INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 2, p. 349-356, 2015

RESEARCH PAPER OPEN ACCESS

Antibacterial activity of methanolic extracts from *Cotoneaster nummularioides*, *Cynodon dactylon* and *Cardaria draba* on typical food-borne pathogens

Fahime Yaghooti, Ali Mohamadi Sani*

Department of Food Science and Technology, Quchan Branch, Islamic Azad University, Quchan, Iran

Key words: Cotoneaster nummularioides, Cynodon dactylon, Cardaria draba, methanolic extracts, Antibacterial activity.

http://dx.doi.org/10.12692/ijb/6.2.349-356 Article published on January 27, 2015

Abstract

The present study aimed at evaluating the in vitro antibacterial activity of methanolic extract of *Cotoneaster nummularioides, Cynodon dactylon* and *Cardaria draba* against different pathogenic microorganisms. The agar disk diffusion method was used to study the antibacterial activity of *C. nummularioides, C. dactylon and C. draba* methanolic extracts against 2 gram-positive and 2 gram-negative bacteria at concentration 300 and 400 mg/ml. The results revealed that the methanol extract of *C. nummularioides* presented the highest zone of inhibition against tested pathogens (7-12 mm inhibition zones). Other plants did not show significant zone inhibition. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) were quantified by micro-dilution method. The leaf extract of *C. nummularioides* against *Bacillus cereus* (PTTC 1015) and *Staphylococcus aureus* (PTTC 1431) strains showed the best activities, with the lowest minimal inhibitory concentration (MIC) of 3.125 mg ml^{-1} and MBC was 102.08 and 108.33 mg ml^{-1} respectively for *Staphylococcus aureus* (PTTC 1431) and *Bacillus cereus* (PTTC 1015). The results showed that the methanol extract of the herb has antibacterial activity and therefore it could be used as a natural preservative ingredient in food and/or pharmaceutical industries.

* **Corresponding Author:** Ali Mohamadi Sani ✉ mohamadisani@yahoo.com

Introduction

The contamination of raw and/or processed foods with micro flora can take place at various stages from the production to the sale and distribution. Thus, food industry at present uses chemical preservatives to prevent the growth of food spoiling microbes. Due to the economical impacts of spoiled foods and the consumers concerns over the safety of foods containing synthetic chemicals, a lot of attention has been paid to naturally derived compounds or natural products (Sahin *et al.* 2004). Essential oils and extracts obtained from many plants have recently gained popularity and scientific interest (Haobin *et al.* 2009) and also Currently there is a growing interest to use natural antibacterial compounds, like essential oils and extracts of various species of edible and medicinal plants, herbs, and spices which have long been used as natural agents for food preservation in food and beverages due to the presence of antimicrobial compounds (Rahman *et al.* 2013).

These bioactive compounds are actually combinations of secondary products present in the plant. They have been used as food preservatives, pharmaceuticals, alternative medicines and natural therapies for centuries. These compounds are mostly alkaloids, steroids, tannins, phenolic compounds, flavonoids, resins and fatty acids. These compounds are odorous, complex, volatile compounds produced by special cells or groups of cells and concentrated in one particular region of plant such as the leaves, bark and stems (Ahmad *et al.* 2013).

The genus Cotoneaster (Rosaceae, Maloideae) consists of approximately 260 species in temperate regions of the northern hemisphere of which 19 occur in most regions of Iran, but its main distribution range includes Alborz Mts. and elevations in northwest Iran (Azerbaijan province) (Heravi *et al.* 2013). *C. dactylon* (L.) Pers. is a perennial grass belonging to family Poaceae that has a variety of medicinal properties (Singh *et al.* 2009). It is native to north and east Africa, Asia and Australia and southern Europe. In Ayurveda *C. dactylon* shows many pharmacological activities like antidiabetic (Jarald *et al.* 2008). *C.draba* (*Brassicaceae*; syn. *Lepidium draba* (L). Link), is native to western Asia, including Iran, and Eastern Europe and is an invasive species in North America, introduced by contaminated seeds in the early 1900s. It can be found in most parts of Iran, in fields and adjacent to water sources and in gardens and bare lands. (Miri *et al.* 2013).

The aims of the present study were to evaluate the potential antimicrobial activities of methanol extracts of *C. nummularioides, C. dactylon* and *C. draba* on typical food-borne pathogens.

Materials and methods

Chemicals and Plant materials

Gentamicin (Sina daroo, Iran), methanol and Dimethyl Sulfoxide (DMSO) (Merck, Germany) were purchased. The aerial part (leaves) of *C. nummularioides* was collected in May 2014 and aerial parts of *C. draba* was collected in May 2014 and also all organ of *C. dactylon* were collected in Avril 2014 from the mountains of North Khorasan Province in Iran. The plants were identified by the Research Center of Natural Products Health (NPH), North Khorasan University of Medical Sciences (Iran).

