International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

ANTIMICROBIAL ACTIVITY OF DIFFERENT EXTRACTS OF *DAUCUS CAROTA* CANOPY

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ABSTRACT

Antimicrobial study of *Daucus Carota* Canopy extracts against bacteria and fungi showed that the ethanolic extract was the most active. Phytochemical investigation of the active ethanolic extract gave rise to 12 natural flavone compounds including the new C-glycoside; diosmetin 6,8-Di-C-α-L-rhamnopyranoside besides the first isolation of luteolin 8-C-β-L-arabinopyranoside and luteolin 6,8-Di-C-α-L-rhamnopyranoside from this plant and the rest 9 known compounds including 3 luteolin glycosides, 1 diosmetin glycosides, 2 chrysoeriol glycosides and 3 aglycones. Their structures were established by chromatographic methods, chemical degradation and various spectroscopic data.

Keywords: *Daucus Carota* canopy, Apiaceae, natural flavone C-glycoside, NMR spectroscopy, Antimicrobial activity.

INTRODUCTION

Plants have the major advantage of being the most effective and cheaper alternative source of drugs [(1)]. They are prospective source of antimicrobial agents in different countries [(2)]. About 60 to 90% of populations in the developing countries use plant-derived medicine. Traditionally, crude plant extracts are used as herbal medicine for the treatment of human infectious diseases [(2–4)]. Plants are rich in a variety of phytochemicals including tannins, terpenoids, alkaloids, and flavonoids which have been found *in vitro* to have antimicrobial properties [(5, 6)]. Plants containing active compounds are able to inhibit the microbial growth. Studying plant based antimicrobial properties provides additional information in developing natural antibiotics and discovering the alternative of antimicrobial drugs for the treatment of infectious diseases [(7)]. The search for newer sources of antibiotics is a global challenge preoccupying research institutions, pharmaceutical companies and academia, since many infectious agents are becoming resistant to synthetic drugs[(8)]. In developing countries were medicines are quite expensive, the investigation on antimicrobial activities from ethnomedicinal plants may still be needed. Apiaceae (umbelliferae) is a cosmopolitan family comprising 455 genera and over 3500 species, which makes this family one of the largest taxon among higher plants [(9)]. The most commonly cultivated member of the family is carrot (*Daucus Carota*). Genus *Daucus* is a member of Apiaceae family which is one of the most important families of angiosperms. The usage of Apiaceae plants, particularly as flavourings, spices, and in traditional medicine, is connected with aromatic compounds that may occur in all parts of these plant[(10)]. Carrots are nutritional heroes; they store a goldmine of nutrients; e. g. carotene as carrots, which the body converts it to vitamin A, an excellent source of vitamins B, C, D and E, thiamine, folic acid and magnesium, as well as calcium pectate (pectin fibre) which have cholesterol-lowering properties. Carrot consumption modifies cholesterol absorption and bile acids excretion and increases antioxidant status and these effects could be interesting for cardiovascular protection. They are used in the diet of cancer patients, the dried flowers are used as a remedy for

dropsy. It is reported to be endowed with medicinal properties, i.e. hypotensive, stomachic, antilipemic, antianaemic, healing, diuretic, anthelmintic, carminative, emmenagogue, ophthalmic and sedative properties[11,12,13]. Its phytochemical screening on its leaves, roots, aerial parts, fruits and seeds showed the presence of many natural compounds such as:- coumarin (e.g aesculetin[14], bergapten[15]), anthocyanins from whole plant[16], phenolic acids (caffeic [17] from aerial parts, leaves and roots, caffeoylquinic [18] from roots and chlorogenic[19] from root and leaves), flavones (e.g apigenin[20] from leaves and luteolin[21] from roots) and their glycosides (e.g apigenin 7-galactomannoside[22] from seeds and luteolin 7-O-β-D-glucoronide[23] from aerial parts), flavanols e.g kaempferol[24] and quercetin[24] from roots and fruits while flavonol glycosides; rutin[24] from roots. The structures of the isolated pure compounds have been elucitated by conventional methods of chemical and physical analysis and confirmed by ^{1}H and ^{13}C-NMR. The objective of this study is to evaluate the antimicrobial activity of *Daucus Carota* canopy different extracts and the isolation and identification of the polyphenolic content of the potent extract. Results showed that the potent one was the ethanolic extract where proved to contain 12 flavone compounds among them the new natural compound; diosmetin 6,8-Di-C-α-L-rhamnopyranoside; which was isolated for the first time in nature.

