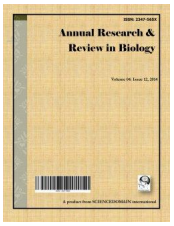

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Evaluation of the Antioxidant, Analgesic and Cytotoxic Activities of *Daucus carota canopy* L Ethanolic Extract

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Authors' contributions

This work was carried out in collaboration between all authors. All authors designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript, managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

In researcher's previous study it has been found that the 80% ethanolic extract of *Daucus carota canopy* L was the most active extract for antibacterial activity and its phytochemical screening led to the isolation and identification of 12 natural flavone compounds including the new C-glycoside; 6,8-Di-C-α-L-rhamnoside diosmetin besides 11 known compounds. The primary objective of the present work is to investigate the 80% EtOH extract of *Daucus carota canopy* L for its antioxidant, analgesic and cytotoxic activities and the new compound for its cytotoxic activity. The ethanolic extract (300 µL, 1 mg/ml) gave the highest effective antioxidant activity with inhibition % 82.09 ± 0.15 using radical scavenging method which is more than the standard BHA (68.2) and less than the standard

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TBHQ (98.2). The cytotoxic activity of ethanolic extract against MCF7 gave IC50 19.4 µg/ml, and the new compounds had IC50 is 8.05 µg/ml compare to Cisplatin which its IC50 is 5.48 µg/ml. Its anti-nociceptive properties show that the ethanolic extract (200 µL, 1 mg/ml) significantly ($p<0.05$) decreased the number of writhes in acetic acid writhing test and increased the response time in hot plate method when comparing with the control group.

Keywords: Daucus carota canopy L; phytochemical; pharmacological activities; antioxidant; analgesic; cytotoxic activity.

1. INTRODUCTION

In the researcher's previous communication [1], it had been found that *Daucus carota canopy* L is a promising plant and it has several medicinal uses as hypotensive, antilipemic, antianaemic, stomachic, healing, anthelmintic, diuretic, emmenagogue, carminative, ophthalmic, emmenagogue and it has sedative properties [2-4], where we previously reported the isolation of 12 natural flavone compounds including the new C-glycoside; 6,8-Di-C-α-L-rhamnoside diosmetin, besides the first isolation of luteolin 8-C-β-L-arabinoside and 6,8-Di-C-α-L-dirhamnoside luteolin from its ethanolic extract [1]. The rest 9 known compounds were identified as: luteolin 7-O-β-D-mannoside-4`-O-β-D-glucoside, 4`-O-β-D-glucoside, 7-O-β-D-mannoside, 6-C-α-L-rhamnoside diosmetin, chrysoeriol 7-O-β-D-arabinoside, 7-O-β-D-glucoside besides the three aglycones: luteolin, diosmetin and chrysoeriol. The present study has been designed to evaluate the potent ethanolic extract of *Daucus carota* canopy for its antioxidant, analgesic and cytotoxic activities and the new compound for its cytotoxic activity.

2. MATERIALS AND METHODS

2.1 General Methods

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; (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).

2.1.1 Drugs and chemicals for antioxidant test

DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate), BHA (Butylated Hydroxyl Anisol) and TBHQ (Tert-Butylated Hydroxyl Quinone); from Sigma-Aldrich Chemie, Steinheim, Germany; as standard antioxidants.

2.2 Potential Cytotoxicity Measurements by SRB Assay

One tumor cell line - MCF7 (breast carcinoma), was obtained from the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Potential cytotoxicity of the 80% EtOH extract of *Daucus carota canopy* L was tested using (SRB) assay of cytotoxic activity according to the method of Skehan [5].

2.2.1 Drugs and chemicals for analgesic test

Acetic acid was purchased from Sigma-Aldrich® Co. and diluted in 0.9% sodium chloride (Sigma-Aldrich®, MO, USA) to prepare 0.6% acetic acid. Diclofenac sodium was purchased from Novartis Co. (Novartis Co., Cairo, Egypt).

