Althobaiti | J Pure Appl Microbiol | 14(3):2165-2171 | September 2020
Article 6571 | https://doi.org/10.22207/JPAM.14.3.57

Print ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Antibacterial Activity And Genotoxicity Effect of Ethanolic Leaves Extract of *Rosmarinus Officinalis*

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Abstract

The recent increasing use of artificial antibiotics has prompted an expansion in resistant strains and high site reactions. Medicinal plants have for quite some time been utilized as traditional medicine to treat pathogenic bacteria. In such manner, consistently numerous scientists are sending a range of plant's secondary compounds to the customer advertise for the treatment of human illnesses. Accordingly, the distinguishing proof of plant spices with antimicrobial impacts can assist with delivering new medications with a wide range of impacts. The aim of the present research was to examine the ability of ethanolic leaves extracts of *Rosmarinus officinalis* plant as antibacterial agent against the Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli.* The zone of inhibition increased with increase in concentration of the test solution. Higher activity of ethanolic extract was found against *S. aureus* (2.4 cm) than *E. coli* (1.8 cm). In addition, the repetitive element PCR (Rep-PCR) significantly showed that several genetic numbers of polymorphic bands were observed in *S. aureus* and *E. coli* treated bacteria with leaves extracts and not observed in the control. These results indicate that these extracts have a genotoxicity effect on the two bacterial genomes. The obtained results demonstrate that *R. officinalis* can be used as a potential source of antibacterial and genotoxicity factors.

Keywords: Rosemary, Re-PCR markers, gene mutations, *S. aureus, E. coli*

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(Received: August 05, 2020; accepted: September 11, 2020)

Citation: Althobaiti F. Antibacterial Activity And Genotoxicity Effect of Ethanolic Leaves Extract of *Rosmarinus Officinalis*. *J Pure Appl Microbiol.* 2020;14(3):2165-2171. doi: 10.22207/JPAM.14.3.57

INTRODUCTION

One of the concerns in the biomedical and medical sciences is the resistance of bacteria to chemical drugs, in cases, where drug resistance is created by changing the drug to fight against pathogenic bacteria[1]. After the discovery of penicillin in the 40s, and its use in treatment, new antibiotics were introduced every day to treat infections[2]. The result was the expansion of the clinical use of natural and synthetic antibiotics in the treatment of clinical infections[3]. Nevertheless, as advances in the production of new chemicals and various antibiotics began to take place, the harmful effects of these drugs gradually began to appear, and since the 1950s numerous pathogenic bacteria have shown resistance to antibiotics, which is still expanding[4]. Therefore, there is a new approach to reduce the spread of pathogenic microbes by using alternative and natural methods such as using essential oils or plant extracts[5]. For many years, natural medications, especially medicinal plants, have been the basis and even in some cases the only treatment, while their raw materials have been used in the pharmaceutical industry[6]. Additionally, the medicinal plants have several characteristics for using to treat bacterial diseases, as natural, low-risk and inexpensive compared to synthetic antibiotics[7-9]. In addition, these herbal remedies are more popular with people[10-12].

The role of natural products in drug production is increasing, not only when biologically active compounds are used directly as therapeutic drugs, but also when used as raw materials for drug synthesis, or as a model the base is used for new biologically active compounds[13-15]. Studies show that only about 10% of the 250,000 species of plants studied worldwide[16]. Therefore, the use of herbal drugs as an alternative to chemical drugs and antibiotics was investigated in many research studies[17-19].

Rosmarinus officinalis (Rosemary) belongs to the Lamiacea family and is popular as a spice and medicinal plant in many countries. Rosemary is listed in the World Series of Weeds, but due to its popularity and therapeutic properties, it is in the top priority[20]. It has antibacterial, antifungal, anticancer, antidiabetic, antiinflammatory, analgesic, antioxidant, and endemic effects on the Mediterranean and Asian region[21-23]. The antimicrobial properties of rosemary are due to phenolic compounds: carnosol, rosmarinic acid, caffeic acid, flavonoids including diosmin, luteolin, zincquanine, and monoprenes such as camphor, cineole and borneol[24].

Fresh and dried rosemary leaves are used for their distinctive aroma in food or herbal tea cooking, while rosemary extracts are commonly used as natural antioxidants that help a person's healthy life[25]. Rosemary is one of the spices that has the highest levels of antioxidants and can help fight bacteria and cancer[26]. Antioxidant properties of rosemary extracts vary due to genetic and growth conditions, region and geographical origin, climatic conditions, extraction process, main plant quality and date of harvest[27]. While the immune-boosting properties of the rosemary plant are sufficiently effective, the plant also works well against bacterial infections, especially those that occur in the stomach. Also, rosemary is associated with the prevention of *Staphylococcus* infection, which kills thousands every year[28].

