African Journal of Microbiology Research Vol. 6(14), pp. 3480-3484, 16 April, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1613
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Evaluation of antimicrobial activities of crude methanolic extract of pods of *Ceratonia siliqua* L. against some pathogens and spoilage bacteria

Anis BEN HSOUNA[1,2], Abdullah Sulaiman ALAYED[2], and Emad M. ABDALLAH[2]*

[1]Bio-pesticides Team (LPIP), Center of Biotechnology of Sfax, University of Sfax, P. B. 1177, 3018 Sfax, Tunisia.
[2]College of Science and Arts, Al-Rass, P. O. Box 53, Qassim University, Saudi Arabia.

Accepted 16 March, 2012

The antimicrobial activity of crude methanolic extract of *Ceratonia siliqua* (Carob) extracted from pods was tested on 13 different references and food isolated bacterial pathogens (8 Gram positive and 5 Gram negative) and 8 different references and food isolated fungal pathogens. Antimicrobial activities of the pods of *C. siliqua* were comparable to those of reference antibiotics (Gentamicin or Amphotericin B). These encouraging results require further investigating in order to discover new antimicrobial agents.

Key words: *Ceratonia siliqua*, antibacterial activity, antifungal activity, total phenolics, total flavonoids.

INTRODUCTION

Pathogenic micro-organisms, including fungi, Gram-positive and Gram-negative bacteria, have been recognised as the main causers of various human infections and food deterioration during storage and processing. To overcome these problems, a wide range of synthetic antimicrobial agents have been used as the sodium or calcium benzoate in food preservation (Mathur and Singh, 2005) or antibiotics in human infectious diseases (Casewell et al., 2003). Recently, the dramatically increasing resistance of micro-organisms towards these conventional antibiotics and food preservatives and their interactions with the food chain urging the need to develop additional natural antimicrobial agents as a public health priority (Chopra, 2007). Numerous numbers of medicinal plants prescribed in traditional medicine were found to have antimicrobial activities against several pathogens and some of them are multi-drug resistant, being a promising alternative source of natural antimicrobials (Abdallah, 2011). *Ceratonia siliqua* L., known in Arab countries as Carob, is a tree indigenous to Mediterranean region. This tree is belonging to sub-family Caesalpinaceae and family Leguminoseae (syn. Fabaceae) (Yousif and Alghzawi, 2000). Information regarding its applications in folk medicine in Arab countries is scanty. However, fruits, pods and leaves of this plant are sold in medicinal plant shops (Attars) all over the Arab counties. It is prescribed in Turkish folk medicine as anti-diarrhoeal and diuretic (Kivak and Mert, 2002). The gum of Carob tree is a galactomannan, a valuable natural food additive for products such as ice cream, sweets and soups. It is also used in the textile and cosmetics industries (Santos et al., 2005). The pods of the carob fruit has long been used as a feed for livestock and in human nutrition, including sweets, biscuits and processed drinks, because of its high sugar content and low price (Khair et al., 2001). To our best knowledge and literary survey, researches on antibacterial and antifungal activities derived from extract of pods of *C. siliqua* are scanty. The aim of this study was to determine the antimicrobial activities using well diffusion agar and broth microdilution methods of crude

*Corresponding author. E-mail: emad`100sdl@yahoo.com. Tel: +966 509949631.

methanolic extract of *C. siliqua* extracted from pods.

MATERIALS AND METHODS

Plant materials

Fresh pods of *C. siliqua* were collected from Chebba (Mahdia, Tunisia, latitude 35.23° and longitude. 11.11°) and a voucher specimen (LBPes C.S. 15.02) was deposited in the Center of Biotechnology of Sfax. The raw material was washed with distilled water and was grounded further to obtain a fine powder.

Preparation of extract

The pods of *C. siliqua* were extracted (100 g) with methanol. The sample was then filtered through filter paper in a Buchner funnel. The filtered solution was evaporated at reduced pressure (Rotary Evaporator Buchi R 200, Switzerland) at 40°C and then dissolved in appropriate solvent. The stock solutions were kept at 4°C in dark bottle until further analysis. The dry extract was stored at -18°C.