2.2.2 Animals for analgesic test

Male Swiss albino mice weighing 20-25 g were purchased from Modern Veterinary Office for Laboratory Animals (Cairo, Egypt). Mice were housed in polyethylene cages under controlled laboratory conditioning (25 ± 1°C temperature, 55% relative humidity, and regular dark/light cycles). Food and water were provided *ad libitum*. All the experimental protocols were approved by The Animal Care and Use Committee at the Faculty of Pharmacy, Suez Canal University.

2.3 Plant Material

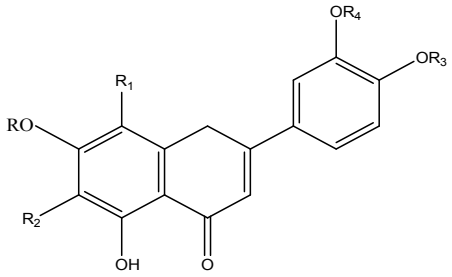
The cultivated *Daucus carota canopy* L was bought from the market, Egypt. A voucher specimen is deposited in the National Research Centre Herbarium.

2.4 Extraction and Fractionation

The *Daucus carota canopy* L (500 g) was coarsely powdered and extracted by successive solvent extraction using separating funnel firstly

with chloroform followed by ethyl acetate and finally with ethanol. The three extracts concentrated to dryness under reduced pressure and controlled temperature (40°C). From the researcher's previous studies the ethanolic extract was the most active for antimicrobial activity. 115 g concentrated ethanolic extract was chromatographed on a polyamide column; the elution started with H_2O followed by aqueous ethanol to give 6 fractions which then purified on subcolumns of Sephadex LH-20 and giving 12 natural flavone compounds where new C-glycoside; 6,8-Di-C-α-L-rhamnoside diosmetin was isolated from the second fraction using 40% EtOH and purified by using Sephadex LH-20 column and *n*-BuOH saturated with H_2O as developing system.

The chemical structure of the isolated compounds was recognized by physical and chemical analysis and confirmed by ^{1}H and ^{13}C NMR spectroscopy as described before. The ethanolic extract was evaluated for its antioxidant, analgesic and cytotoxic activities while the new C-glycoside flavone; 6,8-Di-C-α-L-rhamnoside diosmetin; was evaluated for its cytotoxic activity.



R_1=R_2= C-α-L-rhamnopyranoside, R= R_3= R_4 = H **(1)**
R= β-D-mannopyranoside, R_3= β-D-glucopyranoside, R_1= R_2= R_4= H **(2)**
R_1= R_2= C-α-L-rhamnopyranoside, R= R_4 = H, R_3= OCH_3 **(3)** [new compound]
R= R_1= R_2= R_4 = H, R_3= β-D-glucopyranoside (**4**)
R_1= R_2= R_3= R_4= H, R= β-D-mannopyranoside(**5**)
R= R_2= R_3= R_4= H, R_1= C-β-L-arabinopyranoside(**6**)
R1= R_2= R_3= H, R= O-β-D-arabinopyranoside, R_4 = OCH_3 (**7**)
R1= R_2= R_3 = H, R = O-β-D-glucopyranoside, R_4 = OCH_3 (**8**)
R= R_1= R_3= H, R_2= C-α-L-rhamnopyranoside, R_4= OCH_3 (**9**);
R=R_1=R_2=R_3= R_4= H (**10**);
R=R_1=R_2=R_4= H, R_3= OCH_3 (**11**);
R=R_1=R_2=R_3= H, R_4= OCH_3 (**12**)

Various compounds isolated from *Daucus carota canopy* L

3. EVALUATION OF ANTIOXIDANT ACTIVITY

3.1 DPPH Radical Scavenging Assay

Radical scavenging activity of plant extract against stable DPPH was determined spectrophotometrically in comparison with BHA and TBHQ as standard antioxidants. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were measured at 517 nm on a Shimadzu Spectrophotometer (UV-1601 PC). Radical scavenging activity of plant extract was measured by the method of Brand-Williams [6] as described below.

