



ANTIMICROBIAL ACTIVITIES AND EVALUATION OF GENETIC EFFECTS OF MORINGA PEREGRINA (FORSK) FIORI USING MOLECULAR TECHNIQUES

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ABSTRACT: *Moringa peregrina* (Forsk) Fiori, *Moringa acaeaused* in folk medicine. In the present work, ethanol extracts of different parts of this plant (leaves, seed coat and seed endosperm) along with some standard antibiotic were assessed against three Gram+ve bacteria (*Bacillus subtilius*, *Micrococcus luteus*, *Staphylococcus aureus*) and three Gram-ve bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* & *Klebsiella pneumoniae*). The effect magnitude of these extracts on tested bacteria strains were as follows: leaves extract>seed coat >seed endosperm respectively. Results showed a strong effect of all these parts on *Staphylococcus aureus*. The leaf ethanol extract exhibited remarkable antifungal activities on the tested fungi in order of sensitivity as *C. albicans*>*A.flavus*>*A.fumigatus*>*A.niger*. *C. albicans* was the most sensitive to all used *M.peregrina* parts. Antimicrobial activities of standard antibiotics showed an inhibitory effect on all tested bacteria. However the ciproflaxocin is more effective than streptomycin. The antifungal activities of standard antibiotics showed that the amphotericin is less effective than nystatin. The results of our antimicrobial assay revealed that the different parts extract of *M. peregrina* showed higher inhibitory activity on tested organisms than the standard antibiotics. The antimicrobial effect of *M. peregrina* leaves extract were explored at the molecular level, using random amplification of polymorphic DNA (RAPD) extracted from the control and treated *Staphylococcus aureus* and *Candida albicans*. The results demonstrate polymorphic band pattern for most treated microbes compared with the untreated strains. The present study suggested that *M. peregrina* leaves could have wide antimicrobial activity spectrum.

Key words: Medicinal plants, *Moringa peregrina*, antimicrobial, mutagenicity, RAPD, crude extract.

INTRODUCTION

Medicinal plants are from the most advantageous source of life saving drugs for humans, animals and plants. Bioactive compounds usually extracted from plants are used as medicines, food additives, dyes, insecticides, cosmetics, perfumes and fine chemicals. In some Asian and African countries, 80% of the population are reliant on medicinal plants to maintain their health and to cure their ailment [41]. Synthetic antibiotics, known to enhance antibiotic resistance, which is not the case with natural agents [40]. The *Moringaceae* family consists of 10 species [37] or 12 [29] belong to only one genus called *Moringa*. [31] reported that the most common species are *Moringa peregrina* (forsk) fieri (syn. *Marabica* (Lam.) Pens., *M. arborea verde* (Kenya) *Moringa zeylanica* Sieb; *Balanus myrepsica* Blackm), *Moringa gastenopetala* Cufod, *Moringa borziana* Mattel, *Moringa galongituba* Engl, *Moringa concanensis* Nimmo, *Moringa gaovalifolia* Dinter and A. Berger, *Moringa gadrouhardii*, *Moringa gahildebrandtii*. All plant parts of *Moringa gaceae* family are used in folk medicine. But however a precise elucidation of the beneficial properties this family has not been completed. Leaves of *Moringa* species known to have various biological activities, including antitumor, antioxidant, anti-inflammatory/diuretic, anti-hepatotoxic properties, hypotensive, hypercholesterolemia and hypoglycemic actions [38]. Previous studies have reported that various parts of *Moringa* roots, flowers, bark, and stem including seeds possess antimicrobial properties [26, 6]. Seed extracts of *Moringa oleifera* found to have antimicrobial properties [21, 22]. However, a little review is available on the antimicrobial properties of *Moringa peregrina*. Thus, the present study try to explore the antimicrobial activities of different parts extracts of this species on some bacterial and fungal species and evaluate its genetic effects, at the molecular level using molecular genetics techniques.

