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Monitoring of Antimicrobial Activity of Essential Oils Using Molecular Markers

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ABSTRACT

Technological application of essential oils, as natural antimicrobial agents, to reduce the effect of pathogenic microorganisms, requires new methods of detection. The present work evaluated the parameters of antimicrobial activity of the essential oils of rosemary (*Rosmarinus officinalis*) on two pathogenic strains *Escherichia coli* and *Staphylococcus aureus*. The MBC and MIC values were of 2.5, 25 µl ml^{-1}, and values of 1.25 and 5 µl ml^{-1} for the two strains respectively. In this study, an attempt has been made to evaluate randomly amplified polymorphic DNA (RAPD) analysis for its potential to establish antimicrobial effect of rosemary essential oil. For the preliminary assessment, this study compared the effects occurring at molecular levels in *E. coli* and *Staph. aureus* exposed to rosemary essential oil at the MIC concentrations for the two organisms. The qualitative modifications arising in random amplified polymorphic DNA (RAPD) profiles as a measure of DNA effects were compared with control which showed many differences. In conclusion, the measurement of parameters at molecular levels is valuable for investigating the specific effects of agents interacting with DNA.

Key Words: RAPD-PCR, Rosemary essential oil, *Escherichia coli, Staphylococcus aureus*.

INTRODUCTION

In recent years there has been an increasing interest in the use of natural substances, and some questions concerning the safety of synthetic compounds have encouraged more detailed studies of plant resources. Essential oils are concentrated, hydrophobic liquid containing volatile aromatic compounds from plant. They posses a wide spectrum of pharmacological activities. The antimicrobial effects of essential oils have been documented and used in herbal medicine in many countries [1,2,3]. The main advantage of natural agents is that they do not enhance the "antibiotic resistance", a phenomenon commonly encountered with the long-term use of synthetic antibiotics.

Antibacterial effects of hydrous, methanolic and ethanolic extracts of clove, cinnamon, sage, thyme and rosmarinus on Gram- positive and Gram- negative bacteria had previously been investigated.

The results showed that all of these plants had antibacterial action on methicillin-resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* [4], but they were weakly active against Gram-negative bacteria such as *Pseudomonas aeruginosa* and enteropathogenic *Escherichia coli* [5]. Gram-negative bacteria are more resistant to antibiotics than the Gram-positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism [6].

To reduce health hazards and economic losses due to foodborne microorganisms, the use of natural products as antibacterial compounds seem to be an interesting way to control the presence of pathogenic bacteria and to extend the shelf life of processed food [7,8]. These compounds could be added during the food process. Among these products, essential oils from spices, medicinal plants and herbs have been shown to possess antimicrobial activities and could serve as a source of antimicrobial agents against food pathogens [9,10].

Several references on the antimicrobial and antifungal efficiency of essential oils are available in the literature [11,12].

Rosemary essential oil was used as antibacterial, antifungal, antiseptic agents. It is largely used in traditional medicine**,** in perfumery, phytocosmetic and in liquors manufacturing [13,14]. They have been the subject of considerable studies [15,16]. Their antimicrobial activity is summarized by Burt [17]. The qualitative composition of rosemary EOs is well defined and fixed. Also it used as general stimulant, improves circulation, treats rheumatic pains, and useful for skin care.

Nucleic acid amplification technology has opened new avenues of microbial detection and characterization [18,19], such that growth is no longer required for microbial identification [20,21,22]. In this respect, molecular methods have surpassed traditional methods of detection for many fastidious organisms. The polymerase chain reaction (PCR) and other recently developed amplification techniques have simplified and accelerated the *in vitro* process of nucleic acid amplification. The amplified products, known as amplicons, may be characterized by various methods. Rapid techniques of nucleic acid amplification and characterization have significantly broadened the microbiologists' diagnostic arsenal.

Randomly amplified polymorphic DNA (RAPD), a technique based on the PCR using primers of arbitrary sequence, has been used increasingly for taxonomic, identification and isolate differentiation in a variety of organisms [23,24,25]. It has also been introduced for genetic characterization of many organisms such as *Escherchia coli* and *Staphylococcus aureus* [26,27].

