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خاصیت ضدباکتریایی عصاره و اسانس سرخارگل در مقابل چند باکتری گیاهی

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چکیده سرخارگل *Echinacea purpurea* به دلیل خواص ضدمیکروبی‌اش همواره مورد توجه بوده است. برای ارزیابی اثر عصاره‌های آبی، متانولی، اتانولی، استونی و اسید کلریدریک و نیز اسانس گل این گیاه آزمون‌های بازدارندگی، تعیین حداقل بازدارندگی و کشندگی روی باکتری‌های گیاهی شامل *Rhixobiumtumefaciens*، *Bacillus turengiensis*، *Pectobacterium atrocepticum*، *Pseudomonas fleuorscens*، *Erwinia amylovora*، *Basillus subtilis* و *Rhodococcus faciens* انجام شد. آنتی بیوتیک جنتامایسین به عنوان شاهد مثبت و حلال خنثی دی متیل سولفوکسید به عنوان شاهد منفی قرار داده شدند. در آزمون زیست سنجی، برای هر باکتری آزمایش به صورت یک طرح کاملاً تصادفی با ۲۴ تیمار و سه تکرار در نظر گرفته شد. واکنش باکتری‌ها به عصاره‌های گیاهی سرخارگل متفاوت بود. در مجموع اثر بازدارندگی اسانس گل بیشتر از عصارهٔ اندام‌های مختلف سرخارگل بود. به علاوه، باکتری‌های گرم مثبت به ویژه *R. facience* نسبت به عصاره‌ها اثرپذیری چندانی نشان ندادند، هر چند اسانس گل بازدارنده، باکتری‌ایستا و حتی کشنده بودند. عصاره‌ها بیشتر از آن که باکتری‌کش باشند، باکتری ایستا بودند. تجمع مواد ضدباکتریایی در ریشه و گل بیشتر از ساقه و برگ بود. استون به عنوان کم اثرترین حلال در استخراج مواد ضدباکتریایی عصاره از اندام‌های گیاهی سرخارگل تشخیص داده شد. در کل به نظر می‌رسد سرخارگل از کارآیی بالقوه بالایی برای مقابله با باکتری‌های گیاهی و به ویژه گونه‌های مهاجم و بیماریزا می‌تواند برخوردار باشد.

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Antibacterial potential of purple coneflower extracts and essential oils against some plant-related bacteria

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ABSTRACT Purple coneflower (*Echinacea purpurea*) has been always considered for its antimicrobial potentials. To evaluate the effect of coneflower methanol, ethanol, acetone, HCl and aqueous extracts and also flower essential oils, the bioassay and minimal bacteriostatic and bactericidal concentrations testes were carried out on some plant related bacteria including *Pectobacterium atrosepticum*, *Pseudomonas fluorescens*, *Erwinia amylovora*, *Rhizobium tumefaciens*, *Bacillus subtilis* and *Rhodococcus faciens*. Gentamycin® and dimethyl sulfoxide nutritive solvent were considered as positive and negative controls, respectively. For each bacterium, experiment was considered as completely randomized design with 24 treatments and three replications. Each bacterium reacted in different way against coneflower various organs extracts and flower essential oils. On the whole, coneflower essential oils were more inhibitive than extracts. In addition, Gr^+ bacteria, especially *R. faciens*, did not get affected by coneflower extracts, however, essential oils were inhibitive, bacteriostatic and even bactericide. Antibacterials were accumulated in roots and flowers than leaves and stems. Acetone was the least effective solvent in antibacterials extractions. It seems coneflower has high potential for plant microbes' biocontrol.