- Plant extract was prepared by dissolving a known weight of the plant in 10 ml of methanol (HPLC grade).
- The solution of DPPH0 in methanol (6 x 105 M) was prepared freshly, before UV measurements.
- 2.9 ml of this solution were mixed with 100, 200 and 300 µL of plant solution (1mg/ml) in 1 cm path length cuvette.
- The samples were kept in the dark for 15 min. at room temperature, and then the decrease in adsorption was measured.
- The reference cuvette contained DPPH blank.
- The radical scavenging activity of the samples (antioxidant activity) was expressed as percent inhibition of DPPH radical as the following equation:

  % Inhibition = [(A control –A treatment) / A control)] x 100

Where:

A control: is the absorbance of the control.
A treatment is the absorbance of the treatment.

3.2 Evaluation of Potential Cytotoxicity

The main objective of cancer therapy is to achieve maximum therapeutic destroy of tumor cells using the minimal concentration of the drug. This can be achieved, in principle, via selective antitumor preparations. While 100% selectivity may be impractical, achievement of reasonably high selectivity seems to aim [7].

Potential cytotoxicity of the ethanolic extract of *Daucus carota canopy* L and the new

compound were tested using the method of Skehan [5].

- Cells were plated in 96-multiwell plate (104cells/well) for 24 hrs before treatment with the extract and the new compound to allow attachment of a cell to the wall of the plate.
- Different concentrations of them under test (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer, triplicate wells were prepared for each individual dose.
- Monolayer cells were incubated with them for 48 hr at 37º C and in atmosphere of 5% CO2.
- After 48 hr, cells were fixed, washed and stained with Sulpho-Rhodamine- B stain.
- Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer.
- Color intensity was measured in an ELISA reader.
- The relation between the surviving fraction and both the extract and the new compound concentration is plotted to get the survival curve of each tumor cell line after the specified extract.

Principle:

The ethanolic extract (1-10 µg/ml) of *Daucus carota canopy* L was tested for cytotoxic activity against breast carcinoma cell line (MCF7) as well as the new compound, using the SRB assay. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stains that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content.

3.3 Evaluation of Analgesic Activity

3.3.1 Experimental design

The animals were divided into 4 groups of 6 animals each and dose given as follows:

Group 1: served as control & received 12% tween 80 in distilled water as vehicle at a dose 10 ml/kg body weight orally.

Group 2: served as standard & received Diclofenac sodium in Tween 80 suspension with water at a dose of 10 mg/kg body weight orally.

Group 3: served as test & received *Daucus carota canopy* L extract 100 mg/kg body weight orally.

Group 4: served as test & received *Daucus carota canopy* L extract 200 mg/kg body weight orally.

3.4 Hot Plate Test

The animals were placed individually in Hot plate regulated at temperature (55±0.5ºC) before the treatment & its reaction time was determined. After noting the initial reaction time, the treatment should be given to each mouse. Then each animal was placed in the Eddy's hot plate under regulated temperature. The response time was noted at the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first [8-11]. Mice with baseline latencies of <5s or >30s were eliminated from the study. The reaction time is noted by stop-watch and then the reaction time was determined after 0, 15, 30, 45 & 60 min. After oral administration of standard and test drug [12].

3.5 Acetic Acid Writhing Test

Koster method [13] was used. The mice were injected intraperitoneally with 0.1 ml of 0.6% acetic acid solution 30 min. after treatment with the extract, which induced the characteristic writhing. The number of writhing was observed between (5-15 min). The data were collected and computed according to the following formula:

Percentage Inhibition = [Mean of writhing test (control) – Mean writhing test (test) / Mean number of writhing test (control)] X 100.

3.6 Statistical Analysis for Analgesic

Results are expressed as mean ± S.E.M. Data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test. All statistical tests were done employing the Statistical Package for Social Sciences, version 19 (SPSS Software, SPSS Inc., Chicago, USA) and the differences were considered significant at $P< 0.05$.

