure-3, which shows to be 93.2±2.22 mg CaE/ g of methanol plant fraction.

Table (1)

Preliminary phytochemical screening tests for all *C. cyanoides* fractions

Phytochemical compound	Tests names	Methanol fraction	Hexane fraction	Acetone fraction	Aqueous fraction
Protein & amino acids	Millon's	+	+	+	+
	Ninhydrin	+	+	+	+
Carbohydrate & reducing sugars	Fehling's	+	+	+	+
	Bendict's	+	+	+	+
	Molisch's	+	+	+	+
	Iodine test for starch	-	+	+	-
Glycosides	Lieberman's	+	-	-	-
	Keller-kilani	+	-	-	-
Flavonoids	Alkaline reagent	+	-	-	-
Saponins	Frothing	-	-	-	-
Phytosteroids	Libermann Burchard's	+	+	+	+
Volatile oil	Copper acetate	+	+	+	+
Phenolic compounds	Ferric chloride	+	-	-	-
Tannin	Gelatin	+	-	-	-
Alkaloids	Wagner's	+	+	+	+

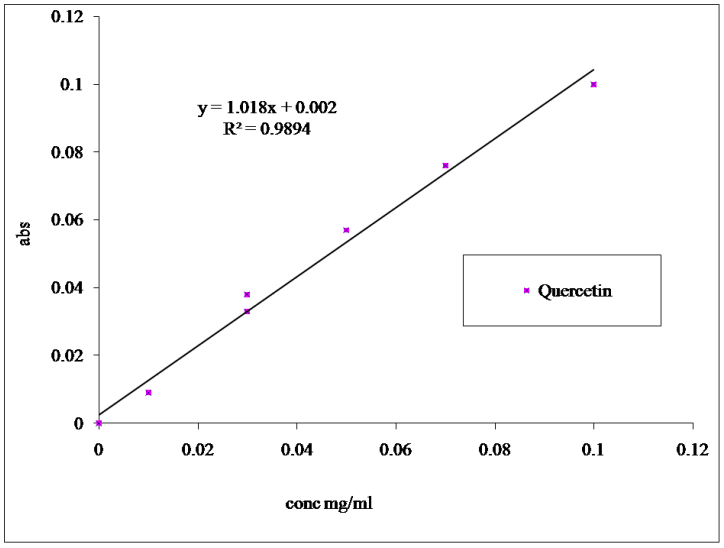

Figure (1): Standard calibration curve of Quercetin

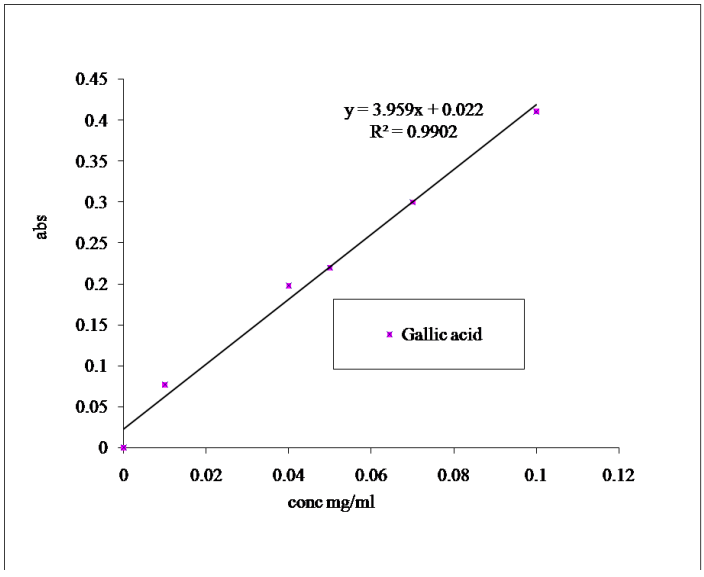