Keywords:
- antimicrobial
- biocontrol
- *Echinasae purpureum*
- MBC
- MIC
- natural products

Introduction Coneflowers are perennial hardy herbs found in central or eastern north of America.[10] Purple coneflower (*Echinacea purpurea*) is main *Echinacea* species commonly used in about 80% of commercial medicinal coneflower products[8] as immunestimulants for conditions such as AIDS, cancer, and chronic fatigue syndrome, viral infections including influenza and the common cold.[8] Since Echinacea is a genus of the *Asteraceae* family, which is known to contain many plants rich in antibacterial polyynes and thiophenes,[6] such compounds might also have contributed to the activities observed. Echinacea has also been shown to have anti-inflammatory, antioxidant, anti-fungal, antiviral and antibacterial activities due to the presence of various active substances such as alkamides, polysaccharides, glycoproteins and derivatives of caffeic acid.[5,12,13,15] There is considerable early literature to suggest that Echinacea exerts have antibacterial activities both in clinical situations and laboratory studies.[1,2] In a recent study, a standardized Echinacea preparation administered daily to normal human subjects resulted in a few significant changes in the aerobic bacterial composition of fecal samples,[4] but the subject of direct antibacterial effects was not addressed. *E. angustifolia* roots or mixtures of *E. purpurea* roots and aerial parts especially ethanol formulations and aerial parts found to have antibacterial activity on certain bacteria, particularly against *Clostridium difficile, Streptococcus pyogenes, Haemophilus influenzae, Legionella pneumophila* and *Propionibacterium acnes.* The Gram-positive organism *P. acne* was sensitive only to *E. purpurea* root and shots in the presence of light. The Gram-positive *C. difficile* was variously sensitive to all extracts.[12] *Echinacea angustifolia* and *E. purpurea* are commonly used in North America for their anti-bacterial effects.[9] *Echinacea* spp. ethanol root and leaf extracts had antimicrobial similar broad activity against Gram positive (*Staphylococcus aureus, Bacillus subtilis, Enterococus faecalis, Clostridium sporogenes*) and Gram negative (*Klebsiella pneumoniae, Salmonella enteriditis, Escherichia coli, Pseudomonas aeruginosa*) bacteria.[14]

The objective of the study was finding antibacterial potential of coneflower extracts and essential oils against some plant-derived bacteria.

Materials and Methods

Bacteria and plant materials preparation

Plant related bacteria including Gram negative positive bacterial strains were received from Iranian Research Institute of Plant Protection (Table 1) and directly transferred on fresh nutrient agar medium incubated in 37 ºC for 24 hours. Purple coneflower was prepared from Medicinal Plant Farm of Ardebil located in Kalkhoran village, dried separately as stem, leaf, root and flowers in the shade laboratory condition, afterward. Extraction was carried out using solvents such as methanol 80%, ethanol 70%, acetone 50%, HCl 50% and water by maceration method. The plant materials separately were powdered using blender and 50 g added to 500 ml of solvent, kept for 48 hours in laboratory condition, shaking by hand time by time, and then filtrated from filter paper No. 1 Whatman. Solvent separation was carried out by Vacuum distillation in rotary set at 40 ºC and the condensed extracts were obtained.[3] The extracts were modified into dry extract by incubation at 45 ºC for 48 hours. Twenty g of dry extract were solved in 1 ml of dimethyl sulfoxide (DMSO) as inactive solvent and sterilized using 0.22 µ syringe filter. To flower essential oil preparation, 50 g of flower powder was used by distillation in water method for 4 hours in clevenger and dehydrated by sodium sulfate, kept at 4 ºC in dark glasses until the bioassay tests time.[7]

Disc diffusion agar method

Muller Hinton Agar (38 g/L) inoculated by 100 µL of 10^6 (CFU/ml) of each bacterium suspension, dispread equally and incubated for 20-25 minutes under the microbial hood for drying then 15 µL of coneflower extract or essential oils were