MATERIALS AND METHODS

Collection and processing of plant materials

Moringa peregrina leaves and seeds were collected in May 2012 from Alboware, (23°56' 39.36"N 39°15'36.37"E) 80 km. northern Almadinah, Saudi Arabia.

Species status of this plant was fervid at Faculty of Sciences Herbarium, King Abdulaziz University, Jeddah. The samples were brought to the laboratory, thoroughly washed in running tap water to remove debris and dust particles and then rinsed in distilled water for five minutes, then air-dried under room temperature until constant weight.

Extracts preparation

Ten grams of dried *Moringia peregrina* parts (leaves and seeds) were catted into small pieces by blender 1-2 mm. Extraction was done by adding 100 ml of ethanol (1:10W/V) left under cold conditions for 48h, then the extracts were filtered through a whatman filter paper(No.1). The extracts solutions were evaporated under reduced pressure at 40°C until dryness, subsequently; the extract was diluted by dimethyl sulf. Oxide (DMSO) and stored under 20°C until analysis according to [8].

Bacterial and fungal strains cultures were prepared for in vitro antibacterial assay of the six Bacteria strains, three Gram positive:

Bacillus subtilis (ATCC11774); *S.aureus* (ATCC29213) and *Micrococcus luteus* (ATCC4698) and three Gram negative *Escherichia coli* (ATCC8739); *Klebsiellapneumonia* (ATCC700603) and *Pseudomon-as aeruginosa* (ATCC27853).

Those strains were provided by Microbiologics® USA. Tested organisms were subcultured on nutrient agar (Oxoid laboratories, UK) slopes. For the antifungal assay, four fungi (*Aspergillus flavus* (ATCC200026); *Aspergillus fumigatus* (ATCC204305); *Aspergillus niger* (ATCC1015) and *Candida albicans* (ATCC10231) were used. The tested organisms were subcultured on nutrient agar medium (Oxoid laboratories, UK) slopes for bacteria and Saboroud dextrose agar slopes (Oxoid laboratories, UK) for fungi were the media used. These stock cultures stored in the dark at 4°C until used.

Antimicrobial activity

Antimicrobial activity was determined using the agar well diffusion assay method as described by Holder and Boyce [19]. DMSO was used as a negative control.

The plates were organized in triplicate. Bacterial cultures were incubated at 37°C for 24 h

Antimicrobial activity was determined by measuring the inhibition zone [4].

DNA extraction and RAPD – PCR amplification conditions.

Genomic DNA was prepared from 18h cultures in exponential phase in Luria–Bertani medium (1000 mL deionized water, 10 g Bactotryptone, 5 g Bacto yeast, 5 g NaCl several drops, 5 M NaOH several drops and 1 M HCl) . Aliquots of 10 ml of each bacterial culture were harvested by centrifugation at 12.000 rpm for 15 min at 4 °c and washed once in sterile distilled water. The pellets were re suspended in 400 µl of lysis buffer containing 2% glucose, 50 m M Tris – HCl (pH 8.0), 25 m M EDTA, 3 mg / ml lysozyme and 200mg / ml RNase. The cell suspension was incubated for 1h at 37°C. Further DNA extraction was performed as described by Sambrook [34]. PCR amplification was carried out in a DNA thermocycler (Biomatra, Germany) for 30 cycles each. The PCR reaction was carried out in a final volume of 25 µL with 1X PCR buffer containing 10 mM Tris – HCl, 25 mM MgCl₂, 1 ml of Template DNA, 0.2 mM deoxy nucleoside triphosphate, 1 to 2 µM (each) primer and 0.5 u of Taq DNA polymerase (Promega). PCR conditions consisted of initial denaturation at 95°C for 2min followed by 95°C for 1min, annealing to primers at 37°C for 1 min, and extension at 72°C for 1 min with a final extension step at 72°C for 5 min. PCR – amplified products were separated using agarose gel electrophoresis in 1% TBE buffer (Tris base 108 g, boric acid 55 g, 0.5M EDTA 20 ml and H₂O₂ to 1 L) dilute accordingly for 1X stock and stained with 0.2 µg /ml ethidium bromide according to Sambrook [34]. Amplified fragments were detected and photographed under UV light. Two random primers were synthesized from University of British Colombia (UBC), Vancouver, Canda (UBC 16, 28 and 89) and their sequences are presented in Tabulation. The random primer names and sequences that used for RAPD analysis are as follows Williams [42].