The aim of the present research was to investigate the antibacterial activity and genotoxicity effect of the rosemary leave extracts against the pathogenic bacteria of *Staphylococcus aureus* and *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains

Two pathogenic bacteria, *S. aureus* TU-23 and *E. coli* TU-39*,* were used in the present study. Both bacteria were identified by 16S rRNA gene as previously reported[29]. The PCR reaction was achieved as follow; 2 µl of template DNA (about 100 ng), 2X Promega PCR Master Mix (Promega®, Lithuania, USA), 10 pmol of specific universal 16s rRNA primers and deionized distilled water (up to 25 µl). The PCR product, about 1260 bp, was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). The resulted sequences of 16S rRNA were aligned with other known 16Sr RNA sequences in GenBank using MEGA program version 7.10 to generate a phylogeny tree.

Plant materials

Rosemary plants (*Rosmarinus officinalis*) were collected from the vicinity of Taif, Saudi Arabia on October 2019. Then the plants were washed with running water and the leaves were

cut into small pieces, then leaved in sun light for three days to dry and the grinded by blender. The powder of leaved was extracted in 70% ethanol to get the crude extract as previously described[29].

Determination of antibacterial activity of rosemary leaves extracts

The agar-well diffusion method was used for investigating the antibacterial activity of rosemary leaves extracts[29]. The two bacteria were cultured in Luria-Bertani (LB) medium for 13 h and harvested at 10000 rpm for 5 min. Then, pellets were resuspended in sterile water and diluted to about 100 CFU/ml. An aliquot of 200 µl of the resulting bacteria was mixed with 50 ml of nutrient agar (NA) medium and then poured into petri dish. Wells with a diameter of 4.6 mm were cut from the agar with a sterile borer. Aliquots of 200 and 300 µl dilution of the leaves extract were used, as well as water as negative control and Streptomycin as positive control at 1.2 µg/ml. The plates were kept at 37°C for 24 h. Antibacterial activity was investigated by measuring the diameter of the inhibition zone (DIZ) of the tested bacteria as previously mentioned[29].

Repetitive-PCR (Rep-PCR) analysis technique

The two bacteria were growth on 5 ml liquid LB medium with different concentrations of rosemary leave extract (0, 10, 20, 30, 50 and 75 ml of the leave extract dilution) at 37°C for 24 h. Next, genomic DNA was extracted using the procedure elucidated by the manufactured (Jena Bioscience, Germany) and Rep-PCR technique was performed using five Rep-PCR primers as followed:

Rep 18 (5'-ACA CAC ACA CAC ACA CG-3'), Rep 19 (5'-AGA GAG AGA GAG AGA GTT-3'), Rep 29 (5'- ACA CAC ACA CAC ACA CT -3'), BOX A1 (5'- CTA CGG CAA GGC GAC GCT GAC G-3'), and (GTG)5 (5'-GTG GTG GTG GTG GTG-3'). PCR was achieved in total volume of 25 µl that containing 2 µl (about 50 ng) of genomic DNA, 12.5 µl of Go Taq® Green Master Mix (Promega, USA), 20 pmol of each primer, and deionized distilled water (up to 25 µl).

RESULTS AND DISCUSSION

The phylogenetic tree of the 16S rRNA of the two pathogenic bacteria are presented in Fig. 1. The 16S rRNA gene sequence of the two species were compared with other *S. aureus* and *E coli* strains that stored in GenBank database. It was documented that ribosomal genes are significantly applicable for classified bacterial species[30]. When re-constructing phylogenetic tree of bacteria species, sequencing of 16S rRNA has been commonly used. As a result, *S. aureus* and *E. coli* are located in same species that collected from GenBank (Fig. 1).

The antibacterial activity of rosemary leaves extracts against *S. aureus* and *E coli* was measured by evaluating the diffusion method in

