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Research Article

Screening of Antibacterial Activity of *Moringa oleifera* Against Pathogenic *Clostridium* spp.

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Abstract: The objective of this study was to evaluate the *in vitro* antibacterial activity of edible parts of *Moringa oleifera* against spore-forming bacteria associated with diarrhea such us *Clostridium difficile* and *Clostridium perfringens*. Ethanolic, methanolic, aqueous and acetone extracts in several presentations of *M. oleifera* (fresh leaf, leaf powder, whole seed powder and seed husk powder) were obtained. Broth microdilution method was used to analyze the activity and to determine the Minimum Inhibitory Concentrations (MICs) and the Minimum Bactericidal Concentrations (MBCs). A two-fold serial dilution of each extract was tested against *C. difficile* and *C. perfringens* during 48 h under anaerobic conditions. The broth microdilution analyzes revealed that red-stemmed leaf showed the lowest MICs (ranging from 3.9 to125 μg/mL), followed by whole seed (MICs 29.29-1875 μg/mL), green-stemmed leaf (MICs 39-2500 μg/mL) and seed husk (MICs 390.6-1562.5 μg/mL). The MBCs values were 1000-2000 μg/mL for red-stemmed leaf fresh, 1250-10000 μg/mL for green-stemmed leaf fresh, 6250-12500 μg/mL for red-stemmed leaf powder, 2500-25000 μg/mL for whole seed and 25000 μg/mL for seed husk. The study revealed that leaves and seeds in different concentrations, irrespective of their presentation, inhibited the growth of the tested strains to varying degrees depending on the solvent employed in extraction. Therefore, it may be concluded that *M. oleifera* may be a potential source for antimicrobial molecule (s) against pathogenic *Clostridium* spp.

Keywords: Antimicrobial effect, *clostridium difficile*, *clostridium perfringens*, leaf, microdilution, seed

INTRODUCTION

Moringa oleifera Lam. (Moringaceae), commonly referred to as "drumstick" is a small- to medium-sized tree, distributed in many countries of the tropics and subtropics. Several parts of this specie have nutritional, therapeutic and prophylactic properties and are used in traditional medicine in treating all sorts of diseases including diarrhea, one of the most common cause of morbidity and mortality worldwide (Fahey, 2005; Goyal *et al*., 2007; Arora *et al*., 2013; Gopalakrishnan *et al*., 2016). It is therefore important to evaluate available natural alternatives to currently used as anti-diarrheal drugs, which are not always free from adverse effects.

Literature suggested that some parts of this specie had potential as an anti-diarrheal agent. Some studies about these anti-diarrheal properties were carried out in animal models. Significant reduction in the severity and frequency of diarrhea were observed with aqueous, methanolic, ethanolic and hydroalcoholic leaves extract of *M. oleifera*, as well as methanolic root extract (Saralaya *et al*., 2010; Lakshminarayana *et al*., 2011; Choudhury *et al*., 2013; Misra *et al*., 2014).

The ethanolic, methanolic, chloroform, aqueous extract of leaves, seeds, root bark, fruits, flowers were also investigated against gram positive and gram negative bacterial and fungal pathogens, with variable antimicrobial activity depending on the solvent employed in extraction (Nikkon *et al*., 2003; Doughari *et al*., 2007; Rahman *et al*., 2009; Bukar *et al*., 2010; Vieira *et al*., 2010; Talreja, 2010; Sayeed *et al*., 2012; Oluduro, 2012; Kalpana *et al*., 2013; Zaffer *et al*., 2014; Fadeyi *et al*., 2015; Shailemo *et al*., 2016).

Even though the observed antimicrobial effects against *Bacillus* spp. there is no data about its activity against other spore-forming bacteria, like the genus *Clostridium* spp. Thus, the present study aims to evaluate the *in vitro* antibacterial activity of edible parts of *M. oleifera* against spore-forming bacteria associated with diarrhea such us *Clostridium difficile* and *Clostridium perfringens*.

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MATERIALS AND METHODS

Plant material: The fresh leaves (green-stemmed leaves and red-stemmed leaves) and seeds of *M. oleifera* were donated by the company Agronature 2000 S.L., Cartagena, Murcia, Spain. Leaves were cleaned with a chlorine solution (150 ppm) and then dried with a laminar air flow. To obtained leaf powder and seed powder, fresh leaves and seeds were dried at 40°C for 7 days and then ground into a fine powder (Karthy *et al*., 2009; Devendra *et al*., 2011).