The detection of DNA damage has been widely studied using a number of laboratory methods, to investigate the effect of mutagens on different organisms. However, molecular markers allow a direct comparison of the effects of genotypes at the DNA level. The explorations of random amplified polymorphic DNA (RAPD) as genetic markers have improved the detection of DNA alterations after the influence of many agents [28]. The different types of DNA damages must be detected by changes in RAPD profiles.

In the context of the information provided above, the objectives of this study have been , to investigate the possibility of mutation changes in two kinds of Gram -ve and Gram +ve bacteria (*Escherchia coli* and *Staphylococcus aureus*) after the influence of rosemary essential oil using RAPD method, to determine whether the RAPD assay could detect changes in bacterial DNA, and to assess how these endpoints (i.e. changes in RAPD profile) compare in terms of detection of antimicrobial activity. The main purpose of this study is to evaluate the potential antimicrobial activity of rosemary essential oil, via the RAPD assay.

Thus a preliminary investigation was undertaken to study the capability of this technique to show mutagenic effects induced by essential oils.

MATERIALS AND METHODS

Preparation of essential oils

The volatile oil of rosemary (*Rosmarinus officinalis*) was extracted from the above ground air dried parts (Shoot system) using steam-distillation apparatus for 3 hours. The oils obtained were separately dried over anhydrous sodium sulphate [29].

Microbial strains and culture conditions

The essential oils were individually tested against the two microorganisms, *Escherichia coli* and *Staphylococcus aureus*. These strains were obtained from Department of Botany, Faculty of Science, Mansoura University. The Bacteriological agar and nutrient broth were provided from Difco.

MIC and MBC determinations

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevent visible growth of the bacteria [11,30]. The minimum bactericidal concentration (MBC) was determined as a concentration where 99.9% or more the initial inoculum is killed [30,31]. The rosemary essential oils was dissolved in Tween-80 (0.05%). The MIC and MBC concentrations were determined by serial dilution with nutrient broth. The bacterial cell number was adjusted at 10^6 bacterial cells/ml (0.1 ml inoculum / tube) for the two strains tested. All samples were incubated at 37°C during 18-24 h. To confirm results of MBC, the experimental suspensions were sub-cultured in nutrient agar plates according to the method described by Ronda [32] and were incubated at 37°C during 18-24 h. We have used this method because it is more sensitive than the agar dilution.

DNA isolation

The isolation of genomic DNA from the two tested organisms (*E. coli* and *Staph. aureus*) was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method [33]. 0.5 gm of fresh samples (organism exposed to oil and control for every organism) was ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 5 ml preheated CTAB buffer, and incubated at 65°C for 1 hour with occasional shaking. The suspension was then mixed with 1/3 volume of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 volume of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or sedimentated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and resuspended in 0.5 ml TE buffer. The enzyme, RNAse A (20µg) was added to the resuspended mixture to digest any contaminating RNA and the tube was incubated at 37 ˚C for 30 min. To remove the enzyme and other contaminating protein, phenol/chloroform extraction was performed.

Random ampl ified poly morphic DN A (R APD) analysis

The polymerase chain reaction (PCR) mixture was prepared as following in 25 ul PCR tubes: genomic DNA 50 ng/ml, 25 pmol dNTPs, and 25 pmol of random primer (Operon Technologies Inc.,) consisted of 0.8 units of *Taq* DNA polymerase. The reaction mixture was placed on a DNA thermal cycler. The PCR programme included an initial denaturation step at 94°C for 2 mins followed by 45 cycles with 94°C for 1 min for DNA denaturation, annealing as mentioned with each primer, extension at 72°C for 30 seconds and final extension at 72 °C for 10 minutes were carried out. A set of five 10-mer primers (Operon technologies Inc., Alameda, California) randomly selected were used in RAPD analysis (Table 1).

Gel Electrophoresis

The amplification products were analyzed by electrophoresis [34]. Along with the PCR amplified products, 100 bp DNA ladders (Promega) as standard marker were subjected to electrophoresis in 2% agarose gel in TAE buffer (for each litre of 50X TAE Stock solution: 242 g Tris Base, 57.1 mL Glacial Acetic Acid and 100 mL 0.5 M EDTA), and stained with ethidium bromide. Molecular size of the marker was 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp. The amplified pattern was visualized on a UV transilluminator and photographed.