Table 1) Bacteria characteristics used in current study

Bacterium	acronym	Gram reaction	activity	plant disease related
Pectobacterium atrosepticum	*Pa*	Gr^-	plant pathogen	potato blackleg
Pseudomonas fluorescens	*Pf*	Gr^-	biocontrol agent	-
Erwinia amylovora	*Ea*	Gr^-	plant pathogen	pear fire blight
Rhizobium tumefaciens	*Rt*	Gr^-	plant pathogen	grape crown gall
Bacillus subtilis	*Bs*	Gr^+	biocontrol agent	-
Rhodococcus faciens	*Rf*	Gr^+	plant pathogen	leafy gall

added on sterilized paper discs located on the solid medium. Gentamicin 10 mg and blank discs immersed in 20 µL of DMSO[1] were considered as positive and negative controls, respectively. Each Perti dish containing three discs including treated, positive and negative control was located in a virtual circle on growth medium with 2 cm interval from the edge. The Petri dishes were incubated in 37 ºC for 24 hours and inhibition zone diameter was measured afterward.[11] For each bacterium, an individual experiment was considered based on completely randomized design with 24 treatments in three replications. Data were analyzed using SPSS ver. 16 and comparing means was done by Duncan multiple rages test at 5% probability level.

Minimal bacteriostatic and bactericidal concentrations (MIC and MBC) test

Nine test tubes for extracts and eight for essential oils containing 1 ml of Mueller Hinton broth (MHb) (21 g/L) were used for MIC test. Two experimental tubes containing both MHb and bacterium and the other one containing MHb only were considered as checks. The remaining tubes were filled by 1 ml of MHb. In the first tube, 1 ml of primary concentration of extracts (40 mg dry extract in 1 ml of DMSO) or essential oils (1 and 2 µg of essential oils in 20 µl of DMSO) were added and vortexed gently for 1 minute. From the first tube, 1 ml of homogenized MHb was transferred into the second tube containing 1 ml of MHb. The procedure was repeated for other tubes. One ml of the homogenized MHb in last time were evacuated. Therefore, the concentrations of 20, 10, 5, 2.5, 1.25, 0.625 and 0.312 mg/ml of extracts and 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 µL/ml of essential oils in 1 µL of MHb were obtained. Each bacterium was added to all tubes but the negative control and incubated at 37 ºC for 24 hours and the growth of bacterium were monitored. The concentration which had no bacterium growth weas considered as MIC. To determine MBC, bacteria were transferred to Mueller Hinton agar and stored at 37 ºC for 24 hours. The concentration with no bacterium growth was considered as MBC (Izadi *et al*. 2012; Shahidi *et al.* 2013).

Results and discussion

All coneflower organs including root, stem, leaf and flower extracts extracted by different solvents had more or less antimicrobial potential on studied bacteria but not on Gr^+ ones, showing the existence of antibacterial substances distributed all over the coneflower organs even in a different manner and amounts. Flower essential oils were also highly antibacterial against all bacteria in both applied concentrations. Flower essential oils applied in 2 µL concentration were significantly more inhibitive on bacterial growth than 1 µL but not against *Bs*.

Effect on *P. atrosepicum*

None of coneflower organs extracts could compete with Gentamicin® in *Pa* growth inhibition. However, flower essential oils were as inhibitive as Gentamicin® in 1 µL of concentration and significantly more growth inhibitive in 2 µL, showing a high potential of essential oils to be considered as a natural promising substances to the bacterium biocontrol. *Pa* can be considered as the most susceptible bacterium against coneflower extracts and essential oils. The only solvent with no effect on antibacterial extraction which is effective on *Pa* from plant organs was HCl. Also, essential oils highly effect on the growth of *Pa* as same as or more than gentamicin. Aqueous coneflower flower and root extracts was the most plant-solvent combination effected on *Pa* growth inhibition. Water seems to be able to release high amounts of antibacterials effective on *Pa* from all parts of coneflower, especially from flower and roots. The other extracts using ethanol, methanol, and acetone could be effective on antibacterial releasing from all coneflower organs effective on *Pa*. But regarding the economic considerations and also higher efficacy of water on plant extract preparation, water will be a good choice for plant extraction procedures in this regard (Table 2). All plant organs extracts and flower essential oils were bacteriostatic on *Pa* but no bactericidal effect can be observed in aqueous extract of any plant organs. Also, root acetone extract was the most bactericidal products affected on *Pa*. Leaf extracts were the less bactericidal on *Pa*. The most bactericidal extract was HCl leaf extract on *Pa*. Acetone was in the second place in plant antibacterial release effective on *Pa* after water,