Figure (2): Standard calibration curve of Gallic acid

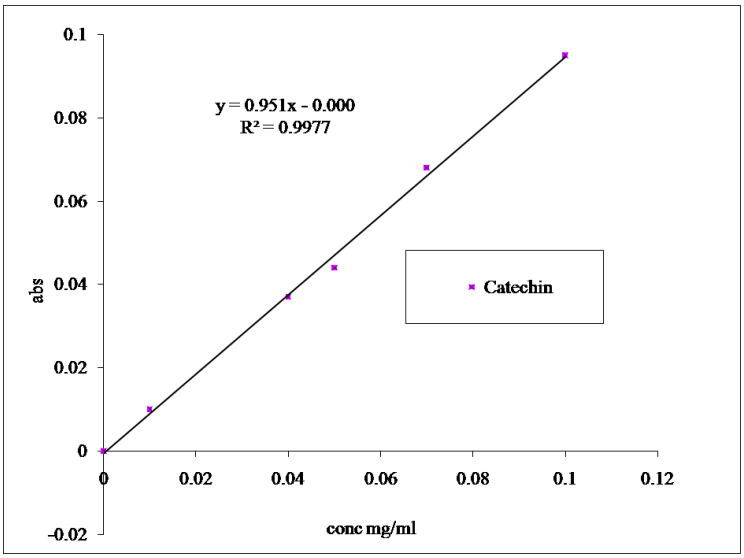

Figure (3): Standard calibration curve of catechin

Determination of antioxidant activity

The antioxidant activity of aqueous, methanol, acetone, and n-hexane fractions of C. cyanoides was determined by DPPH method and Trolox was used as a reference. As depicted in Figure-4, methanol had the highest free radical scavenging activity, followed by aqueous, hexane and acetone fractions. IC_{50} of acetone, hexane, aqueous and methanol fractions was 42.65±2.13 µg/ml, 23.98±1.94, 16.59±1.89 µg/ml and 3.16±1.70 µg/ml respectively. The IC_{50} of Trolox standard reference was 1.09±1.60 µg/ml.

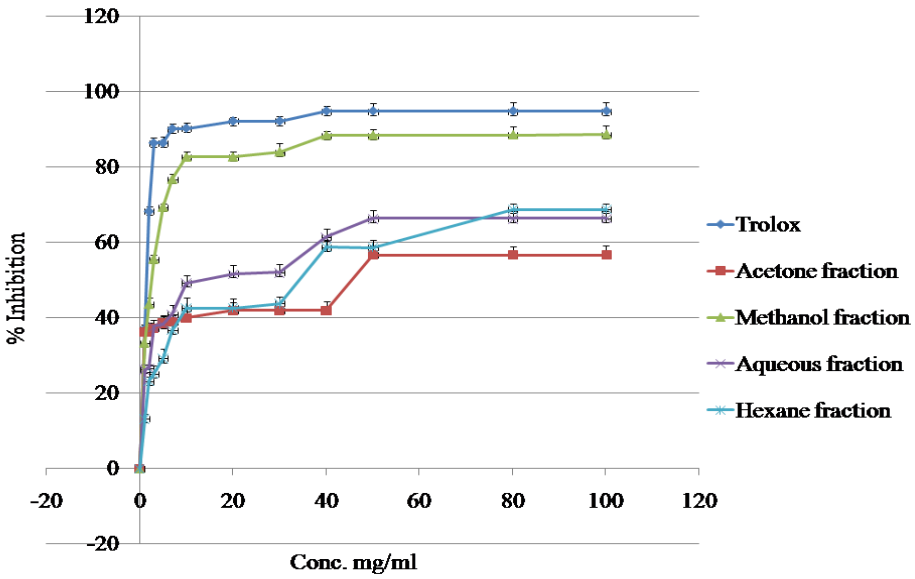

Figure (4): Antioxidant Activities for four fractions of *Centaurea Cyanoides*

Antimicrobial activity:

All C. cyanoides plant fractions showed antibacterial and antifungal activities, but the methanolic fraction was superior in these tests, as shown in Table (2), followed by aqueous fraction except for P. aeruginosa, E. faecium and E. floccosum which needed higher concentration to be inhibited. All fractions had a potent anti MRSA effect ranged from 0.025-0.25 mg/ml. MIC of methanol fraction against S. aureus, E. coli, P. aeruginosa, S. sonnie, E. faecium, MRSA, C. albicans and E. floccosum were 0.025, 0.5, 0.0125, 0.05, 0.025, 0.025, 0.25 and 0.5 mg/ml respectively. MIC of aqueous fraction against S. aureus, E. coli, P. aeruginosa, S. sonnie, E. faecium, MRSA, C. albicans and E. floccosum were 0.125, 2.5, 12.5, 0.25, 25, 0.25, 6.25 and 25 mg/ml respectively. MIC of acetone fraction against S. aureus, E. coli, P. aeruginosa, S. sonnie, E. faecium, MRSA, C. albicans and E. floccosum were 2.5, 25, 3.125, 12.5, 6.25, 0.25, 25 and 6.25mg/ml respectively. MIC of hexane fraction against S. aureus, E. coli, P. aeruginosa, S. sonnie, E. faecium, MRSA, C. albicans and E. floccosum were 2.5, 25, 6.25, 12.5, 6.25, 0.125, 12.5 and 6.25mg/ml respectively.

3.1 Discussion

According to the obtained results from preliminary phytochemical screening for aqueous, methanol, acetone, and n-hexane fractions of C. cyanoides, the methanol fraction was shown to have flavonoids, phenols and tannins. On the other hand, aqueous fraction doesn't show yield of phenolic compounds which may means that the phenolic or glycoside derivatives are not present or in a very low quantities. Therefore, our work was focused to determine methanol fraction quantities. The results of total phenol content, total flavonoid content, and total tannin content were 96.32±2.2 mg of GAE/g, 91.35±1.75 mg QUE/g and 93.2±2.22 mg CaE/g, respectively. These values are far higher than values of total phenol and flavonoid described in earlier studies[23] for other Centaurea species approximately 6 and 2.5 folds, respectively. In addition, they are higher than the total content present in ethyl acetate and chloroform extracts. This suggests that phytochemicals content present in C. cyanoides is higher than content present in other Centaurea species[24]. This prompted us to further analyze its antioxidant and antimicrobial effects.

Table (2)
Antimicrobial activity of *C. cyanoides* methanol, hexane, acetone and aqueous fractions

Bacterial and fungal isolates	MIC value (mg/ml)			
	Methanol fraction	Acetone fraction	Hexane fraction	Aqueous fraction
S. aureus	0.025	2.5	2.5	0.125
E. coli	0.5	25	25	2.5
P. aeruginosa	0.0125	3.125	6.25	12.5
S. sonnie	0.05	12.5	12.5	0.25
E. faecium	0.025	6.25	6.25	25
MRSA	0.025	0.25	0.125	0.25
C. albicans	0.25	25	12.5	6.25
E. floccosum	0.5	6.25	25	25

Most of natural antioxidants are polysubstituted phenolic compounds which appears in all parts of plants[25]. This antioxidant ability of phenolic compounds is due to scavenge free radicals and active oxygen species

such as singlet oxygen and hydroxyl radicals[23]. Free radicals are involved in many disorders like cancer, neurodegenerative diseases, cardiovascular disorders and AIDS. Hence, Antioxidants due to their ability to neutralize free oxygen radicals, have been useful for the management of these diseases[17-28-29]. Methanol fraction of C. cyanoides has been shown to be the best antioxidant activity followed by aqueous, hexane and acetone fractions. The observed antioxidant effect of our methanol fraction was 39 to 87 times more potential than other Centaurea species investigated in earlier studies[30]. The recent studies showed an association between the total phenolic content in medicinal plants and the antioxidant properties suggested that the phenolic compounds contributed significantly to the antioxidant capacity of the medicinal plants[31].