MATERIALS AND METHODS

General methods: ^{1}H and ^{13}C (500, 125 MHz) NMR: Joel spectrometer in DMSO-d_6; UV: Shimadzu spectrophotometer model UV-240 (Kyoto, Japan); column chromatography (CC): Polyamide 6S (Riedel, De Häen), Cellulose (Merck) and Sephadex LH-20 (Pharmacia); paper chromatography (PC): Whatman No. 1 and preparative (PPC) on 3 MM paper using the following solvent systems: (1) BAW (n-BuOH/AcOH/H_2O, 6:1:2); (2) H_2O; and (3) AcOH/H_2O (15:85), (4) 6% AcOH (AcOH: H_2O, 06:94) and (5) Forestal (AcOH: Conc. HCl: H_2O: 30:3:10).

Plant material: The cultivated *Daucus Carota* Canopy was collected from market in Egypt. A voucher specimen is deposited in the National Research Centre Herbarium.

Extraction and fractionation: 500g of powdered air-dried *Daucus Carota* canopy was extracted by successive solvent extraction firstly with chloroform (CF) followed by ethyl acetate (EA) and finally with ethanol (ET). The three extracts were vacuum dried at 40^{o}C and their net weights were calculated to give CF (35 g), EA (57 g) and ET (113 g), respectively. The three extracts were preliminary investigated for their antimicrobial activity which showed that the ethanolic extract was the most active. The two dimension paper chromatography of the ethanolic extract using the solvent systems (1) and (3), respectively, revealed the presence of many components of polyphenolic nature. The concentrated ethanolic extract (113 g) was chromatographed on a polyamide column; elution being performed with water followed by water-ethanol mixtures to give six fractions which were further purified on subcolumns of Sephadex LH-20 and PPC to give rise to 12 pure compounds. Compounds (**1**, 74 mg and **2**, 48 mg) were isolated from fraction **I** which eluted with 20% EtOH then by fractionation over Sephadex LH-20 column using EtOH/H_2O (decreasing polarity) for elution. Compound **3** (86 mg) was isolated from fraction **II** (40% EtOH) and purified by using Sephadex LH-20 column and *n*-BuOH saturated with H_2O as developing system. Applying the third fraction (60% EtOH) on Sephadex LH-20 column and eluted by 50% ethanol to obtain the three pure natural compounds (**4**, 38 mg; **5**, 47 mg and **6**, 62 mg). From the fourth fraction (80% EtOH), compounds (**7**, 46 mg and **8**, 33 mg) were separated in a pure form by applying on Sephadex LH-20 column and eluted by ethanol. The compound **9 (**66 mg), was separated from fraction 5 and purified on PPC using the solvent system BAW for elution. Finally, the three aglycones **10** (48 mg), **11** (36 mg) and **12** (28 mg) were obtained from fraction **VI** using ethanol as eluent and purified on a cellulose column eluted with ethanol. Their chemical structure have been established by conventional methods of chemical and physical analysis and confirmed by ^{1}H and ^{13}C NMR spectroscopy.

Antimicrobial activity of the plant extracts.

Test microorganisms: The following clinical isolates of bacteria and fungi were used for the study: *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Salmonella typhi*, *Candida albicans*, and *C. tropicalis*. Microbial cultures were grown on nutrient agar and potato extrose agar for bacteria and fungi respectively and maintained at 4^{o}C in a refrigerator for further studies.