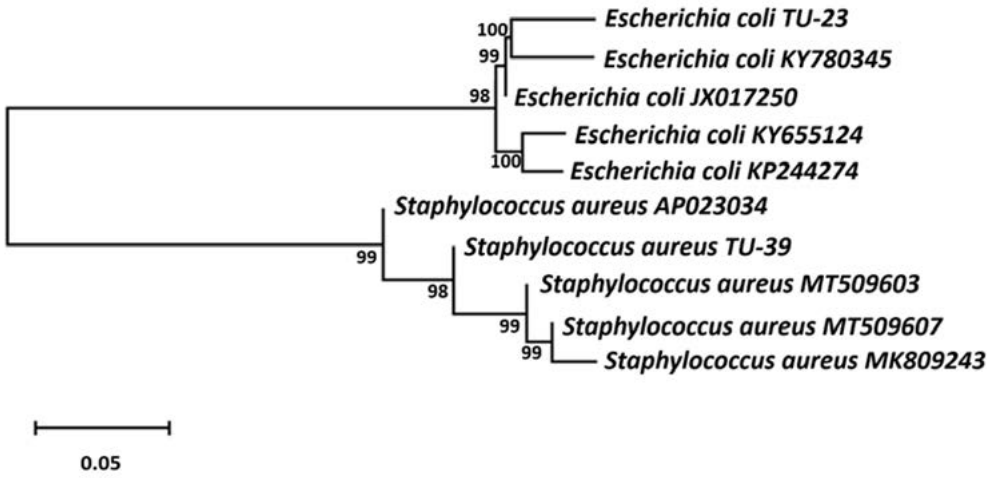

Fig. 1. The phylogenetic relationship tree of *E. coli* TU-23 and *S. aureus* TU-39, which are considered in the present study with related genera, based on the sequence of 16S rRNA gene sequences using neighbor-joining method in MEGA 7 software.

agar. This method allows for better diffusion of extracts in the NA medium and thereby enhances the interaction between the extract and bacteria. The results exhibited that the rosemary leave extracts with amount of 200 or 300 ml dilution of the leaves extract was active and stopped the growth of negative and positive-gram bacteria (Table 1, Fig. 2). Maximum imbibition zone was noticed towards S. aureus (24 mm), while, against *E. coli* was 18 mm (Table 1). This suggests that the rosemary leaves extracts displayed a wide-ranging activity since it was active towards both bacterial species. Similar data were obtained previously[16,21,24].

The results also designated that the ethanol was a good solvent for extracting essential substances from the rosemary leaves. This result is in agreement with the conclusion that stated by Abramovic et al.[31] and Gazwi[32] as the ethanol was the best solvent to extract the phenolic compounds from rosemary leaves.

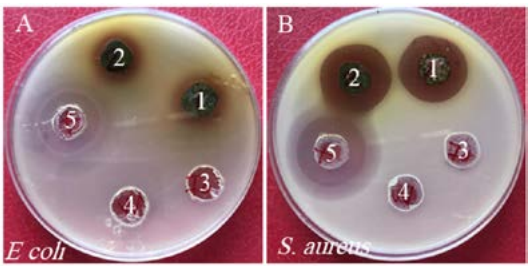

Fig. 2. The inhibition zone of rosemary leaves extracts against *E. coli* (A) and *S. aureus* (B). 1 = 200 ml dilution of the leaves extract, 2 = 300 ml dilution of the leaves extract, 3 = 200 ml 70% ethanol, 4 = distilled water as a negative control, 5= 1.2 µg/ml of Streptomycin.

The high antimicrobial activity of rosemary leaves extracts may be either due to the presence of alkaloids, flavonoids, phenolic compounds, saponins, tannins, steroids, carnosic acid and rosmarinic acid[23,26]. Phenolic compounds and flavonoids are recognised for their antibacterial and antifungal properties[29]. Also, there is some evidence that minor chemical components that located in rosemary leaves have a significant effect as antibacterial activity[33].

To measure bacterial DNA stabilization, both bacteria strains were treated with five dilutions of rosemary leaf extracts ranging from 10 to 75 µl. Rep-PCR data showed that many of the polymorphic sites that presented in *E. coli* and *S. aureus* treated strains compared to those from untreated bacteria (Table 2, Fig. 3). As shown in Fig. 3 and Table 2, rosemary leaves extract produced several polymorphic loci in treated bacteria. Also, several positive or negative polymorphic loci were present or absent in treated bacteria with the rosemary leaves extracts. These results clearly indicate that the rosemary leaves have the ability to induce several point mutations as a result of the

Table 1. Antibacterial activities of rosemary leaves extracts against *E. coli* and *S. aureus*

Isolates	Diameter of inhibition zone (mm)		
	Rosemary leaves extracts (µl)		Streptomycin
	200	300	
S. aureus	20	24	22
E. coli	15	18	21

Table 2. Numbers of polymorphic and monomorphic loci of each Rep-PCR primer and percentage of polymorphism in both bacteria that treated with different concentrations of rosemary leaves extracts

Primer Name	Total loci	Polymorphic loci	Polymorphis loci									
			E. coli					*S. aureus*				
			2	3	4	5	6*	2	3	4	5	6*
Rep 18	11	6	0	2	2	1	0	1	1	2	3	1
Rep 19	13	10	0	0	0	1	1	2	2	2	2	1
Rep 29	10	9	3	3	1	2	1	3	2	4	3	4
BOX A1	12	2	1	1	0	0	0	0	0	0	0	0
(GTG)5	9	5	1	1	1	1	0	0	1	0	0	0
Total	55	32										

*2 to 6 = 10, 20, 30, 50 and 75µl dilution of the leaves extract.