Table 1 . List of random primers used for RAPD analysis.

Primer	Sequence 5'- 3'	Annealing Temp °C / Sec
A1	TGGCGACCTG	36°C
A 2	GAGGCGTCGC	
B1	CCCTACCGAC	
B2	TCGTTCCGC	
B3	CACCTTTCCC	

RESULTS

Antimicrobial Activity

The antimicrobial activity of *Rosmarinus officinalis* essential oil on the two examined bacterial strains was shown in Figure (1). The essential oil had various degrees of inhibition against the two bacterial strains using the drop diffusion method [35].

(a) (b)

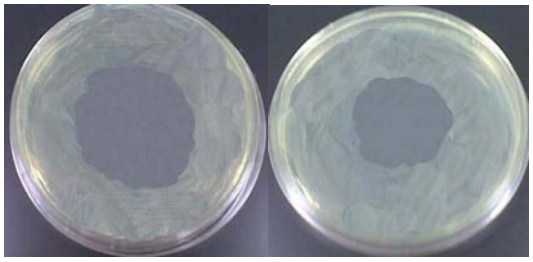

Fig (1). Inhibition of *Staph. aureus* (a) and *E. coli* (b) by rosemary essential oil.

The MIC and MBC values of rosemary EO on the two tested organisms were determined. Rosemary EO exhibited interesting activities against tested bacteria, *E. coli* and *Staph. aureus* with MBC values of 2.5, and 25 µl ml^{-1}, and with MIC values of 1.25 and 5 µl ml^{-1} respectively.

RAPD Profiling

The RAPD assay carried out with 50 ng of control genomic DNA and yielded 5-7 bands in case of *E. coli* and 3-6 bands for *Staph. aureus* respectively (Fig 2a, b, c). In total, 5 oligonucleotide primers were used in the analysis and were able to produce reproducible RAPD bands with template DNA from the tested organisms. The presence of changes in the RAPD profiles obtained from the control and exposed bacterial cells to essential oil depended on the primer used. With respect to exposed cells, the number of bands for each primer varied ranging from one band for primer A2 to ten for primer B3. Polymorphism was evidenced as the presence and / or absence of DNA fragments between the samples (Fig 2a, b). The DNA profiles generated by the five primers revealed differences between control and exposed bacterial cells, with visible changes in the number and size of amplified DNA fragments, and both increases and decreases of DNA band intensities. Arrows on each gel show some of the obvious modifications (Fig 2).

Changes in RAPD profiles arose at oil treatment included increase in band intensity, appearance of new bands, and disappearance of some bands (Fig 2a, b, c). In addition, an appearance of extra bands was the major event arising in the patterns generated by the *Staph. aureus* cells exposed to rosemary essential oil. In contrast, disappearance of bands was well represented in the patterns produced by *E. coli* exposed to EO (Fig 2a, b). Band intensity followed two different tendencies, intensity of some bands increased and others decreased respectively, compared to control intensities.