RAPD Primers	Primer Sequences (5-3)
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPB-06	TGCTCTGCC

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activity of ethanolic extracts of leaves, internal and external seeds of *Moringa peregrine* was assessed against three Gram +ve bacteria (*B.subtilis*, *M.lutues* and *S.aureus*), three Gram – ve bacteria (*E.coli*, *K.pneumonia* and *P. aeruginosa*) at concentrations of 200mg/ml, the leaves extract showed the highest activity against tested organisms followed by the external > internal seeds respectively (Table 1). It can be deduced that *Moringa peregrine* leaf ethanol extract had the broadest spectrum of activity on the tested bacteria.

The magnitude of its inhibition zones against the tested bacteria was as follows: *S. aureus* (27.67mm), *M. luteus* (23.67 mm), *B. subtilis* (20.00mm), *K. pneumonia* (26.67 mm), *P. aeruginosa* (20.67mm) and *E. coli* (19.67 mm) were more sensitive at 200 µl /plates concentration. Napoleon [32] also reported *Enterobacter* spp, *S. aureus*, *P. aeruginosa*, *S. Typhi* and *E. coli* to be sensitive to *Moringa oleifera* leaf ethanol extract at the same concentration. This is justified by the reports of Bukar [10] who indicated that *Moringa oleifera* leaf ethanol extract had activity against four bacterial isolates *Enterobacter* spp, *S. aureus*, *P. aeruginosa*, and *E. coli*. *Moringa perngina* leaf extracts exhibited remarkable antibacterial activities against the tested bacteria in the following sensitivity order *S. aureus* > *M. luteus* > *B. subtilis*. Within the Gram -ve bacteria sensitivity order was *K. pneumonia* > *P. aeruginosa* > *E. coli*. The present results coincided with those of Bukar et al., 2010, whom reported that *M. oleifera* leaf extracts were more potent than *M. oleifera* seeds extracts, which showed high activities against *S. aureus* (24.00mm) and *P. aeruginosa* (13.33mm) where by the internal seeds extract had the least antibacterial activity compared to other parts. *M. peregrina* internal seed extract was active against three bacterial isolates: *M. luteus* (13.33mm), *E. coli* (17.67mm) and *K. pneumonia* (16.33) but *B. subtilis*, *S. aureus* and *P. aeruginosa* were not sensitive. Of note that *P. aeruginosa* has the least sensitive to *M. peregrina* indicating that *M. peregrina* might have limited effect on the proliferation and activities of *P. aeruginosa* which well-known for its strong resistant [9]. The antibacterial properties of the leaf and seed of *M. peregrina* as shown in the present study corroborate the earlier claims by Foidi [14, 2]. The antimicrobial activity of *M. peregrina* seeds is due to the presence of an array of phytochemicals, but most importantly due to the activity of a short poly peptide and benzyl- iso thiocyanate [12, 16]. The peptide may act directly on microorganisms and results in growth inhibition by disrupting cell membrane synthesis or synthesis of essential enzymes [36, 39]. Antimicrobial activities of used standard antibiotics showed an inhibitory effect against all tested bacteria, but results showed that the ciprofloxacin was more effective than streptomycin. This goes in line with Shital [35]. The antifungal activity of different parts of *M. peregrina* extract compared to standard antibiotics are shown in (table 2). The leaf ethanol extract exhibited remarkable antifungal activities against the tested fungi in the following sensitivity order *C. albicans* > *A. flavus* > *A. fumigatus* > *A. niger*. *C. albicans* on one hand, was the most sensitive to all *M. peregrina* parts extracts compared with other tested fungi, on the other hand *A. niger* was the least sensitive fungi. The present results coincided with Dipali [11,17]. The leaves extracts showed the highest activity against tested fungi followed by external > internal seeds respectively (Table 2). The antifungal activities of standard antibiotics showed that the amphotericin is less than nystatin as appropriate antibiotics [18]. The results of our antimicrobial assay revealed that the extract of different part of *Moringa peregrina* showed better inhibitory activity against tested organisms compared with standard antibiotics [17].

