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In vitro antimicrobial activity of carrot callus extracts as affected by tyrosine and tryptophan precursor

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Abstract : Antimicrobial activities of crude ethanolic and water extracts of the calli cultures of *Daucus carota* were investigated. Stem, petiole and root derived calli were obtained on solid MS medium supplemented with 1 mg/l BAP + 2 mg/l NAA and fortified with different concentrations of tyrosine (Tyr) or tryptophan (Trp). The extracts exhibited antibacterial activities with zones of inhibition ranging from: 7 to 30 and 20 to 24 mm in stem callus; 16-32 and 21-27 in petiole callus; 15-20.3 and 5-15 in root callus for ethanol and water extracts respectively in case of gram-positive bacteria. On the other hand only ethanolic extract of stem cultures shows inhibition effect on gram-negative bacteria with zones of inhibition ranging from: 5 to 31 mm. The crude extract did not show any antifungal activity against *Aspergillus niger* strain. Moreover, the zones of inhibition exhibited by the extracts against the test yeast species ranged from 11 to 35 and 12 to 26 mm in stem callus for ethanol and water extracts respectively. Petiole callus extract shows an inhibition activity ranged from13 to21.6mm only with ethanol extract. Addition of 200mg/l tryptophan declared the highest inhibition zone of all calli cultures in ethanolic extract of stem callus (35 mm) followed by petiole callus (32 mm) and root cultures (20.3 mm).
Keywords: *Daucus carota*, crude extract, Antimicrobial activity.

Introduction:

Carrot (*Daucus carota* L.) is one of the most global well-liked root vegetables. It is an important crop of Apiaceae family with small, mostly white flowers set in umbrella-like inflorescence[1]. A long time ago, carrots were applied for health care purposes and regularly used in human nourishment[2]. This vegetable is regarded as a healthy food due to the presence of carotene, vitamins along with adequate fiber content[3, 4, 5, 6,7].

Plants naturally are an affluent supply of active ingredients with healing potential to improve human fitness with controlled hostile effects[8]. Such ingredients has an imperative pharmacological effects and consequently a broaden global market. Many anti hepatotoxic, cardio tonic, nutraceuticals, sweeteners, food additives and animal feed[9,10]. Plants were evaluated in regard to their main bioactive modules which are responsible for different biological activities in conventional and current curative philosophy[11,12, 13].

Plant scientists were pay more attention for plant tissue culture technology as a source of phytochemicals [14]. Furthermore, different callus cultures had established under suitable conditions to enable the in vitro production of antimicrobial substances[15,16]. The antimicrobial activity of extracts from callus cultures of

different plant species has been published. Regarding the commercial application, only few plant cell lines were succeed, particularly those derived from undifferentiated cell cultures[17,18,19].

The aim of this study was to determine the in vitro anti-microbial activity of crude ethanol and aqueous extracts from carrot callus cultures against selected bacteria, fungus and yeast strains.

Material and methods

Plant material and Callus induction:

Seeds of *Daucus Carota* were washed with soap and current tape water, then surface sterilized in a 70% (v/v) Et-OH for 1 min. and followed by 75% Clorox solution of household bleach (5.25 % sodium hypochlorite) with a drop of Tween-20 for 20 min with frequent agitation. After thorough washing in sterile distilled water, seeds were placed on basal MS-medium [20] supplemented with 0.7% (w/v) agar, 3% (w/v) sucrose for seed germination. The pH was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The cultures were incubated under controlled light regime (16-h photoperiod of fluorescent 45µ mol cool white light tubes and 8-h dark) at 25±1 °C. Within five weeks of cultivation, the *in vitro* growing carrot seedlings were used as a source of explants in further experiments.

Petiole, stem and root explants were excised from *in vitro* growing seedlings and divided into three segments before cultured on MS-medium supplemented with 1 mg/l BAP + 2 mg/l NAA. Cultures were kept in a growth room under the same conditions used for seed germination for three weeks and the initiated calli were sub-cultured on the same medium for maintenance and callus increment.

Precursor feeding:

Derived callus from different explants were re-cultured on the same MS-medium but addend with an amino acid precursor, tyrosine or tryptophan were added to culture medium prior to autoclaving at different concentrations (100 and 200 mg/l).The control medium was made without precursor additives. Cultures were incubated under a 16-h/day photoperiod.