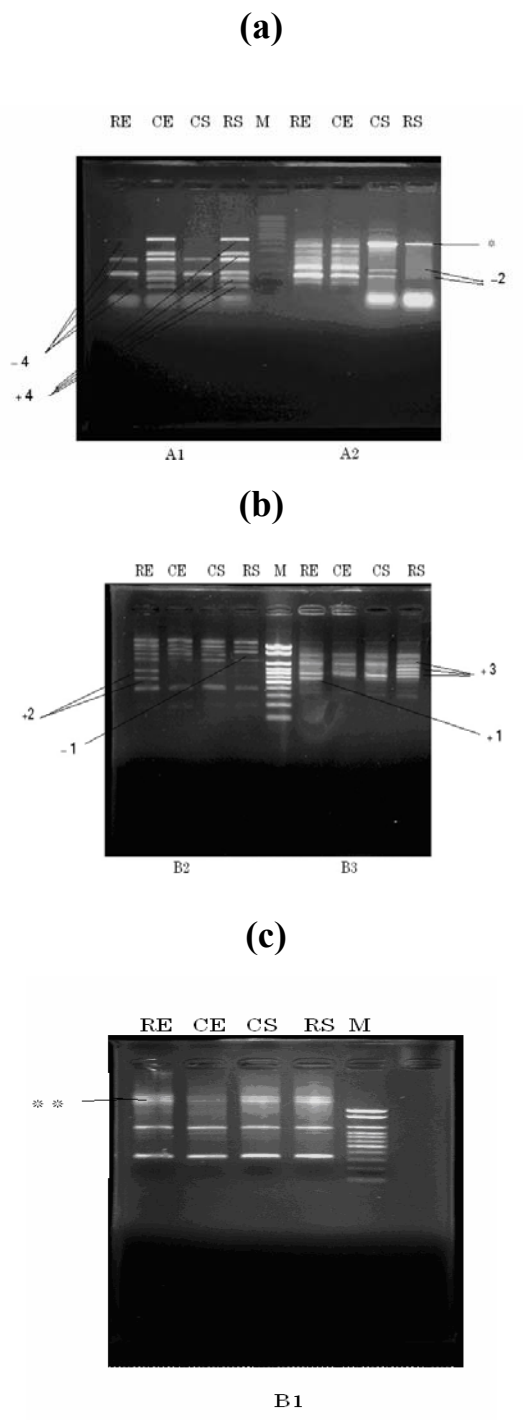

Fig (2). RAPD profiles of *E. coli* and *S. aureus* exposed to rosemary essential oil and control. RAPD profiles were generated using 10-mer primers A1, A2, B1, B2 and B3. M, DNA molecular size marker; CE, Control fingerprint of *E. coli*;

CS, Control fingerprint of *S. aureus*; RE, The fingerprints of exposed *E. coli*; RS, The fingerprints of exposed *S. aureus*.

Selected changes are indicated by arrows in comparison to control patterns. *decrease in band intensities;** increase in band intensities +, appearance of a new band; –, disappearance of a band.

DISCUSSION

Determination of MIC and MBC of rosemary essential oil against tested bacteria showed that *Staph. aureus* was more susceptible to the essential oils than *E. coli*. In accordance with previous findings, it seems that the antibacterial action of the essential oils is more pronounced on Gram-positive than on Gram-negative bacteria [36]. Some studies agree that generally rosemary EO are slightly more active against Gram-positive than against Gram-negative bacteria. Gram-negative organisms are less susceptible because they possess another membrane surrounding the cell wall which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [6].

In the past decade, the tremendous progress made in molecular biology and genetics resulted in the development of modern diagnostic technologies. These technologies include several extremely sensitive and, most importantly, reliable assays for screening different types of biotic and abiotic environmental agents. RAPD- PCR has proved valuable in many areas of biomedical research [37]. In addition, its use in surveying genomic DNA for evidence of various types of damage and mutation suggest that they may potentially form the basis of novel assays for the detection of DNA damage and mutations [38].

The main changes observed in the RAPD profiles have resulted both in the appearance or disappearance of different bands with variations of their intensity as well. These effects may be correlated with structural rearrangements in bacterial DNA caused by different types of DNA damages. The variation in band intensity and disappearance of some bands may correlate with level of photoproducts in DNA templates after treatment, which can reduce the number of binding sites for *Taq* polymerase [39].

These new diagnostic assays also permit the detection and characterization of the antimicrobial effects of essential oils commonly used. The RAPD assay is a sensitive, reliable, and rapid method for assessing DNA damage in individual prokaryotic and eukaryotic cells. The results of this work shows that the RAPD technique could be a promising tool in the *in vitro* detection of alterations in DNA produced by antimicrobial agents, allowing us to see the mechanisms of action of the agent in greater detail. Moreover, the practically unlimited number of informative primers provides a good overall coverage of DNA [40] so that the choice of a set of primers with different sequences will allow increased sensitivity of the assay in the detection of low frequency mutation events. In conclusion, It has to be emphasized that this diagnosis has been made based on analyses utilizing modern and sensitive assays (RAPD assay) performed on the bacterial cells. The observations indicate that rosemary essential oil can induce wide range of adverse reactions in sensitive individuals.

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