Extraction

The plant samples were dried at room temperature under shade (Umer *et al.* 2013), finely ground with a hammer mill, and the powdered sample from each plant was extracted with methanol (1.5 L) (Merck, Germany) for 48 hrs at room temperature (Seukep *et al.* 2013). The extracts were filtered through filter paper, afterwards extracts dried in vacuum at 40°C (Salvat *et al.* 2004) and were kept at 4°C until further uses (E Djeussi *et al.* 2013).

Organisms and Inoculation Conditions

Authentic pure cultures of bacteria were obtained from Persian Type Culture Collection (PTCC). They included gram positive bacteria; *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (PTCC 1431) and gram-negative bacteria; *Salmonella Entrica* (PTCC 1709), *Escherichia coli* (PTCC 1399). They

were maintained on agar slant at 4°C and sub cultured on a fresh appropriate agar plates 24 hrs prior to any antimicrobial test. Mueller Hinton Agar (MHA) was used for the activation of bacteria and the Mueller Hinton Broth (MHB) was used for the MIC determinations (Seukep *et al.* 2013). Finally, suspensions were adjusted to 0.5 McFarland standard turbidity. Bacterial suspensions were standardized to concentrations of 1.5×10^8 CFU ml^{-1} (Library of Congress Cataloging-in-Publication Data, 2005).

Antimicrobial assay

The Methanolic extract of *C. nummularioides, C. dactylon and C. draba* were tested for antimicrobial activity using agar disc diffusion technique to determine the diameter of growth inhibition zones while broth micro-dilution method was used to determine the MIC and MBC (Teke *et al.* 2013).

Disk-diffusion method

The agar diffusion assay was performed according to the modified Kirby-Bauer disc diffusion method (Selim *et al.* 2014). Methanolic extract were dissolved in dimethyl Sulfoxide (DMSO) to a final concentration of 100, 200, 300 and 400 mg ml^{-1} as stock solution and sterilized by filtration through 0.45 µm Millipore filters. The discs (6 mm in diameter) were (Ahmad *et al.* 2013; Rishikesh *et al.* 2012) immediately placed on the surface (Thompson *et al.* 2013) plates (Petri dishes, 80 mm diameters) containing a suitable medium (MHA) seeded with the test organisms (1.5×10^8). The amount of 15 µl of methanolic extract was poured onto discs. These plates were kept at low temperature (4°C) for 15 min to allow maximum diffusion (Rahman and Sultana, 2011). Negative controls were prepared using the same solvent employed to dissolve the extract (DMSO) (10 µl). Gentamycin used as standard antibiotic (positive control) (10 µl) (Assam *et al.* 2010). The test plates were incubated at 37°C for 24 hrs (Mhaske *et al.* 2011; Billah *et al.* 2013; Rishikesh *et al.* 2012). The test materials having antibacterial activity inhibited microorganism growth, and a clear, distinct zone of inhibition surrounding the discs was visualized (Billah *et al.* 2013). Antimicrobial activity was evaluated by measuring the zone of inhibition (Billah *et al.* 2013; Selim *et al.* 2014) ruler to an accuracy of 0.5 mm (Thompson *et al.* 2013) against the test organisms (Selim *et al.* 2014).

Minimum Inhibitory Concentration (MIC) Test

The antibacterial activity of extracts were tested using the micro-dilution antibacterial assay for the minimum inhibitory concentration (MIC) values (Fawole *et al.* 2012) and MBC (Haobin *et al.* 2009). The studied microorganisms included strains of (Mbveng *et al.*, 2012) *Bacillus cereus* (PTCC 1015), *Staphylococcus aureus* (PTCC 1431), *Salmonella enterica* (PTCC 1705) and *Escherichia coli* (PTCC 1399). MIC were determined by the broth micro-dilution method (Coccia *et al.* 2012) in a 96-wells micro-plate (Mbveng *et al.* 2012). All tests were performed in Mueller Hinton broth (MHB) (Haobin *et al.* 2009). The microorganism inoculum was standardized with appropriate culture medium (MHB) to a final concentration of (Coccia *et al.* 2012) 1.5×10^6 CFU ml^{-1} (standardized at 1.5×10^6 CFU ml^{-1} by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV-120-01 spectrophotometer) (Kuete *et al.* 2011). Each extract was dissolved in dimethyl Sulfoxide (DMSO) and added to MHB (Boussaada *et al.* 2008). The final concentration of DMSO was lower than 2.5% and does not affect the microbial growth (Mbveng *et al.* 2012). The extracts were serially diluted to give a concentration of 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg ml^{-1} (Dhiman *et al.* 2011). Then, 100 µl of each concentration was added in a well (96-well micro plate) containing 95 µl of MHB and 5 µl of inoculum (1.5×10^6 CFU ml^{-1}) (Kuete *et al.* 2011). The micro plate was incubated at 37°C ± 1°C for 24 hrs. Dilution of the extract corresponding to respective test organism showing no visible growth was considered as MIC (Umer *et al.* 2013). To determine MBC, 10 µl broth was taken from each well and inoculated in MHB for 24 hrs at 30 or 37°C for bacteria. The MBC is defined as the lowest concentration the methanol extracts at which inoculated microorganism was completely killed (99.99%) (Haobin *et al.* 2009).