[1] Dimethyl sulfoxide

suggesting the antibacterials involving would be more none-polar, regarding none-polarity of water and acetone, but there must certainly be the polars, due to ethanol and methanol extracts efficacy, too. All coneflower extracts especially root and flower ones were highly bacteriostatic on *Pa*. Also, root and flower extracts were more bactericide than others but not aqueous extracts showing water is not successful to extract antibacterials with potential of bacterium killing. Ethanol is the most successful solvent extracting killing antibacterials against *Pa* (Table 3). Coneflower essential oils in both concentrations were more bacteriostatic and bactericide against *Pa* (Table 4).

Effects on *R. tumefaciens*

Root and flower, also suggested being the place of antibacterial materials accumulation. Acetone was the weakest solvent which its extracts from all plant organs had no significant difference with check, with no antibacterial potential. None of plant materials derived from coneflower different organs was as inhibitive as Gentamycin® even in high concentration of essential oils. Also, only ethanol extract of stem had weak antibacterial property on *Rt*. The most effective plant material on *Rt* was essential oils in 2 µL concentration. Essential oils with 1 µL concentration had considerably less antibacterial potential on *Rt* than 2 µL**.** Also, aqueous extracts of coneflower leaf and root were more effective on *Rt* comparing to extracts provided with other solvents. All root and flower extracts extracted by all solvents but acetone was not successful in extraction of antibacterials. Only ethanol and HCL can act as good solvents from root and flower effective on *Rt* (Table 2). All coneflower extracts especially root and flower ones were bacteriostatic on *Rt*. All plant organs were the same in bacteriostatic potential on *Rt*. Also, the solvents act in the same way extracting bacteriostatic materials. Water and ethanol were the most successful solvents but acetone was the weakest one. Methanol and HCL acted in the same way in this regard. Also, acetone was not successful to extract antibacterials with potential of bacterium killing. Root and flower were the most killing coneflower extracts against *Rt*. Ethanol is the most successful solvent extracting killing antibacterials against *Pa* (Table 3). Coneflower essential oils in both concentrations were more bacteriostatic and bactericide against *Rt* (Table 4).

Effects on *P. fluorcens*

Coneflower stem seems to be the organs with least potential of antibacterial property on *Pf*. Only HCl stem extract was slightly inhibitive on *Pf* but other extracts. Also, apparently leaf extracts also were not effective on *Pf* with weakly effect of ethanol extract. Coneflower flower essential oils were more effective than extracts on *Pf*, collectively. Only aqueous root extract could be as inhibitive as 1 µL of essential oils. Acetone also was the weakest solvent to extract antimicrobials effective on *Pf* (Table 2). All coneflower extracts were bacteriostatic on *Pf*. All plant organs extracts possess bacteriostatic materials. However, only root and flower especially roots had bactericidal materials against *Pf*. Only methanol extract of flower was bactericide (Table 3). Coneflower essential oils in both concentrations were bacteriostatic and bactericide against *Pf* (Table 4).

Effects on *E. amylovora*

On *Ea* the most effective extract was surprisingly aqueous one of root but not flower essential oils of coneflower. On the whole, water seems to be a suitable solvent than others extracting antibacterials from root, stem and also flower of coneflowers effective on *Ea*. Flower and root are accumulative antibacterial place of the plant (Table 2). All coneflower extracts especially acetone, ethanol and HCL ones were bacteriostatic on *Ea*. However, only root and flower especially roots stem had bactericidal materials against *Ea* (Table 3). Coneflower essential oils in both concentrations were more bacteriostatic and bactericide against *Ea* (Table 4).