Determination of total phenolics and flavonoids content

Total phenolics content was determined using the Folin-Ciocalteu method (Waterman and Mole, 1994) adapted to a microscale. Gallic acid was used as standard. The absorbance of all the samples was measured at 760 nm using a Bio-Rad SmartSpecTM plus UV–Vis spectrophotometer and the results are expressed in mg of gallic acid equivalent per g of dry plant extract (mg GAE/g). The flavonoids content in extracts was determined spectrophotometrically according to Quettier-Deleu et al. (Quettier-Deleu et al., 2000). The flavonoids content was expressed in mg of quercitin equivalent per g of dry plant extract (mg QE/g).

Microorganisms and growth conditions

Bacteria and fungi were obtained from international culture collections (ATCC) and the local culture collection of the Centre of Biotechnology of Sfax, Tunisia. They included Gram-positive bacteria: *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6536, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 1880, *Listeria monocytogenes* (food isolate 2132) and Gram-negative bacteria: *Salmonella enterica* serotype *Enteritidis* (*Salmonella enteritidis*; food isolate), *Escherichia coli* ATCC 25922, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 10031.

The following fungal strains were also tested, *Aspergillus niger* CTM 10099, *Aspergillus flavus* (food isolate), *Aspergillus nidulans* (food isolate), *Aspergillus fumigatus* (food isolate), *Fusarium graminearum* (ISPAVE 271), *Fusarium oxysporum* (CTM10402), *Fusarium culmorum* (ISPAVE 21w) and *Alternaria alternata* (CTM 10230).

The bacterial strains were cultivated in Muller-Hinton agar (MH) (Oxoid Ltd, UK) at 37°C except for *Bacillus* species which were incubated at 30°C. Fungi were cultured on Potatoes Dextrose agar (PDA) medium and incubated at 28°C. Working cultures were prepared by inoculating a loopful of each test bacteria in 3 ml of Muller-Hinton broth (MH) (Oxoid Ltd, UK) and were incubated at 37°C for 12 h. For the test, final inoculum concentrations of 106 CFU/ml bacteria were used. Fungal spore suspensions were collected from the surface of such fungal colonies by gently scraping with a loop and suspended in 10 ml Potato Dextrose broth (PDB). This suspension was mixed vigorously by vortexing for 15 to 20 min. The spore suspension stock was diluted to obtain a concentration of 10^6 spores/ml (Measured by Malassez blade).

Antimicrobial screening

Antimicrobial activities of the extract of *C. siliqua* were evaluated by means of agar-well diffusion assay according to Güven et al. (2006) with some modifications. Fifteen millilitres of the molten agar (45°C) were poured into sterile petri dishes (Ø 90 mm). A working cell suspensions were prepared and 100 µl was spread onto the surface of the agar plates of Mueller-Hinton agar (Oxoid Ltd, UK) for bacteria and potatoes dextrose agar medium (Oxoid Ltd, UK) for fungi. Once the plates had been aseptically dried, 06 mm wells were punched into the agar with a sterile Pasteur pipette. The methanolic extract of pods of *C. siliqua* was dissolved in methanol/ water (1/9; v/v) to a final concentration of 50 mg/ml and then filtered through 0·22 µm pore-size black polycarbonate filters (Millipore). Thus, 80 µl were placed into the wells and the plates were incubated at 37°C for 24 h for bacterial strains and 72 h for fungi at 28°C Gentamicin (10 µg/wells) was used as a positive control in antibacterial tests while Amphotericin B (20 µg/wells) was used in antifungal activity assays. Negative control consisted of methanol/ water (1/9; v/v) which are used to dissolve the plant extract of *C. siliqua*. Antimicrobial activity was evaluated by measuring the diameter of circular inhibition zones around the well. Tests were performed in triplicates.

Determination of MIC and MFC

Minimum inhibitory concentrations (MICs) of methanolic extract of pods of *C. siliqua* were determined according to Gulluce et al. (2007) with minor modifications against a panel of 21 micro-organisms representing different species of different ecosystems. The test was performed in sterile 96-well microplates with a final volume in each microplate well of 100 µl. For susceptibility testing, 100 µl of Mueller-Hinton broth or potatoes dextrose broth was distributed from the second to the twelfth test wells. A stock solution of the methanolic extract was prepared by dissolving 100 µl of the extract in methanol and then adjusted to a final concentration of 50 mg/ml by Mueller-Hinton broth. The first well of the microplate was prepared by dispensing 160 µl of the growth medium and 40 µl of the essential oil to reach a final concentration of 10 mg/ml and then 100 µl of scalar dilutions were transferred from the second to the ninth well. Thereafter and from each well, 10 µl of the suspension were removed and replaced by the bacterial suspensions to final inoculum concentrations of 10^6 CFU/ml for bacteria and 10^5 spores/ml for fungus. The final concentrations of the extracts adopted to evaluate the antimicrobial activity were 0.039 to 10 mg/ml. The 10th well was considered as growth control containing Mueller-Hinton media for bacterial strains while potato dextrose broth (PDB) was used for fungi, since no essential oil solution was added. The plates were then covered with the sterile plate covers and incubated at 37°C for 24 h for bacterial strains and 72 h for fungi at 28°C. The MIC was defined as the lowest concentration at which the microorganism does not demonstrate visible growth after incubation. As an indicator of microorganism growth, 25 µl of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (0.5 mg/ml) dissolved in sterile water were added to the wells and incubated at 37°C for 30 min (Eloff, 1998). Where microbial growth was inhibited, the solution in the well remained clear after incubation with MTT.

