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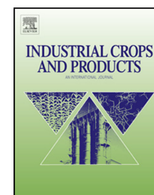

In vitro antimicrobial evaluation and phytoconstituents of *Moringa oleifera* pod husks

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ABSTRACT

Phyto-medicine is regaining interest owing to its advantages over conventional drugs and increasing cases of drug resistance. This work evaluates *in vitro* antimicrobial properties and establishes the active phytoconstituents of microbiologically unexplored *Moringa oleifera* pod husks against Gram positive, Gram negative bacteria and yeast pathogens. The MIC values varied with different organisms except in few cases such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium* 1, *Escherichia coli*, *Klebsiella pneumoniae* 1 which belong to the two major groups of bacteria where a similar MIC value of 400 mg/l was observed. Interestingly, the clinical isolate of MRSA exhibited the lowest MIC of 300 mg/l. Most of the Gram negative bacteria and *Candida tropicalis* were killed instantly at MIC concentrations ranging from 800 mg/l to 8000 mg/l. The acetone extract induced longer PAE ranging from 18.3 h to 20.3 h and 20 h to 21.6 h in some Gram positive such as *Staphylococcus epidermidis*, *Enterococcus faecalis* and Gram negative bacteria such as *Klebsiella pneumoniae* 2, and *Salmonella typhimurium* 2 respectively as compared to ciprofloxacin. The biosafety assays for mutagenicity and toxicity tested negative at 300 mg/l and 4000 mg/l concentrations. The major phytoconstituents established were alkaloids, flavonoids, tannins, diterpenes, triterpenes, and cardiac glycosides. Flavonoids and diterpenes, exhibited inhibitory properties against all test organisms except *Klebsiella pneumoniae* 2 and *Candida tropicalis*. In conclusion, the identified active phytochemicals have exhibited antimicrobial potential against a wide range of medically important pathogens including MRSA, a drug resistant bug. Hence, *M. oleifera* pod husks which are usually considered as agri-residues hold the potential for development of drugs or drug leads of broad spectrum activity including multidrug resistant bugs which are currently of great concern.

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1. Introduction

Phyto-medicine is regaining interest owing to the advantages of using readily available antimicrobial compounds from plant sources which are renewable in nature, less expensive, acceptable due to long history of use, fewer side effects, and better patient tolerance. It has been estimated that between 60% and 90% of the population of developing countries use traditional medicines almost exclusively and consider it to be a normal part of primary healthcare (WHO, 2002). Increasing bacterial resistance is prompting resurgence in research of the antimicrobial role of herbs against resistant strains (Alviano and Alviano, 2009).

The active phytochemicals in plants, especially fruits have been associated with numerous health benefits (Lachance and Das, 2007) and are used as ingredients in many pharmaceutical preparations today. There are a number of classes of secondary metabolites from fruits and vegetables that exert biological activities and can potentially be used to promote human health. These include alkaloids, amines, cyanogenic glycosides, terpenoids, flavonoids, glucosinolates, non-protein amino acids, phenylpropanes, polyacetylenes, polyketides, saponins and steroids (Thompson and Thompson, 2010). A vast number of medicinal plants have been recognized as valuable sources for such natural antimicrobial compounds and *M. oleifera* Lam. is one such species which belong to the family Moringaceae a monotypic of single genera with around 33 species of which 4 are accepted, 4 are synonym and 25 have not been assessed (The plant list, Version 1 (http://www.theplantlist.org)). The various extracts of its morphological parts such as seeds cotyledon, seeds' coat, stem bark, leaves, root bark have been reported to have antimicrobial potential (Arora et al., 2013). There is no major work on antimicrobial

Abbreviations: Ph, pod husks of *Moringa oleifera*; MIC, minimum inhibitory concentration; VCC, viable cell count; SA, *Staphylococcus aureus*; SE, *Staphylococcus epidermidis*; SF, *Shigella flexeneri*; ST, *Salmonella typhimurium*; EC, *Escherichia coli*; KP, *Klebsiella pneumoniae*; PA, *Pseudomonas aeruginosa*; EF, *Enterococcus faecalis*; MRSA, methicilin resistant *Staphylococcus aureus*; CA, *Candida albicans*; CT, *Candida tropicalis*.

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activity of pods' husks except other pharmacological potentials such as Cholesterol reduction (Mehta et al., 2003), hypotensive (Faizi et al., 1998), antiurolithiatic (Vijayalakshmi Satish et al., 2010), anti-inflammatory (Cheenpracha et al., 2010), hepatoprotective (Paliwal et al., 2011) and bioenhancer (Shanker et al., 2007). However, recently Onsare et al. (2013) have reported preliminary work on the antimicrobial activity of aqueous extract of pods' husks against Gram positive, Gram negative pathogenic bacteria and yeast strains. It's on this basis, the present work was designed to further establish antimicrobial potential of its organic extract, its safety, phytochemical groups responsible for the bioactivity which, to the best of our knowledge have not been reported earlier.

2. Material and methods

2.1. Test sample, chemicals and microbial cultures

The dry pods were collected from *M. oleifera* tree at the botanical garden of Guru Nanak Dev University, Amritsar India which had been identified and deposited in the Botanical Herbarium of the same University under Accession number 6746 HERB (Onsare et al., 2013). The chemicals and standard antibiotics used in this work were purchased from Sigma and Hi-Media, Mumbai, India.