Preparation of extract: Ethanolic, methanolic, aqueous and acetone extracts in several presentations of *M. oleifera* (fresh leaf, leaf powder, whole seed powder and seed husk powder) were obtained according to Karthy *et al*. (2009), Vijay and Samrot (2010), Onuoha and Alisa (2013) and Shailemo *et al*. (2016), with few modifications. The weighed amount of *M. oleifera* part (25 g of leaves or 40 g of seeds) were mixed with 100 mL extraction solvent and were macerated with intermittent shaking at room temperature (26°C) (Table 1). The percolates were filtered with filter paper (Whatman No. 1) and concentrated under vacuum at 40-60°C using arotary evaporator. The extracts were weighed, dissolved in the minimal volume of solvent and stored in a refrigerator at 4°C for antibacterial activity test.

Test bacterial strains and growth conditions: Typed strains of *Clostridium difficile* (CECT 531) and *Clostridium perfringens* (CECT 376) were obtained from Spanish Culture Collection Type at the University of Valencia, Spain. Test strains were routinely cultured on Brain Heart Infusion (BHI) agar (Oxoid) under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) at 37°C in an anaerobic chamber (bioMérieux) for 24-48 h.

Screening extracts for antibacterial activity: To determine the susceptibility of the strains to *M. oleifera* extracts, broth microdilution assays were performed according to the Clinical and Laboratory Standards Institute methodology (CLSI, 2010) with few modifications. Briefly, a series of two-fold dilutions of each extract were made in a 96-well microtitre plate in BHI broth. Each dilution was performed in duplicate wells. Suspensions of tested organisms cultured on BHI agar during 24 h were adjusted to 0.5 McFarland (1.0×10^8 CFU/mL) in 0.85% saline solution (bioMérieux) with the Densimat photometer (bioMérieux). Then it was inoculated in each well of microtitre plate to correspond to a final inoculum concentration of approximately 1.0×10^7 CFU/mL. Growth was measured spectrophotometrically by optical density at 600 nm (OD600) with Microplate Reader (BioTek) at 48 h after anaerobic incubation at 37°C. The difference in OD600 measurements in the control samples between 0 and 48 h of incubation was defined as 100% growth. Differences in OD600 measurements at 0 and 48 h of incubation in the tested samples were calculated as percentages of the value obtained from the control. The Minimum Inhibitory Concentration (MIC) was determined as the lowest extract concentration resulting in a growth reduction at least of 0.1% compared to the respective control samples. The Minimum Bactericidal Concentration (MBC) was determined as the lowest concentration of extract resulting in a growth reduction >99.9%. Each plate tested was performed by duplicate in separated experiments.

Statistical analysis: The values of MIC and MBC obtained were calculated from the median values of percentages from, at least, three duplicates.

RESULTS AND DISCUSSION

Minimum inhibitory and bactericidal concentrations of the different extracts against tested pathogens are shown in Table 2 and 3, respectively.

The inhibitory effect varied greatly according to the extraction solvent employed, as shown in data. Antibacterial activity of ethanolic extract, for both leaves and seeds, was observed. It should be noted that red-stemmed leaves were exhibited more antimicrobial activity than green-stemmed leaves, showing lower MICs and MBCs. The acetone extract the fresh green-stemmed leaf was also shown to be active at lower concentrations than ethanolic extract. However, the aqueous extract only showed antibacterial activity for whole seeds. Aqueous extract the fresh green-stemmed leaf was not active against *Clostridium* spp.

Table 1: Conditions of extract preparation

Plant part	Plant presentation		Extraction solvent	Sample	Maceration time	Solvent
Green-stemmed leaf	Fresh	15	Acetone	25 g sample + 100 mL solvent	18 h	Acetone
		3	Ethanol 95%	25 g sample + 100 mL solvent	7 days	Ethanol 95%
		6	Cold water	25 g sample + 100 mL solvent	7 days	DMSO 3%
Red-stemmed leaf	Fresh	8	Ethanol 95%	25 g sample + 100 mL solvent	7 days	Ethanol 95%
	Powder	20	Ethanol 95%	25 g sample + 100 mL solvent	7 days	Ethanol 95%
Whole seed	Powder	26	Water	40 g sample + 100 mL solvent	72 h	DMSO 3%
		24	Ethanol 100%	40 g sample + 100 mL solvent	72 h	Ethanol 50%
		29	Methanol 99.9%	40 g sample + 100 mL solvent	72 h	DMSO 30%
Seed husk	Powder	31	Methanol 99.9%	40 g sample + 100 mL solvent	72 h	DMSO 30%

Table 2: Minimum inhibitory concentration of *Moringa oleifera* Lam. leaf and seed extracts against *Clostridium* spp.