Controls used in the study: Bacitracin (10µg/disc) was used as positive control for *S. aureus*; Erythromycin (10µg/disc) was used for *P. aeruginosa*, *E. coli*, *M. luteus*, and *S. typhi*; Chloramphenicol (30µg/disc) was used for *K. pneumoniae*; Fuconazole (10µg/disc) was used for *C.*

albicans and *C. tropicalis*. Sterilized distilled water was used as negative control for the study.

Antimicrobial assay: Antimicrobial activity of the tested extracts was determined by the agar well diffusion method [25]. All test organisms were inoculated on MHB (Mueller Hinton broth) and PDB (Potato Dextrose broth) for 8 hours. Microbial isolates were inoculated on MHA (Mueller Hinton agar) plates and PDA (Potato Dextrose agar) plates by using sterile cotton swabs for bacteria and fungi respectively. Agar surface was bored by using sterilized gel borer to make wells (7 mm diameter). 100 µl of the tested extracts and 100 µl of sterilized distilled water (negative control) were poured in to separate wells. The standard antibiotic disc was placed on the agar surface as positive control. Bacterial plates were incubated at 37^{o}C for 24 hours and fungal plates were incubated at room temperature for 48 to 72 hours. Experiment was performed in triplicates.

Determination of relative percentage inhibition: The relative percentage inhibition of the test extracts and the isolated compounds with respect to positive control was calculated by using the following formula [26, 27].

Relative percentage inhibition of the test extract = 100 x (X-Y)/ (Z-Y)

Where,

x: total area of inhibition of the test extract

y: total area of inhibition of the solvent

z: total area of inhibition of the standard drug

The total area of the inhibition was calculated by using area = πr^2; where, r = radius of zone of inhibition.

Statistical Analysis: The values of antimicrobial activity of the test extracts of *Daucus Carota* canopy are expressed as mean ± standard deviation of the response of 3 replicates determinations per sample.

Spectral data

New natural 6,8-Di-C-α-L-rhamnopyranoside diosmetin:

R_f-values x100: 33 (1), 21 (2), 24 (3); UV λ_{max} nm (MeOH): 242sh, 250, 268, 290, 345; +NaOMe: 272, 302sh, 385; + NaOAc: 270sh, 283, 328, 397; +NaOAc/H_3BO_3: 252sh, 270, 350; + $AlCl_3$: 266sh, 272, 295, 360, 392; +$AlCl_3$ / HCl: 262sh, 275, 295, 350, 382; sh (shoulder); ^{1}H-NMR: δ(ppm) 7.66 (d, J= 2.5 Hz, H-2'), 7.55 (dd, J= 2.5 & 8.5 Hz, H-6'), 7.07 (d, J= 8.5 Hz, H-5'), 5.43 (br, H-1``, H-1``` of the two rhamnose moieties), 3.07-3.6 (m, rhamnose sugar protons), 0.84, 0.87 (d, J= 6 Hz, CH_3 of rhamnose). ^{13}C-NMR spectral data: Aglycone moiety: δ(ppm) 163.8 (C-2), 103.5 (C-3), 180.9 (C-4), 161.6 (C-5), 108.3 (C-6), 164.2 (C-7), 104.6 (C-8), 123.5 (C-1`), 113.2 (C-2`), 146.7 (C-3`), 150.9 (C-4`), 112.0 (C-5`), 118.5 (C-6`), 55.6 (OCH_3); 6-C-rhamnoside; 77.5 (C-1``), 75.7 (C-2``), 74.1 (C-3``), 72.6 (C-4``),71.8 (C-5``), 16.1 (C-6``); 8-C-rhamnoside; 77.9 (C-1```), 74.9 (C-2```), 74.5 (C-3```), 72.3 (C-4```), 72.1 (C-5```), 16.5 (C-6```).