Statistical analysis

Experimental results concerning this study were represented as

Table 1. Total phenolic and flavonoid contents of *C. siliqua* crude extracts.

Extract	Total phenolics (mg GAE /g)	Total flavonoids (mg QE/g)
Methanol	465.5 ± 1.3	24.6 ± 2.4

Each value represents the mean ± S.D. of three experiments. (mg GAE /g): mg of gallic acid equivalent per g of dry plant extract. (mg QE/g): mg of quercitin equivalent per g of dry plant extract.

Table 2. Antibacterial activity of the methanolic extract of pods of *C. siliqua* against foodborne, spoiling bacteria and determination of the Minimum Inhibitory Concentrations (MICs).

Bacterial strain	Inhibition zones diameter (mm)[a]		MIC (mg/ml)
	CsMetOH[b]	Gentamicin[c]	
Gram positive			
Bacillus subtilis ATCC 6633	15 ± 0.2	20 ± 0.2	0.625 ± 0.8
Bacillus cereus ATCC 14579	16 ± 0.3	20 ± 0.4	0.625 ± 0.5
Staphylococcus aureus ATCC 25923	20 ± 0.6	25 ± 0.8	0.312 ± 0.2
Staphylococcus aureus ATCC 6536	22 ± 0.8	16 ± 0.6	0.312 ± 0.4
Staphylococcus epidermis ATCC 12228	17 ± 0.1	20 ± 0.5	1.25 ± 0.8
Enterococcus faecalis ATCC 29212	15 ± 0.4	12 ± 0.2	1.25 ± 0.5
Micrococcus luteus ATCC 1880	19 ± 0.5	20 ± 0.7	0.625 ± 1.2
Listeria monocytogenes (food isolate 2132)	13 ± 0.4	15 ± 0.0	2.5 ± 0.9
Gram negative			
Salmonella enteritidis (food isolate)	10 ± 0.6	18 ± 0.8	2.5 ± 0.9
Escherichia coli ATCC 25922	12 ± 0.2	21 ± 1.0	1.25 ± 0.6
Escherichia coli ATCC 8739	11 ± 0.3	20 ± 1.2	2.5 ± 0.6
Pseudomonas aeruginosa ATCC 9027	10 ± 0.1	18 ± 0.7	2.25 ± 0.4
Klebsiella pneumoniae ATCC 10031	13± 0.2	12 ± 0.5	1.25 ± 0.8

Values are given as mean ± S.D. of triplicate experiment. [a]Diameter of inhibition zones of methanolic extract of pods of *C. siliqua* including diameter of disc 6 mm . [b]CsMetOH: *Ceratonia siliqua* methanolic extract from pods. [c] Gentamicin (10 µg/well).

mean ± S.D. of three parallel measurements. Analysis of variance was performed by Excel procedure.

RESULTS AND DISCUSSION

Currently, due to the dramatic failures of synthetic antibiotics to overcome the developing pathogens, medicinal plants emerge as alternative source for new natural antimicrobial agents (Abdallah, 2011). It is known that phytochemical compounds of medicinal plants such as alkaloids, flavonoids, phenols, glycosides, saponins, sterols etc. have curative properties (Mallikharjuna et al., 2007). In this investigation, the methanol extract of *C. siliqua* of pods exhibited presence of considerable amounts of total phenolics (465.5 ± 1.3 mg GAE /g) and Total flavonoids (24.6 ± 2.4 mg QE/g) (Table 1). Flavonoids and phenolic compounds are present in different quantities in most vascular plants (Taleb-Contini et al., 2003). They are a subject of medical research, being having may pharmacological benefits, including antioxidant, anti-inflammatory, antiallergic, hepatoprotective, antiviral, antimicrobial and anticarcinogenic activities (Najafi et al., 2010; Linuma et al., 1994; Manach et al., 2005). Interestingly, Flavonoides are also reported as promising antimicrobial against methicillin-resistant *S. aureus* (Linuma et al., 1994).