The world-wide usage of antibacterial medications has caused the emergence and spread of antibiotic-resistant strains of bacterial pathogens, such as MRSA. This draws the attention for new antimicrobial approaches; therefore, we investigated the effect of various methanol, acetone, hexane, and aqueous fractions of C. cyanoides on bacterial and fungal growth, which has never been investigated earlier. It appeared that all of C. cyanoides fractions have antibacterial and antifungal activities against S. aureus, E. coli, P. aeruginosa, S. sonnie, E. faecium, MRSA, C. albicans and E. floccosum. Methanolic fraction had the best antimicrobial activity followed by aqueous fraction which is in support importance of aqueous fraction in traditional medicine. In comparison with earlier studies where other Centaurea species were investigated, the results of this study showed stronger antimicrobial activity. For example, methanol fraction of C. cyanoides possesses 20-80 times stronger antimicrobial activity than the investigated methanol fraction of Centaurea species against S. aureus[32]. It is even stronger than commercial antibiotics such as amoxicillin[33].

4. Conclusion

C. cyanoides methanolic and aqueous fractions possesses a potential antimicrobial activity, while methanol fraction possess high antioxidant activity. Aqueous and methanol fractions of Centaurea species showed strong antimicrobial activity against S. aureus, S. sonnie, MRSA, and E. coli, which appear to be higher than the other Centaurea species investigated earlier. This is most probably due to the high content of phytochemicals such as flavonoids, phenols, and tannins. However, this may prove its traditional uses in the treatment of ophthalmic infectious diseases. The obtained antimicrobial and antioxidant biodata from C. cyanoides may be a possible choice for future development of medicines treatment of various infections but require further validation.

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ملخص

تم عمل تجزيئ لنبتة القنطريون الكحلي باستخدام الماء، والميثانول، والأسيتون، والهكسان لتقييم المكونات الكيميائية النباتية، والأنشطة المضادة للأكسدة والمضادة للبكتيريا لدعم استخدامها في الطب التقليدي. تم تقييم نشاط مضادات الأكسدة باستخدام طريقة دبف. بينما تم تقييم النشاط المضاد للميكروبات باستخدام طريقة المايكروداليوشن التخفيف الدقيق وطريقة تخفيف الأجار. وقد تبين أن جميع مكونات عملية التجزيئ بالمحاليل تحوي البروتين والأحماض الأمينية والكربوهيدرات والسكريات المختزلة والزيوت الطيارة والستيرول والقلويدات. في حين احتوى الجزء الميثانولي على فلافونويدات (٩١,٣٥ ± ١,٧٥ ملغ)، فينولات (٩٦,٣٢ ± ٢,٢ ملغ) والعفصيات (٩٣,٢ ± ٢,٢٢ ملغ) بجانب المواد الكيميائية النباتية المذكورة سابقاً. الجزء المذاب في الميثانول أظهر أعلى نشاط لمضادات التأكسد (IC_{50}=3.16 ± 1.70 ميكروجرام/ مل) متبوعًا بالجزء المذاب في الماء (IC_{50}=16.59±1.89 ميكروجرام/ مل)، والجزء المذاب بالهكسان (IC_{50}=23.98±1.94 ميكروجرام/ مل) والجزء المذاب بالأسيتون (IC_{50}=42.65±2.13 ميكروجرام/ مل). أظهرت جميع الأجزاء المذابة في المحاليل الأربعة أنشطة لمضادات الميكروبات بشكل ملحوظ. الجزء الميثانولي للقنطريون الكحلي يمتلك نسبة عالية من مركبات الفلافونويد، والعفصيات والفينولات، أظهر نوع النبتة نشاطا قويا كمضادة للأكسدة ومضادة للميكروبات.

الكلمات الدالة: سنتوريا سيانويد (قنطريون كحلي)، مضادات الميكروبات، مضادات الأكسدة، المكونات الكيميائية النباتية.

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