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IN VITRO ANTIBACTERIAL ACTIVITY OF *ROSMARINUS OFFICINALIS* METHANOLIC AND AQUEOUS EXTRACTS

Rachad Alnamer[1, 2*], Katim Alaoui[2], Latifa Doudach[2], El Houcine Bouidida[3], Fatiha Chibani[4], Mosa'd AL-Sobarry[1], Abdelaziz Benjouad[1] and Yahia Cherrah[2]

[1]Laboratory of Biochemistry and Immunology, Department of Biology, Faculty of Science, Mohammed V Agdal University, Agdal, Rabat, Morocco
[2]Laboratory of Pharmacology and Toxicology, Department of Drugs Sciences, Faculty of Medicine and Pharmacy, Mohammed V Souissi University, ERTP*, Rabat, Morocco
[3]National Laboratory of Drugs Controlled, Rabat, Morocco
[4]Laboratory of Microbiology, Hospital Ibn Baja, Taza, Morocco

***Corresponding author e-mail:** *yemtiger1@yahoo.com*

ABSTRACT

Rosemary (Rosmarinus officinalis L., family *Lamiaceae*) had been reported in traditional medicine, to exhibit antimicrobial properties. Therefore, this study is aimed at determining the antibacterial activity of *R. officinalis* leaves against pathogenic microorganisms by determination the minimal inhibitory concentration and to serve as criteria to recommend the ethno pharmacological uses of the plant. Plant leaves were dried, powdered and extracted by cold maceration with methanol for 48h. The extracts were screened against 24h broth culture of bacteria seeded in Muller Hinton Agar at concentration 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56mg/ml in sterile distilled water and incubated at 37°C, for 18h and measuring the inhibition zone diameter (IZD). Minimum inhibitory concentrations (MICs) against three Gram-positive bacteria (*Micrococcus luteus*, *Staphylococcus aureus* and *Bacillus subtilis*), three Gram-negative bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*) were determined for the methanolic and aqueous extracts of *Rosmarinus officinalis*. The methanolic extract showed pronounced antibacterial than the aqueous extract against all of the tested microorganisms. Methanolic extract inhibited with minimal inhibitory concentration of 1.56, 1.56, 3.13, 1.56, 3.13 and 3.13mg/ml against *Micrococcus luteus, Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli*, respectively, while the aqueous extract inhibited with minimal inhibitory concentration of 6.25 mg/ml against all tested bacterial strains both Gram-positive and Gram-negative bacteria. The methanolic and aqueous extracts of *R. officinalis* demonstrated activities against certain bacteria confirming the use of the plant in ethno pharmacology.

Keywords: *Rosmarinus officinalis*, minimal inhibitory concentration, antibacterial screening, bacterial strains, medicinal plant.

INTRODUCTION

Over the past decade herbal medicine has become a topic of global importance, making an impact on both world health and international trade. Medicinal plants continue to play central roles in the healthcare system of large proportion of the world's population. This is particularly true in the developing countries, where herbal medicine has a long and uninterrupted history of use. Recognition and development of medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations. [1] Continuous usage of herbal medicine by a large proportion of the population in the developing countries is largely due to the high cost of western pharmaceuticals, health care, adverse effects that

follow their use (in some cases) and the cultural, spiritual point of view of the people of the countries. [1] In western developed countries however, after a downturn in the pace of herbal use in recent decades, the pace is again quickening as scientists realize that the effective life span of any antibiotic is limited. [2] Worldwide spending on finding new anti-infective agents (including vaccines) was expected to increase 60% from the spending levels in 1993. New sources, especially plant sources, are also being investigated. Secondly, the public is becoming increasingly aware of problems with the over-prescription and misuse of traditional antibiotics. In addition, many people are interested in having more autonomy over their medical care. All these makes the knowledge of chemical, biological and therapeutic activities of medicinal plants used as folklore medicine become necessary. [3] In Morocco, there is more than 42,000 species of plants; divided into 150 families and 940 genus used in traditional medicine. [4, 5]

Rosmarinus officinalis L., commonly referred to as rosemary, belongs to the mint family. It is a common dense, evergreen, aromatic shrub grown in many parts of the world. [6] The fresh and dried leaves are frequently used in traditional Mediterranean cuisine as an additive. They have a bitter, astringent taste, which complements a wide variety of foods. A tisane can also be made from them. They are extensively used in cooking, and a distinct mustard smell gives off while they are burned, therefore, they often are used to flavor foods while barbecuing.