Results and discussion

Results of disc-diffusion test

The results antibacterial activity of methanolic extracts determined by diameters of inhibition zones are presented in Table1. These results indicated that the diameters of inhibition zones varied from 6-12 mm and 19–29 mm for the various concentration of extracts and gentamycin respectively. Among the three extracts, the methanolic extract from aerial parts (leaves) of *C. nummularioides* had substantial of antimicrobial activity against 2 bacteria (*B. cereus* and *S. aureus)* species tested. On the other hand, the methanolic extracts from aerial parts of *C. draba* and all organ of *C. dactylon* plants showed no antibacterial activity and inhibition zone diameter and did not show antibacterial activity against all the tested bacterial strains et the 4 concentrations of 100, 200, 300 and 400 mg ml^{-1}. The maximal inhibition zones for bacterial strains, which were sensitive to the methanolic extract of *C. nummularioides* in the range of 7-12 mm respectively.

Table 1. Inhibition zone in diameter (mm) for methanol extract of *C. nummularioides.*

Microorganism	Concentration of methanolic extract (mg/ml)				Positive control	Negative control
	100	200	300	400	Gentamicin	DMSO
B. cereus	6	6	8	12	29	6
S. aureus	6	6	7	11	19	6
S. enterica	6	6	6	6	20	6
E. coli	6	6	6	6	21	6

Results of MIC and MBC

The MIC and MBC values of *C. nummularioides* summarized in Table 2 and results of aerial parts of *C. draba* and all organ of *C. dactylon* plants showed in Table 3 and 4, which shows that the methanolic extracts were able to prevent the growth of all the four studied microorganisms, including gram-positive and gram-negative bacteria, within the concentration range of 3.125 to 266.66 mg ml^{-1} the tested bacteria.

Table 2. MIC and MBC for methanolic extract of *C. nummularioides* (mg ml^{-1}).

Microorganism	MIC	MBC
B. cereus	3.125±0	108.33±61.11
S. aureus	4.16±1.38	102.08±65.28
S. enterica	75±33.33	400±0
E. coli	66.67±22.22	233.33±111.11

Table 3. MIC and MBC for methanolic extract of *C. draba* (mg ml^{-1}).

Microorganism	MIC	MBC
B. cereus	100±0	400
S. aureus	50±0	>400
S. enterica	166.66±44.44	>400
E. coli	200±0	>400

Discussion

Among methanolic extracts of *C. nummularioides, C. draba* and *C. dactylon*, the extract obtained of *C. nummularioides* showed stronger results and MIC values was ranges of 3.125- 66.667 mg ml^{-1}. While the strongest bacteria was *E. coli* (MIC=66.667 mg ml^{-1}) and the most sensitive bacteria against this extract plant was *B. cereus* (MIC= 3.125 mg ml^{-1}), But the lowest MBC values was for *S. aureus* (102.08 mg ml^{-1}). The lowest MIC values for *C. draba* and *C. dactylon* were respectively 50 mg ml^{-1} (*S. aureus)* and 16.67 mg ml^{-1} for *B. cereus* bacteria. MBC values methanolic extract of *C. dactylon* was 216.667 mg ml^{-1} for *B. cereus*. Among the extracts, the most antibacterial effect was obtained from the *C. nummularioides* extract.

Table 4. MIC and MBC for methanolic extract of *C. dactylon* (mg ml^{-1}).

Microorganism	MIC	MBC
B. cereus	16.67±5.55	216.667±122.22
S. aureus	33.33±11.111	>400
S. enterica	266.66±88.88	>400
E. coli	266.66±88.88	>400

Since pre-historic times, man has gone in different ways to search for cures and relief from various diseases by using numerous plants, plant products and plant-derived products. Recently, there is a scientific interest and a certain popularity with regard to screening essential oils and extracts from plants used medicinally all over the world. Historically, many plants essential oils and crude extracts have been used as topical antiseptics, or have been reported to have antimicrobial properties (Hossain *et al.* 2012).