4. RESULTS AND DISCUSSION

4.1 Antioxidant Activity

The DPPH inhibition results of *Daucus carota canopy* L ethanolic extract (100, 200 and 300 µL, 1mg/ml) when compared with standards Butylated Hydroxyl Anisol and Tert-Butylated Hydroxyl Quinone are explained in Table 1, were

the 300 µL show a maximum scavenging activity with inhibition % 87.97 ± 0.12, while, the 200 and 100 µL showed inhibition % 86.96 ± 0.26 and 85.93 ± 0.14 respectively.

In general, we can demonstrate that the three concentrations of *Daucus carota canopy* L extract had potent antioxidant activity, more than Butylated Hydroxyl Anisol (with% inhibition 68.2), and less than Tert-Butylated Hydroxyl Quinone (with % inhibition 98.2). Polyphenolic compounds are a class of antioxidant agents which proceed as free radical terminators [14]. The ethanolic extract (300 µL, 1mg/ml) of *Daucus carota canopy* L had the maximum antioxidant activity proved that the phenolic compounds in it are referred to its antioxidant activity of this plant. Many studies have confirmed the radical scavenging properties of the plant phenolic compounds and ensured the relationship between phenolic compounds and antioxidant activity [15].

4.2 Cytotoxic Activity

The in vitro anticancer activities of *Daucus carota canopy* L ethanolic extract and the new compound were evaluated against breast cancer cell line (MCF7) in comparison with Cisplatin as standard drug. The drug dose which decreases survival to half (IC_{50}) was determined in this study. The results for ethanolic extract, new compound and that for Cisplatin are shown in Figs. 1, 2 and 3.The results showed that the *Daucus carota canopy* L ethanolic extract has cytotoxic activity, as its IC_{50} is 20.3 µg/ml, and the new compound has IC_{50}: 8.05 µg/ml, while that for the standard (Cisplatin) is 5.48 µg/ml.

4.3 Analgesic Activity

The acetic acid writhing test and hot plate methods in mice proved that the central mechanism of compound giving analgesic effect. Hot plate method acts to improve the brain efficacy so it is helping in producing the centrally acting anti-nociceptive response that principally focus on effects related to the spinal cord level. By using hot plate method on mice, *Daucus carota canopy* L ethanolic extract revealed anti-nociceptive properties at the high dose (200 mg/kg), which significantly ($P< 0.05$) elevated hot plate time at 30, 45 and 60 min. in comparison with the control group (Table 3), in which the analgesic activity was indicated at the dose of 200 mg/kg. On using the acetic acid writhing test, acetic acid-induced abdominal constriction which is a sensitive method to assess analgesics which acting peripheral [16]. Additionally, acetic acid causes pain because of the liberation of endogenous substances e.g. histamine, serotonin, bradykinins, prostaglandins (PGs), and substance P, end products [17]. Table 3 revealed that on using acetic acid method, we got the same results, by comparing the high dose 200 mg/kg of the plant ethanolic extract with the control and the other groups, the extract showed analgesic effect as the number of writhing response is decreased significantly ($P<0.05$) by this comparison. Analgesic effect showed in this study may be due to the presence of the previously isolated 12 naturally flavones [9]. From the previous study, it could be suggested that Daucus carota canopy L ethanolic extract might be used to relieve pain.

Table 1. DPPH inhibition (%) of different concentrations of *Daucus carota* extract in comparison with BHA and TBHQ

Drug	DPPH inhibition (%)
Standard antioxidant (BHA)	68.2
Standard antioxidant(TBHQ)	98.2
Daucus carota L extract, 100 µL,1mg/ml	85.93 ± 0.14
Daucus carota L extract, 200 µL,1mg/ml	86.96 ± 0.26
Daucus carota L extract, 300 µL,1mg/ml	87.97 ± 0.12

Table 2. Effect of *Daucus carota canopy* L extract on hot plate reaction in mice