Historically, rosemary has been used as a medicinal agent to treat renal colic and dysmenorrheal. It has also been used to relieve symptoms caused by respiratory disorders and to stimulate the growth of hair. Extracts of rosemary are used in aromatherapy to treat anxiety-related conditions and to increase alertness. [7-9] Essential oils and various extracts of plants have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases. [7, 10] Particularly, the antimicrobial and antivirus activities of plant oils and extracts have formed the basis of applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. [11] Because of the possible multiple resistances and side effects of the synthetic antimicrobial, increasing attention has been directed towards natural antimicrobial. [12] There are many studies on the antimicrobial activity of secondary metabolites in recent years, but to our knowledge, fewer comparative studies on antimicrobial activity of *R. officinalis* L. methanolic and aqueous extracts have been reported. [13]

To the best of our knowledge, this is the first study to provide data that the methanolic and aqueous extracts of *Rosmarinus officinalis* evaluated against a wide range of bacteria. Thus, the aim of this study is to evaluate the antimicrobial activity of rosemary (*Rosmarinus officinalis*) methanolic and aqueous extract, and to, therefore, determine the scientific basis for its use in traditional medicine in the treatment of infection diseases.

MATERIALS AND METHODS

Plant material: *Rosmarinus officinalis* was collected based on ethnopharmacological information, from villages around the region Rabat-Salé-Zemour-Zaers, with the agreement from the authorities and respecting the United Nations Convention of Biodiversity and with assistance of traditional medical practitioner. The plant was identified with botanist of scientific institute (Pr. M. Ibn Tatou). A voucher specimen (RAB12560) was deposited in the Herbarium of Scientific Institute, University Mohammed V-Rabat-Morocco.

Preparation of extract

Methanolic extract: Stems and leaves of *R. officinalis* were successively extracted with methanol by maceration at room temperature (25°C) over period of 48h. 500 g of plant material and one liter of methanol were used in the extraction. Methanol containing the extract was then filtered through Whatman paper and the solvent was vacuum-distilled at 65°C in a rotary evaporator. The remaining extract was finally dried in the oven at 30°C for 2h to ensure the removal of any residual solvent. Final extract was a yellow powder in percentage dray weight 22.8%; this methanolic extract was kept in deep freeze at -20°C until use.

Aqueous extract: 500 g of plant material was extracted by infusion in boiled water (500 ml) for three days. The respective aqueous extracts were separated from its residues by gravity filtration and then lyophilized (Free Zone® Dry 4.5, USA). For each study, the lyophilized aqueous extract was carefully prepared under the same condition used throughout the studies (time, temperature and the amount of plant material and water used for extraction under reflux and lyophilization) and each time the quality of extraction was checked by the yield of the lyophilization material. The final crude extract was obtained as dark brown powder in

percentage from dry weight (18.7%). For assuring stability, the lyophilized material was stored at -20°C.

Antibacterial Activity Test

Bacterial strains: The following 6 microorganisms (three Gram-negative and three Gram-positive bacteria), were used in this study: *Escherichia coli* ATCC 54127, *Pseudomonas aeruginosa* ATCC 15442, *Klebsiella pneumoniae* ATCC 53153, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633 were used for antibacterial testing. The cultures of bacteria were maintained in their appropriate agar slants at +4°C throughout the study and used as stock cultures. The bacteria were obtained from the **L**aboratory of **M**icrobiology, **N**ational **L**aboratory of **V**eterinary **D**rugs **C**ontrolled, Rabat, Morocco.

Determination of antibacterial activity by the paper disc diffusion method: The methanolic and aqueous extracts of *R. officinalis* were tested for antibacterial activity by the paper disc diffusion method. Molten (4 5°C) sterile Muller Hinton Agar (10ml) in a flask was inoculated with a broth culture (1%, containing 10^6-10^7 cfu/ml) of the respective bacterial strains and poured over plates containing 10ml Muller Hinton Agar in sterile 9cm Petri dishes.

Fifty microliters of dilutions of the methanolic and aqueous extracts were pipette on sterile filter paper disc (Whatman No.1. 5mm in diameter), which were allowed to dry in an open sterile Petri dishes in a biological safety cabinet with vertical laminar (Nuaire Petri dishes Laminar Flow Products, USA). 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.56mg/ml of the methanolic and aqueous extracts solutions were applied to the discs. Discs were placed on the surface of the inoculated plates and incubated at 37°C for 18h.

Zones of inhibition of microbial growth around the paper disc containing the extracts were measured and recorded after the incubation time. The inhibitory zone was considered the shortest distance (mm) from the outside margin of the paper disc to the initial point of the microbial growth. All analyses were applied in triplicate. [14, 15] Discs impregnated with sterile distilled water served as negative control. Penicillin and Ampicillin which purchased from Sigma (St. Louis, USA) was used as a positive control. [17-19]

Determination of Minimum Inhibitory Concentration (MIC): Test strains were suspended in Muller Hinton Agar (MHA). The suspension was adjusted spectrophoto-metreically to match the turbidity of a McFarland 0.5 scale (*i.e.* to give a final density of 10^7cfu/ml).