	Min. inhibitory concentration (µg/mL)				
	Leaves				
	Aqueous	Acetone	Ethanol		
Clostridium spp	Green-stemmed	Green-stemmed	Green-stemmed	Red-stemmed	Red-stemmed powder
Clostridium difficile	nd	39.06	156.25	3.91	48.82
Clostridium perfringens	nd	156.25	2500	125	48.82
	Min. inhibitory concentration (µg/mL)				
	Seeds				
	Aqueous	Methanol		Ethanol	
Clostridium spp	Whole seed powder	Whole seed powder	Seed husk powder	Whole seed powder	
Clostridium difficile	625	48.82	390.6	1875	
Clostridium perfringens	312.5	48.82	1562.5	29.29	

nd: No detected; Min.: Minimum

Table 3: Minimum bactericidal concentrations of *Moringa oleifera* Lam. leaf and seed extracts against *Clostridium* spp.

	Min. bactericidal concentrations (µg/mL)				
	Leaves				
	Aqueous	Acetone	Ethanol		
Clostridium spp.	Green-stemmed	Green-stemmed	Green-stemmed	Red-stemmed	Red-stemmed powder
Clostridium difficile	nd	2500	10000	1000	12500
Clostridium perfringens	nd	1250	10000	2000	6250
	Min. bactericidal concentrations (µg/mL)				
	Seeds				
	Aqueous	Methanol		Ethanol	
Clostridium spp.	Whole seed powder	Whole seed powder	Seed husk powder	Whole seed powder	
Clostridium difficile	7500	2500	25000	25000	
Clostridium perfringens	15000	2500	25000	25000	

nd: No detected; Min.: Minimum

at 50000 µg/mL, which was the highest concentration tested. The methanol extract of seeds also exhibited antibacterial activity and the whole seed showed lower concentrations than husk seed.

Antimicrobial effect of *M. oleifera* against *Bacillus* spp. another spore-forming genus, has been observed in literature, although there are few data about its MICs and MBCs (Nikkon *et al*., 2003; Rahman *et al*., 2009; Talreja, 2010; Sayeed *et al*., 2012; Zaffer *et al*., 2014; Ode and Abiodun, 2015; Shailemo *et al*., 2016). *Bacillus cereus*, *B. subtilus* and *B. megaterium* showed susceptibility to juice and ethanol extract of fresh leaves (Rahman *et al*., 2009). Aqueous extract of leaves also exhibited antimicrobial activity, contrasting with the strains tested in our study.

The observed antimicrobial effect of extracts could be explained by the rich composition of important phytoconstituents of *M. oleifera*. Compounds such as 4-(4′-O-acetyl-α-Lrhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate and 4-(α-Lrhamnopyranosyloxy) benzyl glucosinolate, as well as carotenoids, niaziridin and niazirin, have been attributed as the active antimicrobial principlesof *M. oleifera* (Caceres *et al*., 1991; Karim and Azlan, 2012).

This chemical composition of the plant could be influenced by geographical location, altitude or temperature. For this reason, further studies are necessary for chemical characterization and extraction of the active principles and comprehension of the mechanism of antibacterial action of *M. oleifera* extracts.

CONCLUSION

The extracts of the tested edible parts of *M. oleifera* showed varying degree of antibacterial activities against the tested bacterial species. Regardless extraction solvent, red-stemmed fresh leaves exhibited the greatest inhibitory effects. Whole seed showed more antibacterial activity than husk seed.

The ethanolic extract of leaves (especially from red-stemmed leaves) and seeds exhibited a good inhibitory effect. Traditionally, people use an alcoholic decoction of various herbs to cure the disease and according to the present study, preparing an extract

with this organic solvent provided a considerable antibacterial activity.

The inhibitory effect of *M. oleifera* extracts observed against pathogenic *Clostridium* spp. can introduce the plant as a potential candidate for drug for the treatment of infection caused by these pathogens, as well as a sanitizer or preservative in foods against these foodborne microorganisms often implicated in the spoilage of foods and foodborne illnesses.

CONFLICT OF INTEREST

The authors declare no financial, commercial or academic conflicts of interest.

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