Group	Dose (mg/kg) P.O.	0 mint	15 Mint	30 mint	45 Mint	60 mint
Group I	Vehicle	11 ± 0.37	12 ± 0.56	16 ± 0.63	13 ± 0.63	12 ± 0.76
Group II (Diclofenac sodium)	10	13 ± 0.42*	20 ± 1.28*	19 ± 0.97	13 ± 0.63	15 ± 0.97
Group III (extract)	100	13 ± 0.63*	16 ± 0.63	14 ± 0.84	11 ± 0.21	14 ± 0.97
Group IV (extract)	200	18 ± 0.42$^{*\bullet\Delta}$	23 ± 1.69$^{*\Delta}$	26 ± 2.01$^{*\bullet\Delta}$	26 ± 1.32$^{*\bullet\Delta}$	21 ± 1.90$^{*\bullet\Delta}$

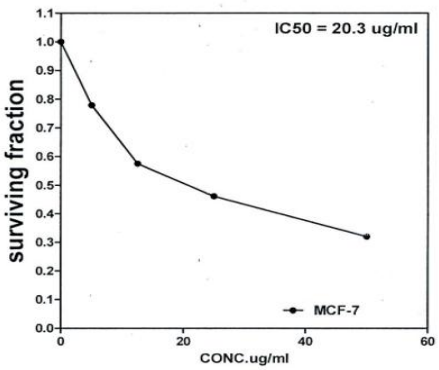


Fig. 1. Cytotoxic activity of the ethanolic extract of *Daucus carota canopy* L

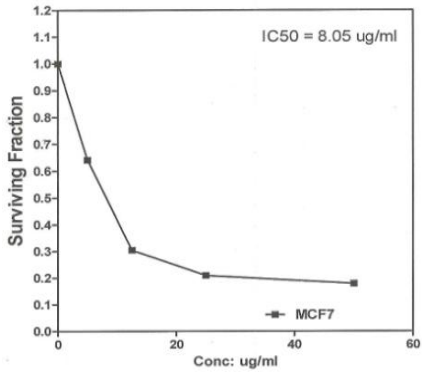


Fig. 2. Cytotoxic activity of the new compound extracted from *Daucus carota canopy* L

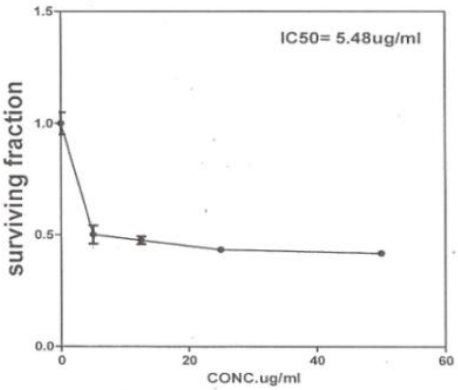


Fig. 3. Cytotoxic activity of the standard drug (Cisplatin)

Table 3. Effect of *Daucus carota* canopy L extract on acetic acid-induced writhing in mice

Treatment	Dose (mg/kg, .o.)[a]	Writhing (n)[b]	Inhibition (%)
Control (Vehicle, 10 ml/kg, p.o.)	…..	27 ± 0.42	……
Diclofenac	10	19 ± 0.45*	29.63
Daucus carota L Extract	100	22 ± 0.37*•	18.51
Daucus carota L Extract	200	15 ± 0.5*•Δ	44.44

[a] *Administered 30 min before 0.6% acetic acid (60 mgykg, i.p.).*
[b] *Counted for 20 min after acetic acid injection; values are mean"S.E.M.; n=6.*

5. CONCLUSIONS

It was finally concluded that the ethanolic extract (300 µL, 1 mg/ml) gave the highest effective antioxidant activity with inhibition % 82.09 ± 0.15 using radical scavenging method and its cytotoxic activity against MCF7 gave IC_{50} 19.4 µg/ml while for new compound 6,8-Di-C-α-L-rhamnoside diosmetin is 8.05 µg/ml compare to Cisplatin which its IC50 is 5.48 µg/ml. The anti-nociceptive properties for ethanolic extract (200 µL, 1 mg/ml) show that decreased number of writhes in acetic acid writhing test and increased the response time in hot plate method when comparing with the control group.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee".

COMPETING INTEREST

Authors have declared that no competing interests exist.

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