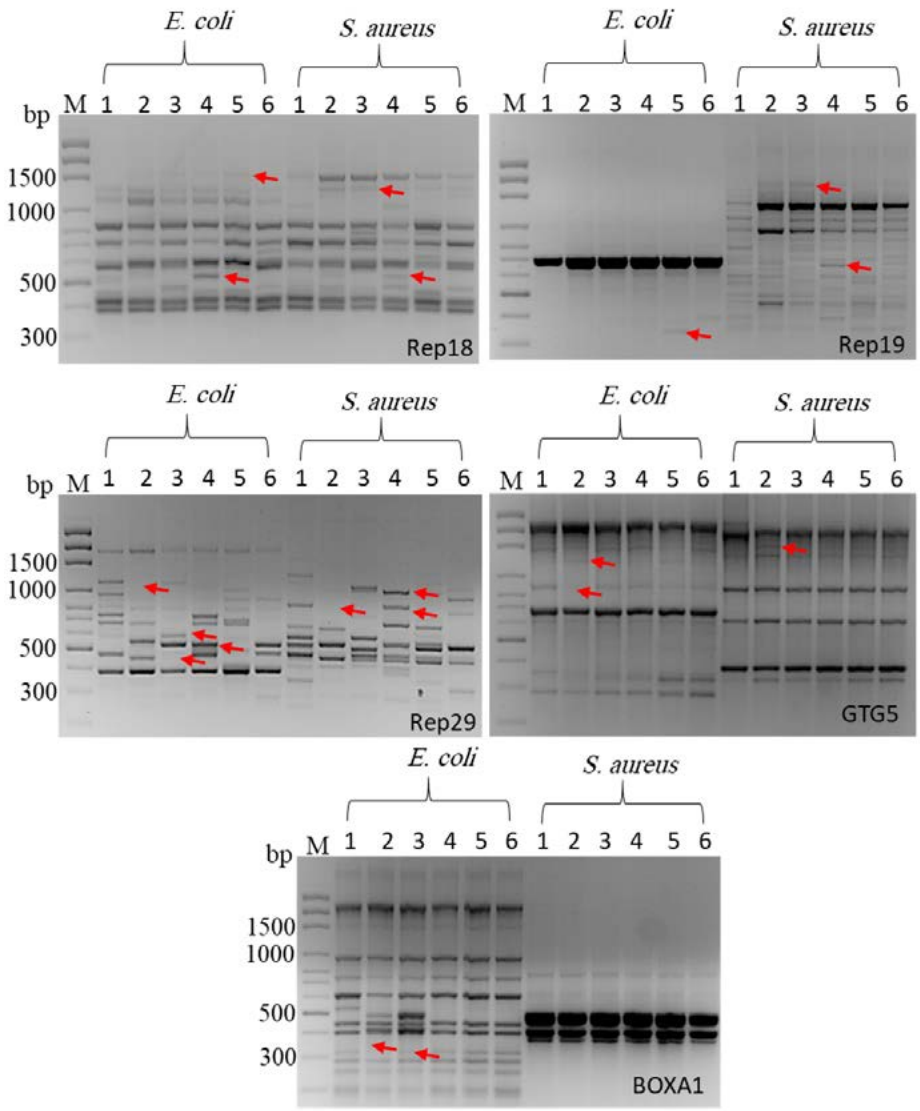

Fig. 3. Rep-PCR profile of the *E. coil* and *S. aureus* treated with five different concentrations of rosemary leaves extracts. 1 = non treated cells, 2 to 6 = 10, 20, 30, 50 and 75 ml dilution of the leaves extract using five Rep-PCR markers. M: is 100 bp DNA ladder.

deletion or addition of nucleotides as evidenced by the disappearance or appearance of many genetic sites and, consequently, the change in the primers matching sites compared to control (Fig. 3). These results are consistent with the results that previously found[29]. Some of the chemical ingredients in rosemary extracts may act as interaction factor or can generate free radicals that interact with genomic DNA to produce deletions in many nucleotides. Similar data were previously mentioned with different medicinal plants[34,35].

CONCLUSION

The antibacterial activities of rosemary leaf extract can be shown as biochemical compounds that act in treated bacteria as a genetic toxin or mutagenic agent that causes addition or deletion in several DNA nucleotides. The ability to

develop antibacterial agents from medicinal plants seems satisfactory, because, in addition to this activity, they may produce fewer side effects and act on strains resistant to conventional antibiotics.

ACKNOWLEDGMENTS

None.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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