Extraction procedure:

Hot extraction method:

fifteen gram of carrot callus was extracted in 100 ml of solvent (ethanol and distilled water) by soxhlet extraction technique overnight at respective solvent temperatures [21]. The obtained extracts were subjected to vacuum evaporator to evaporate excess solvents. After that, the dried crude extract yields were weighed and used for further experimental studies.

Antimicrobial activity

The antimicrobial activities were carried out according to the conventional agar diffusion test [22] using cultures of gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus thuringiensis*), gram-negative (*Psedumonas floureceans*, Escherichia coli), *Aspergillus niger*, *Saccharomyces cervisiae* and *Candida albicans*. The bacterial strains were cultured on nutrient medium, while the fungi and yeast strains were cultured on malt and yeast media, respectively. Broth media included the same contents except for agar. Bacteria and yeast were incubated for 24 h., and fungi for about 48 h. For preparation of inoculated plates, 1 ml of inoculant was added to 50 ml of agar media (50 ºC) and mixed by simple inversion. The agar was poured into 12 Cm Petri dishes and allowed to cool to room temperature. Five gram of each type of callus were extracted by ethanol or distilled water as described above and used as tested samples. Microorganism plates were undisturbed for 30 min to allow distribution of the tested callus extract of all samples (100µL) into the agar medium. The microbial growth inhibition zone was measured after incubation at 30ºC, at the appearance of clear microbial free inhibition zones, beginning within 24 h for yeast, 24-48 h for bacteria and 48-72 h for fungus. Antimicrobial activities were calculated as a mean of three replicates ± SE.

Results and Discussion:

In the present study, different explants of *Daucus carota* were grown on MS medium containing 1 mg/l BAP + 2 mg/l NAA for callus induction (Fig.1).

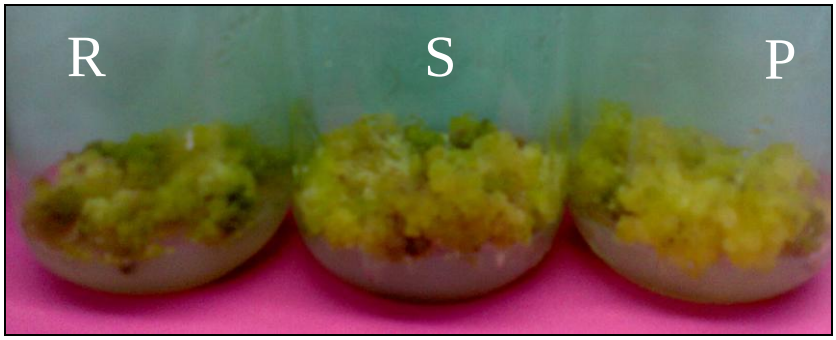

Figure 1: Callus derived from *Daucus Carota* different explants (R: root; S: stem; P: petiole), cultured on MS - medium supplemented with 1 mg/l BAP + 2 mg/l NAA after four weeks cultivation.

Derived cultures were used to extract the phytoconstituents of *D. carota* callus cultures after addition of tyrosine or tryptophan as precursors at different concentrations (100 and 200 mg/l). Table (1) shows the highest extraction yield with stem callus at control treatment (2.1 %) followed by ethanolic extracts (1.7 %) at 100 mg/l tryptophan precursor and lastly aqueous extracts (0.15%) at 100 mg/l tryptophan precursor. Phytoconstituents derived from root callus cultured on MS- medium fortified with either of tyrosine or tryptophan (100 and 200 mg/l respectively) recorded (0.99, 0.95, 1.2 and 1.0%, respectively) values with organic extraction and (0.09, 0.01, 0.12 and 0.05%, respectively) with aqueous extraction which are less than control (1.4, 0.23%, respectively). On the other hand, Extracts derived from petiole callus recorded values (0.96, 0.73, 1.1 and 0.87%, respectively) with ethanolic extraction ;(0.1, 0.09, 0.11 and 0.05%, respectively) with aqueous extraction and (1.2, 0.2%, respectively) with control.

It could be concluded that, additions of tyrosine and tryptophan to the culture medium were noticed to reduce the extraction yield percentage of organic and aqueous extracts compared with the control. Therefore, all previous result indicating that ethanol as protic organic solvent is more appropriate for extraction of carrot callus Phytoconstituents than water as protic non-organic solvent. Consequently, the difference in extraction yield may be attributed either to the solvent used for extraction and / or to the plant part used to initiate *Daucus carota* calli cultures.

Table (1): Extraction yield (%) of *Daucus carota* calli cultures derived from stem, root and petiole explants cultured on MS-Medium + 1 mg/l BAP + 2 mg/l NAA.