The result of the anti-bacterial activity tests of *C. siliqua* pods methanolic extract are presented in Table 2. The methanolic pod extract of *C. siliqua* showed significant antibacterial activity against all tested pathogens when compared to the reference antibiotic (Gentamicin). The most susceptible bacteria were *S. aureus* ATCC 6536, *S. aureus* ATCC 25923, *M. luteus* ATCC 1880, *Staphylococcus epidermis* ATCC 12228, respectively. Ranging Between 22 to17 mm Inhibition zone). Then *B. cereus* ATCC 14579, *B. subtilis* ATCC 6633, *E. faecalis* ATCC 29212, *K. pneumoniae* ATCC 10031 and *Listeria monocytogenes* (food isolate 2132), respectively (16 to 13 mm Inhibition zone). While, the least susceptible bacteria were *Escherichia coli* ATCC 25922, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027 and *S. enteritidis*

Table 3. Antifungal activity of the methanolic extract (concentration) of pods of *C. siliqua.*

Fungal strain	Inhibition zone (mm)	Amphotericin B[a]	MIC (mg/ml)
Aspergillus niger (CTM 10099	18 ± 0.3	15 ± 0.9	1.25 ± 0.4
Aspergillus flavus (food isolate)	14 ± 0.6	10 ± 0.3	1.25 ± 0.3
Aspergillus nidulans (food isolate)	13 ± 0.2	0	1.25 ± 0.8
Aspergillus fumigatus (food isolate)	12 ± 0.7	0	1.25 ± 0.1
Fusarium graminearum (ISPAVE 271)	15 ± 0.6	14 ± 0.5	0.156 ± 0.1
Fusarium oxysporum (CTM10402),	15 ± 0.4	14 ± 0.2	0.156 ± 0.2
Fusarium culmorum (ISPAVE 21w)	15 ± 0.3	12 ± 0.7	0.156 ± 0.3
Alternaria alternata (CTM 10230)	11 ± 0.2	14 ± 0.6	2.5± 1.2

Values are given as mean ± S.D. of triplicate experiment. [a] Amphotericin B (20 µg/well).

(food isolate) respectively (12 to 10 mm Inhibition zone).

Surprisingly, *S. aureus* ATCC 6536 was much susceptible to *C. siliqua* methanolic extract rather than the reference antibiotic Gentamicin. The MIC tests confirmed these findings (Table 2). Accordingly, pods of *C. siliqua* methanolic extract could be a promising antibacterial, it should be fractioned in order to extract its active ingredients which may be a candidate alternative of the sodium or calcium benzoate in food preservation and as antibacterial agent against some pathogens. Current findings are important, as most of these bacteria have been reported as multi drug resistant (MDR), such as *K. pneumonia* and *E. coli* (Wiener et al., 1999), *S. enterica* (White et al., 2001), *P. aeruginosa* (Paterson, 2011) and *S. aureus* (Diep et al., 2008).

In addition, the result of the anti-fungal activity tests of *C. siliqua* pods methanolic extract (Table 3) exhibited .significant antifungal activity against all tested fungi in comparison with the reference antifungal agent (Amphotericin B). The most susceptible fungi was *A. niger* (CTM 10099z), then *F. culmorum* (ISPAVE 21w), *F. oxysporum* (CTM10402), *F. graminearum* (ISPAVE 271*),* *A. flavus* (food isolate), *A. nidulans* (food isolate), *A. fumigatus* (food isolate). The least susceptible fungi was *A. alternata* (CTM 10230). It is notable that almost all tested fungal strains were much susceptible higher than that of the reference antifungal agent (Amphotericin B). It is known that, foods are prone to biodeterioration by moulds and fungi during post-harvest processing, transport, and storage (Chauhan, 2004). Contamination by *Aspergillus* species and with their respective mycotoxins is considered as a challenge for the pharmaceutical and food industries. Plant extracts are widely claimed to have a broad-spectrum antifungal activity and are considered as an important source for the search of lead compounds.