Results and Discussion

Antimicrobial study of the *Daucus Carota* canopy extracts against bacteria and fungi showed that the ethanolic extract was more active than the other two extracts. In fact, the ethanolic extract showed strong inhibitory activity against *S. aureus*, *M. luteus*, *C. albicans*, *C. tropicalis*, *P. aeruginosa* and *E. coli* while, *S. typhi* and *K. pneumoniae* were found to be resistant towards the ethanolic extract of *Daucus Carota* canopy. Due to these results phytochemical investigation of the ethanolic extract was carried out which showed the presence of a number of flavonoid compounds, to which the antimicrobial activity may be related [28]. Thus, the active ethanolic extract was then applied on CC using polyamide 6s as an adsorbent and eluted by water followed by water-ethanol mixtures to obtain six fractions. These fractions were further purified using subcolumns or PPC to give rise to 12 pure compounds which are identified as following: luteolin 6,8-Di-C-α-L-dirhamnopyranoside **(1)**, luteolin 7-O-β-D-mannopyranoside-4`-O-β-D-glucopyranoside **(2)**, diosmetin 6,8-Di-C-α-L-rhamnopyranoside **(3),** luteolin 4`-O-β-D-glucopyranoside **(4)**, luteolin 7-O-β-D-mannopyranoside **(5)**, luteolin 8-C-β-L-arabinopyranoside **(6)**, chrysoeriol 7-O-β-D-arabinopyranoside **(7)**, chrysoeriol 7-O-β-D-glucopyranoside **(8)**, diosmetin 6-C-α-L-rhamnopyranoside **(9)**, luteolin **(10)**, diosmetin **(11)** and chrysoeriol **(12)**. Among which compound 3 is a new natural product which is discussed here. The purified compound **(3)** was first identified through R_f-values and colour reactions with the UV spectral data in MeOH and with diagnostic shift reagent indicated a flavone type with a free 5,7-dihydroxyl groups while it is substituted at 4` , where a bathochromic shift ( 40 nm) was produced in band (I) upon the addition of NaOMe with a decrease in intensity and stable indicating that 4` was occupied. The addition of NaOAc led to a bathochromic shift (33 nm) in band (II) indicating a free 7 OH group which was confirmed by the appearing of a shoulder at (325) in band I on the addition of NaOMe. No shift was produced in band I upon the addition of NaOAc/H_3BO_3 i.e the absence of 3`, 4`-dihydroxyl group in ring B. Chemical investigations of compound (1) illustrated that it was a C-glycoside type, where it not hydrolysed by normal complete

acid hydrolysis and it gave the aglycone diosmetin and the sugar rhamnose (which was identified by Co-PC) when subjected to periodate oxidation[29]. Its structure was further confirmed by the ^{1}H-NMR[30] spectra whereby two doublet signals appeared at δ7.66 and 7.55ppm assigned to H-2`, H-6` a doublet signal at 7.07 assignable to H-5` the signal of H-6 and H-8 were absent; besides , the presence of a broad signal at δ 5.43 ppm assigned to two rhamnose moieties at position 6 and 8 with the doublet signals of the methyl group of rhamnose at 0.84, 0.87 (J= 6.5 Hz). The suggested structure was thoroughly confirmed by applying ^{13}C-NMR analysis[31]. The chemical shifts of the carbon signals were found identical with those reported for a 6,8-disubstituted diosmetin where a downfield shift of C-6 and C-8 at 108.3, 104.6 ppm respectively, than that of the unsubstituted one (C-6 at δ 99.0 and C-8 at δ 94.0 ppm) and an upfield shift of C-5 and C-7 at δ 150.0 and 156.8 ppm, respectively, than that of the unsubstituted one (C-5 at δ 161.7 and C-7 at δ 164.4 ppm) also the presence of the two anomeric carbone signals at 77.5 ,77.9 ppm in addition to the two methyl group of rhamnose moiety at 16.5, 16.1 ppm ensured the proposed structure.