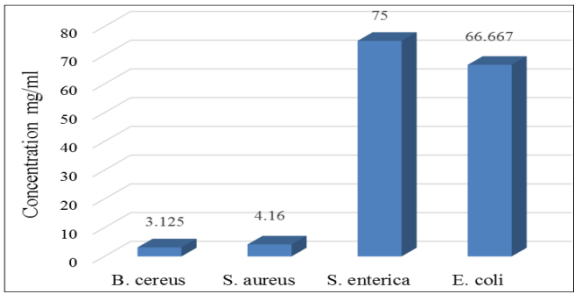

Fig. 1. MIC for methanolic extract of *C. nummularioides* (mg ml^{-1}) against different bacteria.

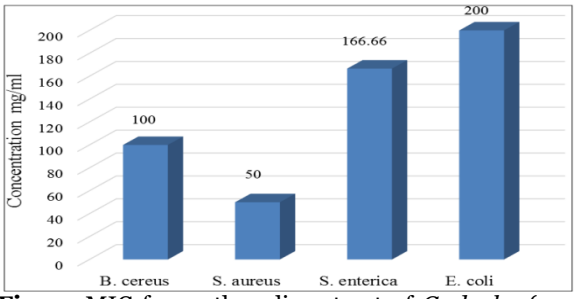

Fig. 2. MIC for methanolic extract of *C. draba* (mg ml^{-1}) against different bacteria.

The gram-positive bacteria were found to be more sensitive towards the plants methanol extracts than gram-negative bacteria. Antibacterial activity of MeOH extracts and its polar fractions could also be attributed to the presence of several types of compounds such as flavonoids and phenolic acids (Rahman *et al.* 2011). Generally, the higher resistance among Gram-negative bacteria could be ascribed to the presence of their outer phospholipidic membrane, almost mpermeable to lipophilic compounds. The absence of this barrier in Gram-positive bacteria allows the direct contact of the essential oils hydrophobic constituents with the phospholipids bilayer of the cell membrane, where they bring about their effect, causing either an increase of ion permeability and leakage of vital intracellular constituents, or impairment of the bacteria enzyme (Selim *et al.* 2014; Delamare *et al.* 2007).

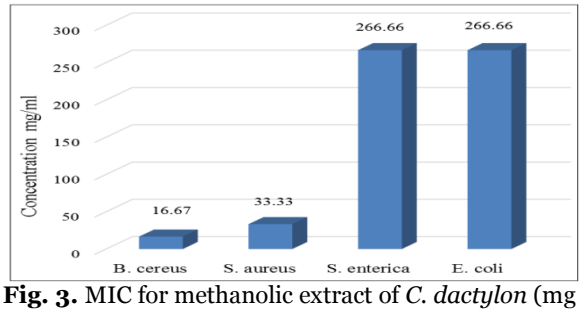

Fig. 3. MIC for methanolic extract of *C. dactylon* (mg ml^{-1}) against different bacteria.

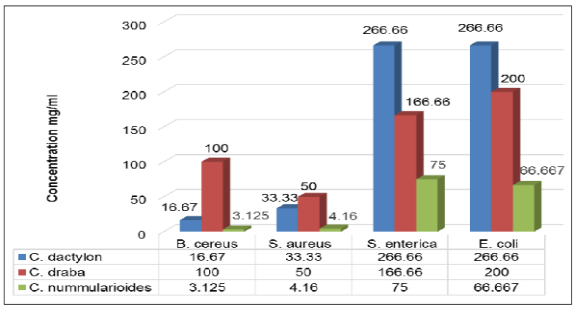

Fig. 4. Comparison Chart MIC (mg ml^{-1}) of methanolic extracts obtained from plants tested.

The results of the antibacterial screening showed that MeOH extracts of this plants had potential activity against some of the representative food-borne pathogens. Antibacterial activity of MeOH extract and its fractions could also be attributed to the presence of

several types of compounds such as flavonoids and phenolic acids (Rahman *et al.* 2011).

Conclusion

Among methanolic extracts of *C. nummularioides, C. draba and C. dactylon*, the extract obtained from *C. nummularioides* showed stronger results. The extract from this plant was showed antimicrobial activity against *S. aureus, B. cereus, S. enterica* and *E. coli* food borne pathogen. Therefore it can be concluded methanolic extracts of this plants especially *C. nummularioides* in appropriate combination, can act as an effective food preservative. Of course, this was the first study to compare the antimicrobial properties of methanolic extracts three plants on food-borne pathogens.

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