This is the first study on antimicrobial activity of pods of *C. siliqua* from Tunisia. Moreover, data regarding antimicrobial activity of this plant is scantly world wide. An investigation on leaves of *C. siliqua* from Turkey reported its significant effect on a broad range of microbial pathogens (Kivak and Mert, 2002). The findings of this study highlight its interesting antimicrobial activity which requires further investigations to isolate its active antimicrobial principles.

Conclusion

This study describes the antimicrobial activity of pods of *C. siliqua* (Carob), a popular plant in Tunisia of many applications in food and in traditional medicine. The methanolic extracts of its pods exhibited potent antimicrobial activity against a broad range of microorganisms. Its antimicrobial potency could be related to presence of phenolic compounds and flavonoids in considerable quantities in pods. Pods of *C. siliqua* are a promising source for new antimicrobials.

REFERENCES

Abdallah EM (2011). Plants: An alternative source for antimicrobials. J. Appl. Pharma. Sci., 1(6): 16-20.

Casewell M, Friis C, Marco E, McMullin P, Phillips I (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health, J. Antimicrob. Chemother., 52: 159–161.

Chauhan RKS (2004). Mycotoxins the “hidden killers” in food and feed: an overview and strategy for future. J. Ind. Bot. Soci., 83: 11–21.

Chopra I (2007). The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern?. J. Antimicrob. Chemother., 59(4): 587-590.

Diep BA, Chambers HF, Graber CJ (2008). Emergence of Multidrug-Resistant, Community-Associated, Methicillin-Resistant *Staphylococcus aureus* Clone USA300 in Men Who Have Sex with Men. Ann. Inter. Med., 148(4): 249-257.

Gulluce M, Sahin F, Sokmen M, Ozer H, Daferera D, Sokmen A, Polissiou M, Adiguzel A, Ozkan H (2007). Antimicrobial and antioxidant properties of the essential oils and methanol extract from *Mentha longifolia* L. ssp. longifolia. Food Chem., 103: 1449-1456.

Güven K, Yücel E, Çetintaş F (2006). Antimicrobial activities of fruits of *Crataegus* and *Pyrus* species. Pharmaceut. Biol., 44: 79-83.

Kivak B, Mert T (2002). Antimicrobial and Cytotoxic Activities of *Ceratonia siliqua* L. Extracts. Turk J. Biol., 26: 197-200.

Linuma M, Tsuchiya H, Sato M, Yokoyama J, Ohyama M, Ohkawa Y, Tanaka T, Fujiwara S, Fujii T (1994). Flavanones with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. J. Pharm. Pharmacol., 46(11): 892-895.

Mallikharjuna PB, Rajanna LN, Seetharam YN, Sharanabasappa GK

(2007). Phytochemical Studies of *Strychnos potatorum* L.f.- A Medicinal Plant. E-J. Chem., 4(4): 510-518.
Manach C, Williamson G, Morand C, Augustin SA, Rémésy C (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Rev. of 97 bioavailability studies, Am. J. Clin. Nutr., 81(1): 230S-242S.
Mathur S, Singh R (2005). Antibiotic resistance in food lactic acid bacteria, a review. Inter. J. Food Micro., 105 (3): 281-295.
Najafi S, Sanadgol N, Nejad BS, Beiragi MA, Sanadgol E (2010). Phytochemical screening and antibacterial activity of *Citrullus colocynthis* (Linn.) Schrad against *Staphylococcus aureus*. J. Med. Plant Res., 4(22): 2321-2325.
Paterson DL (2011). The Epidemiological Profile of Infections with Multidrug-Resistant *Pseudomonas aeruginosa* and *Acinetobacter* Species. Clin. Infect. Dis., 43(2): S43-S48.
Taleb-Contini SH, Salvador MJ, Watanabe E, Ito IY, Oliveira DCR (2003). Antimicrobial activity of flavonoids and steroids isolated from two *Chromolaena* species. Brazilian J. Pharma. Sci., 39(4): 403-408.
White DG, Zhao S, Sudler R (2001). The isolation of antibiotic-resistant *Salmonella* from retail ground meats. Engl. J. Med., 345: 1147-1154.
Wiener J, Quinn JP, Patricia A (1999). Multiple antibiotic-resistant *Klebsiella* and *Escherichia coli* in nursing homes. J.A.M.A., 281 (6): 517-523.
Yousif AK, Alghzawi HM (2000). Processing and characterization of carob powder. Food Chem., 69 283-287.