Callus Source	Treatment (mg/l)	yield (%; w/w)	
		Ethanol extraction	Aqueous extraction
Stem	Control	2.1	0.18
	100 Tyr	1.1	0.15
	200 Tyr	0.81	0.095
	100 Trp	1.7	0.16
	200 Trp	1.4	0.003
Petiole	Control	1.2	0.2
	100 Tyr	0.96	0.1
	200 Tyr	0.73	0.09
	100 Trp	1.1	0.11
	200 Trp	0.87	0.05
Root	Control	1.4	0.23
	100 Tyr	0.99	0.09
	200 Tyr	0.95	0.01
	100 Trp	1.2	0.12
	200 Trp	1.0	0.05

Where: - Control: precursor free medium; Tyr: tyrosine; Trp: tryptophan

To improve the production of active ingredients, numerous organic compounds were supplemented to the culture medium[23]. The concept is that an intermediate compound of a metabolic route, be expecting to raise the yield of end products[24].

Frequently used plants were shows a potential biological performance against a variety of microbial infections[25]. The basis for finding the natural products and their separation using different individual polar solvents is important for extraction of single compounds[26]. Many crude extracted compounds showed significant results in disease administration than those of a single isolated active fraction or purified ingredients [27]. The reaction of diverse groups of active ingredients in the crude extract possibly will enhance the curative achievement[28]. The available results deal with the fact that organic extracts offered extra influential antimicrobial activity as compared to aqueous extracts [29].

Antimicrobial activity of *Daucus carota* calli extract:

Extraction of *Daucus carota* calli cultures were examined to their antimicrobial activity against some Gram-positive bacteria as *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus thuringiensis;* Gram-negative bacteria as *Pseudomonas floureceans*, *Escheria coli*; fungus as *Aspergillis niger* and yeast as *Sccharomyces cervisiae* and *Candida albicans*.

1- Stem callus extracts:

Ethanolic extracts of stem callus cultured on MS-medium containing tyrosine or tryptophan were shown in table (2) to reveal their inhibitory effects as antimicrobial activity. Except for *Aspergillus niger,* stem cultures of *Daucus carota* extracted in ethanol inhibited all microbial strains under study. Gram-positive bacteria gave inhibition zone varied from 7 mm (*Bacillus thuringiensis;* control) to 30 mm (*Bacillus thuringiensis;* 200 mg/l tryptophan) with ethanolic extracts. Furthermore, the inhibition zones of Gram-negative bacteria were ranged from 5mm (*E. coli*; control) to 31mm (*E. coli*; 100 mg/l tryptophan). On the other hand, distilled water extract did not show any antibacterial or antifungal activity against almost all the tested strains (Table 2).

Table (2): Zone of inhibition produced by ethanolic and aquous stem extracts of *Daucus carota* calli cultures against some pathogens.

Extract	Microbial culture	Diameter of zone of inhibition (Mean ± SD) (mm)				
		Control	100 Tyr	200 Tyr	100 Trp	200 Trp
Ethanol	*Bacillus subtilis*	12±2.8	22±2.7	18±2.3	17±1.8	19±2.1
	Staphylococcus aureus	10±1.4	19±2.1	19±2.1	10±0.97	9±1.1
	Pseudomonas floureceans	15±1.1	21±1.8	16±1.9	18±1.4	17±1.3
	Escheria coli	5±0.74	15±1.1	10±1.2	31±2.9	22±2.7
	Bacillus thuringiensis	7±0.82	21.5±2.7	22±3.1	25±3.1	30±3.9
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	32±3.9	22.5±3.1	30±3.7	11±1.4	22±1.8
	Candida albicans	20±1.5	22.4±2.9	23.6±2.7	23.5±3.1	35±4.3
Aquous	*Bacillus subtilis*	-	-	-	-	-
	Staphylococcus aureus	-	-	-	-	-
	Pseudomonas floureceans	-	-	-	-	-
	Escheria coli	-	-	-	-	-
	Bacillus thuringiensis	20±1.9	21±2.7	22±1.8	22±2.7	24±2.9
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	12±1.5	20±2.3	14±1.5	17±1.2	26±2.2
	Candida albicans	20±1.9	15±1.1	16.5±1.5	17.5±1.9	19.4±2.1

Where:-

Control: precursor free medium; 100 Tyr: 100 mg/l tyrosine; 200 Tyr: 200 mg/l tyrosine; 100 Trp: 100 mg/l tryptophan; 200 Trp: 200 mg/l tryptophan; (-) No activity. Values are means of three replicates ± standard deviation.