Table 1. The antibacterial activity of different parts extract of *Moringa perngina* concentration 100 mg/ml compared to antibiotics against the tested bacteria.

	Gram positive bacteria			Gram negative bacteria			
	<i>B. subtilis</i>	<i>M. luteus</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>K. pneumonia</i>	<i>P. aeruginosa</i>	
	ATCC11774	ATCC4698	ATCC29213	ATCC8739	ATCC700603	ATCC27853	
Leaves	20.00±0.00	23.67±0.33	27.66±0.33	19.67±0.33	26.67±0.00	20.67±0.33	Between Strains SD=0.5735
Internal seed	0.00±0.00	13.33±0.33	00.00±0.00	17.67±0.33	16.33±0.33	0.00±0.00	
External seed	18.67±0.33	20.33±0.33	24.00±0.00	19.33±0.33	20.67±0.33	13.33±0.00	
Streptomycin	25.00±0.00	23.67±0.33	19.00±0.00	23.00±0.00	25.00±0.00	22.00±0.00	
Ciprofloxacin	34.00±0.00	46.00±0.00	38.00±0.00	44.00±0.00	42.00±0.00	42.00±0.00	
Total	19.53	25.40	21.73	24.73	26.13	19.60	
Std.Deviation	11.54	11.36	12.97	10.14	9.02	14.14	

Molecular characterization of *S. aureus* and *C. albicans* by RAPD analysis

Three random primers OPA-05, OPA-06 and OPB-06 were used for RAPD analysis of the tested bacteria.

The genetic effects of the ethanolic leaves extract of *M. peregrina*

Table 2. The antifungal activity of different parts extract of *Moringa perngina* concentration 100 mg/ml compared to antibiotics against the tested fungi.

Plant parts	<i>A.flavus</i>	<i>A.fumigatus</i>	<i>A.niger</i>	<i>C.albicans</i>	Between Strains LSD=0.57735
Leaves	23.33±0.58	22.67±0.58	18.67±0.58	24.67±0.58	
Internal seed	17.33±0.58	21.33±0.58	14.67±0.58	20.67±0.58	
External seed	21.67±1.15	22.33±0.58	17.67±0.58	22.67±0.58	
Amphotericin	29.00±0.00	25.33±0.58	24.33±0.58	27.33±0.58	
Nystatin	31.67±0.57	27.00±1.00	29.00±1.00	28.33±1.15	
Total	24.60	23.73	20.87	24.73	
Std.Deviation	5.35	2.25	5.34	3.01	

Table 3. RAPD analysis of the polymorphic amplified fragments of *S.aureus* and *C.albicans* isolates using three primers