Effects on *B.subtilis and R. faciens*

Plant extracts had no inhibitive effect on these Gr+ bacteria. Only essential oils could be inhibitive on both bacteria more on *Rf* especially in 2 µL of concentration (Table 2). Based on MIC tests on *Bs*, extracts showed strong bacteriostatic potential with no bacteriocidal property. However, they were neither bacteriostatic no bactericide on *Rf* (Table 3). But essential oils had the same bacteriostatic and bactericidal effect on both bacteria. They were four times more bacteriostatic than bactericide. (Table 4).

Table 2) Inhibition zone diameter caused in bacteria colonies by coneflower various organs extracts in disc diffusion agar method

Bacterium	solvent	inhibition zone diameter (mm)							
		plant organs extracts				essential oils		Gentamycin	check
		root	stem	leaf	flower	1 µL	2 µL		
Pectobacterium atrosepticum	acetone	13.16 de	8.16 g	9.33 g	12.50 e	21.5 b	26.50 a	22.33 b	6.40 h
	ethanol	10.33 f	10.33 f	10.66 f	10.33 f				
	methanol	10.66 f	8.33 g	8.33 g	10.83 f				
	HCl	6.40 h	6.40 h	6.40 h	6.40 h				
	water	14.50 d	10.16 f	10.16 f	19.33 c				
Rhizobium tumefaciens	acetone	6.40 g	6.40 g	6.40 g	6.40 g	9.00 ef	13.00 b	19.00 a	6.40 g
	ethanol	10.66 cd	8.33 f	8.50 f	11.00 cd				
	methanol	8.66 f	6.40 g	6.40 g	10.33 cde				
	HCl	10.33 cde	6.40 g	6.40 g	10.16 cde				
	water	14.00 b	6.40 g	10.5 cde	11.66 c				
Pseudomonas fluorcens	acetone	6.50 d	6.40 g	6.40 g	6.40 g	10.33 d	14.33 b	24.33 a	6.40 g
	ethanol	10.33 g	6.40 g	6.40 g	9.33 c				
	methanol	10.16 f	6.40 g	6.40 g	10.33 d				
	HCl	8.16 f	8.33 f	6.40 g	8.33 c				
	water	12.66 de	8.16 g	8.16 f	6.40 g				
Erwinia amylovora	acetone	10.50 c	6.40 e	8.16 d	8.83 d	10.16 c	10.66 c	23.00 a	6.40 e
	ethanol	6.40 e	6.40 e	6.40 e	6.40 e				
	methanol	8.50 d	6.40 e	6.40 e	8.33 d				
	HCl	8.16 d	6.40 e	6.40 e	10.16 c				
	water	14.50 b	8.16 d	6.40 e	10.50 c				
Bacillus subtilis	acetone	6.40 c	6.40 c	6.40 c	6.40 c	10.16 b	10.33 b	21.00 a	6.40 c
	ethanol	6.40 c	6.40 c	6.40 c	6.40 c				
	methanol	6.40 c	6.40 c	6.40 c	6.40 c				
	HCl	6.40 c	6.40 c	6.40 c	6.40 c				
	water	6.40 c	6.40 c	6.40 c	6.40 c				
Rhodococcus faciens	acetone	6.40 d	6.40 d	6.40 d	6.40 d	11.16 c	16.00 b	23.33 a	6.40 d
	ethanol	6.40 d	6.40 d	6.40 d	6.40 d				
	methanol	6.40d	6.40 d	6.40 d	6.40 d				
	HCl	6.40 d	6.40 d	6.40 d	6.40 d				
	water	6.40 d	6.40 d	6.40 d	6.40 d				

Data followed by common letter(s) have no significant difference in 5% level of probability.