Antimicrobial activity: Ethanolic extract of *Daucus Carota* canopy exhibited broad spectrum of antimicrobial activity against Gram positive bacteria, Gram negative bacteria and fungal cultures. Antimicrobial activity was measured as zone of inhibition and represented as mean ± standard deviation (n=3). Extract exhibited maximum antimicrobial activity against *S. aureus* (20.2 ± 1.75), followed by *M. luteus* (12.61 ± 1.72). *C. albicans* (9.21 ± 1.02), *C. tropicalis* (6.61 ± 1.32), *P. aeruginosa* (5.49 ± 2.03) and *E. coli* (3.39 ± 1.53). Results are summarized in Table 1. *S. typhi* and *K. pneumoniae* were found to be resistant towards the ethanolic extract of *Daucus Carota* canopy.

CONCLUSION:

It was finally concluded that the antimicrobial activities of the three extracts chloroform (CF), ethyl acetate (EA) ethanol (ET) of *Daucus Carota* canopy which were tested against the Gram positive bacteria, Gram negative bacteria and fungal cultures, proved that the ethanolic extract was the efficient extract due to its polyphenolic content. *Staphylococcus aureus* and *Micrococcus luteus* were the most susceptible microorganisms, whereas *Salmonella typhi* and *Kelebsiella pneumonia* were the most resistant bacteria regarding the *Daucus Carota* canopy extracts. Moreover the phytochemical screening of the more active ethanolic extract lead to isolation of 12 compounds of flavone nature including 1 new compound (3); diosmetin 6,8-Di-C-α-L-rhamnopyranoside; for the first time in nature besides the first isolation of luteolin 8-C-β-L-arabinopyranoside and luteolin 6,8-Di-C-α-L-dirhamnopyranoside from this plant.

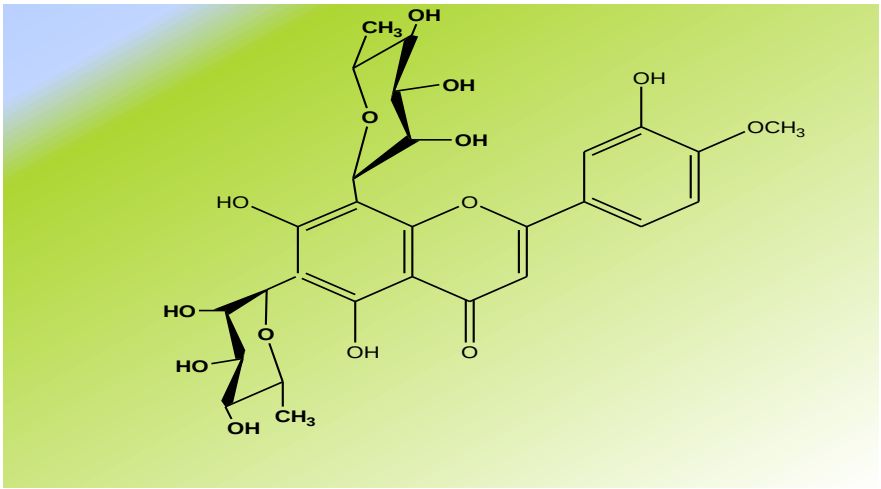

Figure 1: Structure of the new compound 6,8-Di-C-α-L-rhamnopyranoside diosmetin (3)

Table 1: Antimicrobial study of the *Daucus Carota* canopy extracts:

***Daucus Carota* canopy**	Pseudomonas aeruginosa	Salmonella typhi	Kelebsiella pneumoniae	Escherichia coli	Staphylococcus aureus	Micrococcus luteus	Candida albicans	Candida tropicalis
Chloroform extract	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ethyl acetate extract	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ethanolic extract	5.49±2.03	n.a.	n.a.	3.39±1.53	20.2±1.75	12.61±1.72	9.21±1.02	6.61±1.32
PC	14.0±1.0	16.0±4.35	15.66±3.78	33.66±1.52	14.33 ± 0.57	27.66±2.08	15.66±1.52	21.66±1.52
NC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Pc: positive control, Nc: negative control; Values are expressed as mean ±stander deviation of the tree replicates
Zone of inhibition not include the diameter of the well.

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