Stem callus extract of *D. carota* showed a distinguished growth inhibition activity of *Saccharomyces cervisiae* (control; 32mm) compared with media containing precursor (tyrosine and tryptophan, 100, 200 mg/l of each) with ethanolic extraction. Maximum inhibiting activity for *Candida albicans* growth was observed with 200 mg/l tryptophan (35mm) when applying ethanol extraction followed by 200 mg/l tyrosine (23.6mm) then 100 mg/l tryptophan (23.5mm) and 100 mg/l tyrosine (22.4mm), while control recorded the minimum value (20mm). Distilled water extraction shows less inhibition activity of yeast strains compared with ethanolic one (Table 2).

2- Petiole callus extract:

Data in Table (3) revealed that the crude ethanolic extract of petiole callus showed growth inhibition for all microbial strains except *Pseudomonas floureceans, Escheria coli* and *Aspergillus niger.* Table 3 values revealed that the crude extract of petiole calli had a wide array of antibacterial activities against both gram-positive and gram-negative bacteria. Of the three gram-positive bacteria, 200 mg/l tryptophan showed a superior performance (32mm) against *Bacillus subtilis*. *Bacillus subtilis* was the most sensitive (32 mm) to the crude extract with 200 mg/l tryptophan, followed by L. *Bacillus thuringiensis* (23 mm) with 100 mg/l tryptophan, despite the fact that *Staphylococcus aureus* being the most resistant (21.5 mm) at control treatment. In general, the gram-positive bacteria were more susceptible to petiole crude extract than gram negative ones.

Table (3): Zone of inhibition produced by ethanolic and aquous petiole extracts of *Daucus carota* calli cultures against some pathogens.

Extract	Microbial culture	Diameter of zone of inhibition (Mean ± SD) (mm)				
		Control	100 Tyr	200 Tyr	100 Trp	200 Trp
Ethanol	*Bacillus subtilis*	30±3.4	27±3.1	25±2.8	24±2.4	32±3.7
	Staphylococcus aureus	21.5±2.8	18±2.1	16±1.9	17±1.6	19±2.2
	Pseudomonas floureceans	-	-	-	-	-
	Escheria coli	-	-	-	-	-
	Bacillus thuringiensis	22.3±2.9	22±2.9	20±2.1	23±2.7	21±2.4
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	15±1.7	21.6±2.2	15.3±1.8	13±1.4	20±2.6
	Candida albicans	20±2.4	15±1.7	16.5±1.3	17.5±1.9	19.4±2.3
Aquous	*Bacillus subtilis*	-	-	-	-	-
	Staphylococcus aureus	-	-	-	-	-
	Pseudomonas floureceans	-	-	-	-	-
	Escheria coli	-	-	-	-	-
	Bacillus thuringiensis	23±2.8	21±2.1	22.5±2.3	26±3.1	27±2.9
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	-	-	-	-	-
	Candida albicans	-	-	-	-	-

Where:-

Control: precursor free medium; 100 Tyr: 100 mg/l tyrosine; 200 Tyr: 200 mg/l tyrosine; 100 Trp: 100 mg/l tryptophan; 200 Trp: 200 mg/l tryptophan; (-) No activity. Values are means of three replicates ± standard deviation.

Zones (21.6 mm) of *Saccharomyces cervisiae* were recorded by ethanolic extracts of petiole callus grown on 100 mg/l tyrosine. The highest inhibitory activity for the growth of *Candida albicans* was observed in precursors-free medium (20mm) followed by 200 mg/l tryptophan (19.4mm) then 100 mg/l tryptophan (17.5mm) and 200 mg/l tyrosine (16.5mm) while, 100 mg/l tyrosine recorded the least inhibition value (15mm).

Of the two gram-negative bacteria, only *Bacillus thuringiensis* was appear to be sensitive (27 mm) to the aqueous crude extract of petiole callus at 200 mg/l Tryptophan. Aqueous extract did not show any antifungal activity regarding *Aspergillus niger* strains (Table 3).

3- Root callus extract:

The obtained results in Table (4) detailed that ethanolic extracts from root callus had no inhibiting activity for all tested microbes except *Bacillus subtilis* that showed an inhibition zones (20.3mm ;control) followed (19.5mm; 200 trp) then (18mm; 200 tyr). It is observed that 100 mg/l of both of tyrosine and tryptophan had a similar performance (15mm) of each. With less inhibition activity, the same pattern was noticed with *Bacillus subtilis* in case of distilled water extraction (Table 4).