Table 3) Minimal inhibitory and bactericidal concentrations of coneflower various organs extracts with different solvents

Bacterium	solvent	MIC				MBC				MBC/MIC			
		root	stem	leaf	flower	root	stem	leaf	flower	root	stem	leaf	flower
Pectobacterium atrosepticum	acetone	0.75	1.25	1.25	0.75	1.25	-	20	5.00	1.7	-	16	6.7
	ethanol	0.75	0.75	1.25	0.75	5.00	5.0	20	10.00	6.7	6.7	16	13.3
	methanol	1.25	1.25	1.25	1.25	2.50	-	-	10.00	2.0	-	-	8.0
	HCl	0.75	1.25	0.25	0.75	-	5.00	10	2.50	-	4	8	2.0
	water	1.25	1.25	1.25	1.25	-	-	-	-	-	-	-	-
Rhizobium tumefaciens	acetone	2.50	2.50	2.50	2.50	-	-	-	-	-	-	-	13
	ethanol	0.75	0.75	0.75	0.75	10.00	20.00	20	10.00	13.0	26	26	16
	methanol	1.25	1.25	1.25	1.25	20.00	-	-	20.00	16.0	-	-	8.0
	HCl	1.25	1.25	1.25	1.25	20.00	-	-	20.00	8.0	-	8	26.0
	water	0.75	0.75	0.75	0.75	10.00	-	10	10.00	26.0	-	-	-
Pseudomonas fluorcens	acetone	0.75	1.25	0.75	0.75	-	-	-	-	-	-	-	-
	ethanol	1.25	0.37	0.37	0.37	2.50	-	-	-	2.0	-	-	-
	methanol	1.25	1.25	1.25	1.25	2.50	-	-	5.00	2.0	-	-	4.0
	HCl	0.75	1.25	0.25	0.75	5.00	-	-	-	4.0	-	-	-
	water	1.25	1.25	1.25	1.25	-	-	-	-	-	-	-	-
Erwinia amylovora	acetone	0.75	0.75	0.75	0.75	20.00	-	-	-	16.0	-	-	-
	ethanol	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
	methanol	5.00	5.00	5.00	5.00	-	-	-	2.50	-	-	-	0.5
	HCl	0.75	0.75	0.75	1.25	-	-	-	20.00	-	-	-	16.0
	water	2.5	2.5	2.5	2.5	10.00	-	-	20.00	8.0	-	-	8.0
Bacillus subtilis	acetone	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
	ethanol	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
	methanol	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
	HCl	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
	water	0.75	0.75	0.75	0.75	-	-	-	-	-	-	-	-
Rhodococcus faciens	acetone	-	-	-	-	-	-	-	-	-	-	-	-
	methanol	-	-	-	-	-	-	-	-	-	-	-	-
	ethanol	-	-	-	-	-	-	-	-	-	-	-	-
	HCl	-	-	-	-	-	-	-	-	-	-	-	-
	water	-	-	-	-	-	-	-	-	-	-	-	-

Table 4) Minimal inhibitory and bactericidal concentrations of coneflower flower essential oils

Bacterium	MIC		MBC		MBC/MIC	
	1 µL	2 µL	1 µL	2 µL	1 µL	2 µL
Pectobacterium atrosepticum	0.125	0.0625	0.25	0.25	2	4
Rhizobium tumefaciens	0.250	0.125	1.00	0.5	4	4
Pseudomonas fluorcens	0.125	0.125	0.25	0.5	2	4
Erwinia amylovora	0.125	0. 250	0.5	1.0	4	4
Bacillus subtilis	0.125	0.125	0.5	0.5	4	4
Rhodococcus faciens	0.125	0.125	0.5	0.5	4	4

Conclusions Plant related studied bacteria affected in differently from coneflower various organs extracts and flower essential oils. Collectively, essential oils were more inhibitive than extracts. Plant extracts had no effects on growth inhibition of Gr^+ bacteria including *Bs* and *Rf*, although flower essential oils was inhibitive, bacteriostatic and bactericide. All Gr^- bacteria were affected by coneflower extracts. Accumulation of antibacterial substances in flower and roots is highly suggested. On the whole, coneflower essential oils were more inhibitive than extracts. It seems coneflower has high potential for plant microbes' biocontrol.

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