Primer name	Fragment		<i>S.aureus</i>		<i>C.albicans</i>	
	No	Bs	Control	Treated	Control	Treated
OPA-05	1	1100	-	-	+	-
	2	900	-	-	+	-
	3	700	+	-	-	-
	4	590	+	-	+	-
	5	480	-	-	+++	+++
	6	400	+	Faint-	-	-
	7	370	-	-	+	-
	8	350	+	-	+	++
	9	300	+	-	-	-
	10	180	+	-	+	-
	11	120	+	-	+	-
Total	11		7	5	8	8
Percentage polymorphic band			63.64%	45.45%	72.73%	72.73%
OPA-06	1	1200	-	-	+	-
	2	890	-	-	+	-
	3	630	-	-	+	-
	4	460	+	-	+	-
	5	390	-	-	+	-
	6	430	+	-	-	-
	7	300	+	-	-	-
	8	290	-	-	-	-
	9	240	+	-	+	-
	10	210	+	-	-	-
	11	160	-	-	+	-
	12	110	+	-	-	-
Total	12		6	5	7	7
Percentage polymorphic band			50.00%	41.67%	58.33%	58.33%
OPB-06	1	940	-	-	+	-
	2	780	-	-	+	-
	3	700	-	So faint+	-	-
	4	600	-	-	-	-
	5	500	-	-	+	-
	6	410	-	-	-	-
	7	370	-	So faint+	-	-
	8	290	+	-	+	-
	9	150	+	-	+	-
Total	9		2	4	6	5
Percentage polymorphic band			22.22%	44.44%	66.67%	55.55%

The genetic effect of *M.peregrina* ethanolic leaves extract on *S. aureus* and *C.albicans* was investigated, as those two species exhibited the highest sensitivity to this extract. This was executed using RAPD analysis. The RAPD analysis results illustrated in (Tables 2 and Figure1) show polymorphic number of genetic bands, which were the electrophoretic products of PCR for treated *S. aureus* and *C.albicans* compared with those of untreated bacteria.