The reference bacterial and yeast strains used in this study were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh – India. They were selected from Gram positive bacteria, Gram negative bacteria and Yeast strains to represent a broad spectrum of potential pathogens that pose significant threats in the medical field (Table 1).

The cultures were maintained on nutrient agar slants except *Enterococcus faecalis*, *Candida tropicalis* and *Candida albicans* which were maintained on trypticase soya agar (TSA), Sabouraud agar and yeast malt agar respectively. All these were sub-cultured regularly and preserved on solid media at 4 °C as well as in 10% glycerol suspensions at −20 °C and −80 °C.

2.1.1. Inoculum preparation

Each inoculum was standardized as follows; a loopful of 3–4 isolated colonies was inoculated into 5 ml of suitable broth and incubated at 37 °C to activate for 4 h base on previous studies (Kaur and Arora, 2008). This incubation period is optimal for obtaining cells which are at exponential phase since at this stage, there are not underlying factors which can give erroneous results in antimicrobial studies. These actively growing microbial cells were then adjusted with their respective broths so as to obtain a turbidity visually comparable to that of 0.5 McFarland standard which is prepared by mixing 0.5 ml of 1.75% (w/v) barium chloride dihydrate ($BaCl_2{\cdot}2H_2O$) with 99.5 ml of 1% (v/v) sulphuric acid (H_2SO_4). This turbidity is equivalent to approximately 1×10^8 colony forming units per ml (CFU/ml).

2.1.2. Sterilization of plant material

The dry pods were split open to remove the seeds and the husks were cut into small pieces (Fig. 1) and surface sterilized by soaking them in 1% mercuric chloride ($HgCl_2$) for 5 min and rinsing them 3–4 times using sterile distilled water and lastly drying at 50 °C. The dried plant material was ground into fine powder using an electric grinder.

2.2. Extraction of plant material and antimicrobial activity by agar diffusion assay

Seven organic solvents with increasing polarity such as hexane, petroleum ether, butanol, chloroform, acetone, ethyl acetate, methanol and water were used for extract preparation. Five grams of the sample was mixed with the known volume of solvent in the ratio of 1:20 (w/v) and extracted three times (for 24 h at ambient temperature (30 °C) with rotatory shaking at 80 rpm for each extraction). Each extracted material was vacuum filtered through Whatman filter paper No. 1 and the miscella (filtrate) thus obtained was combined and evaporated in a rotavapour and the residue was reconstituted in 6 ml of 30% DMSO (predetermined). The extracts were used for antimicrobial testing by agar diffusion assay. The organic extract (acetone) exhibiting maximum antimicrobial activity was selected for subsequent studies.

Table 1
Reference microbial strains and their clinical implications.

Organism	Reference	Clinical implication
Gram positive bacteria		
Enterococcus faecalis	MTCC 439	UTI[b], Endocarditis, Bacteremia, Meningitis (Hidron et al., 2008; Murray, 1990)
MRSA[a]	Clinical isolate	Skin infections (resistant to Beta-lactam antibiotics)
Staphylococcus aureus	MTCC 740	Staph infections in immune-compromised patients (Kluytmans et al., 1997)
Staphyloccocus epidermidis	MTCC 435	Nosocomial in immune-compromised patients (Otto, 2009)
Gram negative bacteria		
Escherichia coli	MTCC 119	UTI, neonatal meningitis and rarely haemolytic-uremic, peritonitis, mastitis, septicaemia (Todar, 2007)
Klebsiella pneumoniae 1	MTCC 109	Bacteremia and Metastatic infection (Ma et al., 2005; Tsay et al., 2002)
Klebsiella pneumoniae 2	MTCC 530	
Pseudomonas aeruginosa	MTCC 741	Nosocomial infection in immune-compromised patients (Pier and Ramphal, 2005)
Salmonella typhimurium 1	MTCC 98	Enteritis, typhoid fever, salmonellosis (Holden, 2002)
Salmonella typhimurium 2	MTCC 1251	
Shigella flexneri	MTCC 1457	Shigellosis (Clemens et al., 1999)
Yeast strains		
Candida albicans	MTCC 227	Oral and genital infections (dEnfert and Hube, 2007)
Candida tropicalis	MTCC 230	Candidiasis/candidaemia (Rajendra et al., 2010)

[a] Methicillin-resistant *Staphylococcus aureus*.
[b] Urinary tract infection.

2.3. Minimum inhibitory concentration

The MIC of the acetone extract of *Moringa oleifera* pod husks was determined by agar dilution method (Arora and Kaur, 1999). A stock solution of 20,000 mg/l was prepared and dispensed into suitable agar plates in varying concentrations (0.4–4 mg ml/l). The plates were inoculated with 0.1 ml of a 4 h activated test organisms (adjusted to 0.5 McFarland standard) and incubated for 24 h at 37 °C for bacterial strains and 24–48 h at 25 °C in case of yeast strains. The lowest concentrations exhibiting complete inhibition of the microbial growth was taken as the MIC. The experiment was performed in duplicate and repeated three times with standard antibiotics (Ciprofloxacin 6.7 mg/l and Amphotericin B 750 mg/l) as positive controls.

2.3.1. Total activity potency

Total activity potency Eloff, 2004 is the volume at which test extract can be diluted without losing the ability to kill

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