Table (4): Zone of inhibition produced by ethanolic and aquous root extracts of *Daucus carota* calli cultures against some pathogens.

Extract	Microbial culture	Diameter of zone of inhibition (Mean ± SD) (mm)				
		Control	100 Tyr	200 Tyr	100 Trp	200 Trp
Ethanol	*Bacillus subtilis*	20.3±3.1	15±1.9	18±1.9	15±1.3	19.5±2.1
	Staphylococcus aureus	-	-	-	-	-
	Pseudomonas floureceans	-	-	-	-	-
	Escheria coli	-	-	-	-	-
	Bacillus thuringiensis	-	-	-	-	-
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	-	-	-	-	-
	Candida albicans	-	-	-	-	-
Aquous	*Bacillus subtilis*	5±0.8	11±1.2	12.8±1.4	11.5±1.1	15±1.9
	Staphylococcus aureus	-	-	-	-	-
	Pseudomonas floureceans	-	-	-	-	-
	Escheria coli	-	-	-	-	-
	Bacillus thuringiensis	-	-	-	-	-
	Aspergillus niger	-	-	-	-	-
	Saccharomyces cervisiae	-	-	-	-	-
	Candida albicans	-	-	-	-	-

Where:-

Control: precursor free medium; 100 Tyr: 100 mg/l tyrosine; 200 Tyr: 200 mg/l tyrosine; 100 Trp: 100 mg/l tryptophan; 200 Trp: 200 mg/l tryptophan; (-) No activity. Values are means of three replicates ± standard deviation.

Plant tissue and cell culture has great potential as an alternative source to conventional farming or wild plant harvest in the industrialized production of plant metabolites under controlled conditions [24].

The addition of amino acids to the nutrient media in plant cell and tissue culture is an important aspect for motivating plant cell growth. As well, the amino acids are playing a significant role in the biosynthetic pathway of the majority of secondary metabolites [30]. In the present study, tyrosine or tryptophan were proved to have a role in the antimicrobial activity of calli cultures of *D. Carota*. Similar trends were observed in *Taxus* cell suspension cultures [31].

Screening of crude plant extracts leads the way for detection of novel bioactive ingredients, and clarification of their structures to find out new synthetic medications [32]. Active ingredients derived from different plants serving as a prototype to develop less toxic and more effective drugs in controlling the growth of microorganism [33, 34, 35]. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or yeast. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds [36,37,38]. The search

for antimicrobials from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones.

In the present investigation, different crude extracts of *Daucus carota* was evaluated regarding their antimicrobial activity against certain Gram positive and Gram negative bacteria, fungus and yeast strains which was regarded as human pathogenic microorganism.

The results illuminated the sensitivity of Gram positive bacteria to carrot callus extracts than Gram negative ones, probably due to the differences in cell structure. Gram positive bacteria have more mucopeptide in their cell wall composition while Gram negative bacteria have only a thin layer of mucopeptide and most of their cell structure is lipoprotein and lipopolysaccharides. Thus, Gram negative bacteria are more resistant [39, 40]. Moreover, Tian et al. investigated the antibacterial effects of aqueous and ethanolic extract of *Galla Chinesis* plant native to China and they reported that Gram positive bacteria (*B. cereus*, *S. aureus*, *B. subtlis*) are more sensitive than Gram negative bacteria (*Escherichia coli*) to plant extracts. This result is consistent with the findings of this study[41].

Furthermore, it was reported that ethanolic extract was more effective and has a greater inhibitory effect compared to the aqueous extract[42]. Heidari-Sureshjani et al. also reported that ethanolic extract of *Kelussia odsoratissima* plant was exhibited broad spectrum activity against food borne and food spoilage bacteria, as compared to aqueous extract[43].

Discrepancy in chemical composition of callus tissues and intact plants has been described for number of plant species[44, 45, 46]. In the present research we suggest that the varied antimicrobial effect of the different tested extracts could be associated with the active ingredients present in each extract at a different concentration.

Conclusion:

Carrot is a valuable plant with wide range of antimicrobial activities. Evidently, there are no satisfactory information's to confirm the antimicrobial properties of carrot callus cultures. This study looks into the *in vitro* antimicrobial activity of these plants against eight pathogenic microorganisms that cause the most common cases of infectious diseases of disadvantaged areas. However, further works have to conduct for detection of antimicrobial ingredients accumulated in callus extracts and to carry out pharmacological studies.

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