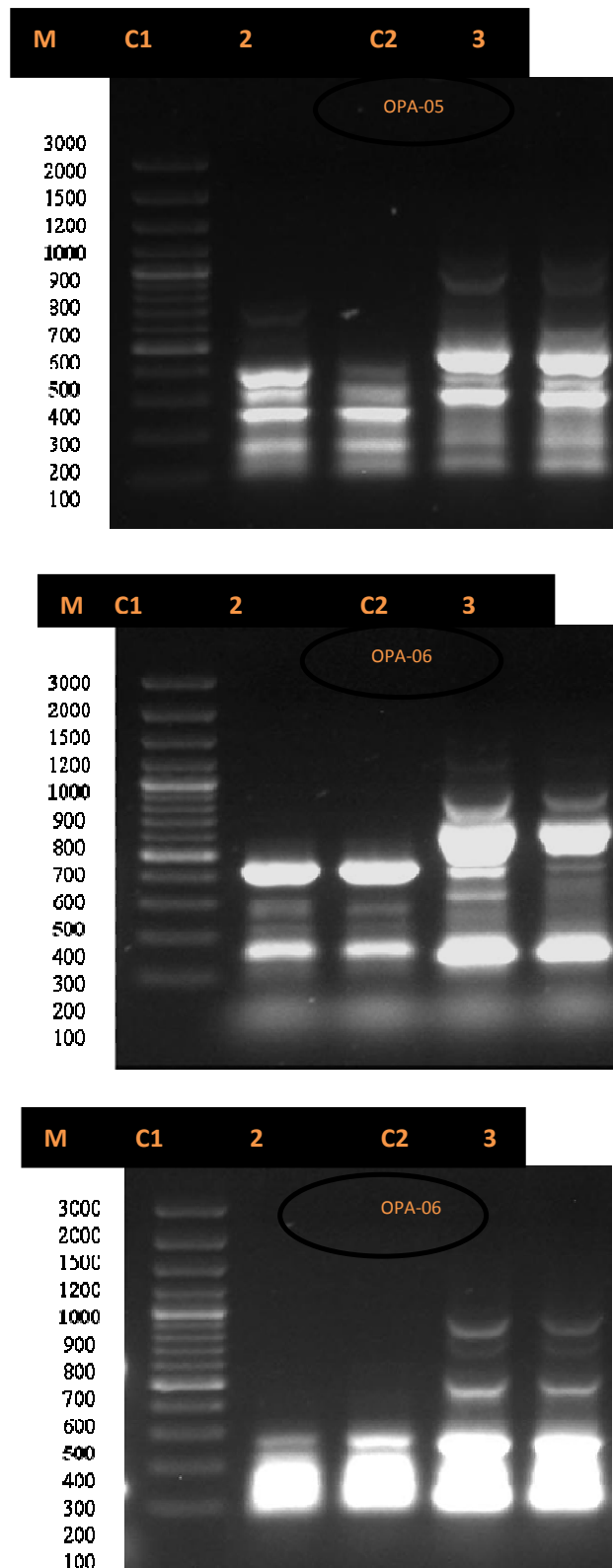


Figure 1: RAPD profile of *S.aureus* and *C.albicans* after the treatment with leaves extract of *Moringa perngina* : whereas;C1 ,untreated *S.aureus*;2,treated *S.aureus* ;C2, untreated *C.albicans* ;3 treated *C.albicans*

Table (1) showed that the highest number of polymorphic bands among treated *S. aureus* was generated in reactions with the primers OPA-05 and OPA-06 which was five genetic bands and represented 45.45% of the total bands. While, among treated *S. aureus* and *C. albicans*, the reaction with the primers OPA-05 resulted in the highest number of polymorphic bands (five and eight) that represent 45.45% and 72.73% of the total obtained bands (11) Table (2). The results of RAPD analysis revealed a polymorphic banding pattern when comparing the untreated *S. aureus* and *C. albicans* with those treated by *Morin gaperegrina* leaves extract (Figs.1 and 2). This observation gives good evidence to the ability of *Morin gaperegrina* leaves extract to induce point mutation as a result of deletion compromising at least one nucleotide as revealed by disappearance of many genetic bands and change in restriction endonuclease sites as compared with untreated *S. aureus* and *C. albicans*. These results suggested molecular changes as a deletion in one or more loci which affect gene expression and interruption in biochemical pathways of DNA and protein synthesis, consequently as Alkaloids in *Morin gaperegrina* leaves extract often do. These results are consistent with the results obtained by [1, 30, 15]. Some of the components of *R. stricta* may act as intercalation agent or generate free radicals which are interacted with plasmid DNA to account for the observed deletions, as suggested by similar results obtained by Ansah [5, 7] in their study with *Cryptolepis sanguinolenta* and *Aspergillus terreus*. However, the interpretation of the molecular events responsible for differences in the RAPD patterns is not an easy task since different DNA alterations can induce similar type of changes. [23]. Exposure of an organism to these plant extracts may result in the formation of covalently bound adducts between the chemical or its metabolites and the DNA; faulty repair of these adducts often results in mutations and, sometimes, cytogenetic changes. In this context, present results showed that the fragments were displayed in the control and disappeared in the treated bacteria strains. Therefore it can be stated that RAPD could be of great help in the explanation of the antibacterial mode of action of ethanol extract of *Morin gaperegrina* leaves extract on DNA synthesis. The effects seen in the present study may reflect a chemical and physiological change in tested *S. aureus* and *C. albicans* strains, this change of gene expression clearly showed the effect of change in nucleic acid, and may also reveal a new genotype [28]. Therefore more rigorous investigation is needed in this matter. Disappearing bands are likely to be due to changes in oligonucleotide priming sites, originated from rearrangements and less likely from point mutations and DNA damage in the primer binding sites [33, 24, 1, 25]. Total or partial changes in DNA sequence, due to mutation and/or large deletions create in new priming sites can be induced by effect-induced genotoxicity eg, see Enan [13] and Maryam [27]. Our findings support this claim that DNA polymorphisms detected by RAPD can be considered as a powerful biomarker assay for detection of the level of DNA damage in various treated *S. aureus* and *C. albicans* strains to ethanol extracts of *Morin gaperegrina* leaves, as suggested by similar results obtained by Adel [3] in their study on the effect of *Rhazya stricta* leaf extract and [20] in their study with *Conocarpus erectus*.

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