The viability indicator MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide, Sigma, Aldrich) and the quick microplates method were used for the determination of the minimum inhibitory concentration (MIC)**.** [19-22] Serial dilutions of extracts were dissolved in distilled water and made in a concentration range from 1.56mg/ml to 200mg/ml in sterile test tubes. Each test tube was inoculated with 20µl from each various dilutions of the methanolic and aqueous extracts of *R. officinalis* were added to 5ml of Muller Hinton Agar both in tubes containing 10^7cfu/ml of live bacterial cells. The tubes were then incubated under optimal conditions at 37°C for 18h. After incubation, as an indicator of bacterial growth 1 mg/ml of MTT was added to each well, after incubation periods ranging from 3 to 5h at 37°C, control without plant extracts with MTT and bacterial inoculums were used as negative control. The bacterial suspension changed to blue when bacterial growth occurred. The highest dilution (lowest concentration), showing no visible growth was regarded as the MIC. Cell suspension (0.1ml) from the tubes showing no growth was sub cultured on Muller Hinton Agar plates in triplicate to determine if the inhibition was reversible or permanent. All tests were performed in triplicate.

Statistical analysis: Results of the research were tested for statistical significance by one-way ANOVA. Differences were considered statistically significant at the $P < 0.05$ level.

RESULTS AND DISCUSSION

The methanolic and aqueous extracts were evaluated for antimicrobial activity against Gram-positive (*S. aureus, M. luteus* and *B. subtilis*), Gram-negative (*P. aeruginosa, K. pneumoniae* and *E. coli*) bacteria. Rosemary methanolic was found to be the most active against all of the bacterial strains. *R. officinalis* methanolic extract displayed good activities, inhibiting the growth of *E. coli*, *P. aeruginosa* and *K. pneumoniae* with IZD of 28, 24 and 20 mm respectively but with less activity against three Gram positive bacteria (*S. aureus, M. luteus* and *B. subtilis*), with inhibition zone diameter of 20 mm ($P < 0.001$). The inhibition zone diameter for the rosemary methanolic extract ranged from 20 to 50 mm, while IZD for aqueous extract ranged from 18 to 30 mm ($P < 0.01$) (Table 2 and 3). The results of the paper disc diffusion support and extend previous finding that rosemary contains numerous biologically

active compounds and some of these have been frequently used in folk medicine for their antibacterial properties. In addition, the MIC values support the finding of the paper disc diffusion method (Table 1, 2 and 3). Phytochemical studies have identified active components in the methanolic and aqueous extract of this plant, such as flavonoids including diosmetin, diosmin, luteolin, apigenin, quercetin and kaempherol, phenols such as caffeic and rosmarinic acids, and terpenoids like, carnosol, carnosic acid, rosmanol, and oleonolic and ursolic acids. [23-33] The biological activity of rosemary against the tested bacteria could be attributed to the presence of flavonoids, phenolic acids (caffeic, chorogenic and rosmarinic) and essential oils (camphor and cineole) and diterpenes (carnosol). [8, 9, 31-34] Biologically active components are believed to disturb permeability of the cytoplasm membrane and thereby facilitate the influx of antibiotic. [35] The results presented in this report highlight the potential of rosemary extract as a source of antibiotic resistance modifying compounds. The MICs for the rosemary methanolic extract ranged from 1.56 to 6.25 mg/ml for all test microorganisms ($P < 0.001$), while MICs for aqueous extract ranged from 3.13 to 6.25 mg/ml ($P < 0.01$) (Table 1). These differences in the susceptibility of the test microorganisms to the test samples could be attributed to variation in the rate of samples' penetration through the cell wall and cell membrane structures. [36] In general, the methanolic extract showed greater antimicrobial activity than aqueous extract (Table 1). Taking the least IZD of the standard (Penicillin and Ampicillin) as the breaking point, most of the extracts passed the breaking point (Table 2 and 3).

It is quite difficult to attribute the antimicrobial effect of an extract to one or a few active principles, because extracts always contain a mixture of different chemical compounds. In addition to the major components, also minor components may make a significant contribution to the antimicrobial activity of extracts. Following the results above, we could infer that the antimicrobial activity of rosemary methanolic and aqueous extract is the synergistic effect of their compositions. It provided evidence that rosemary methanolic and aqueous extracts may become the potential natural antimicrobial in the field of food and pharmaceutical industries.

CONCLUSION

To sum up, this work represents that the methanolic and aqueous extracts from stems and leaves of *Rosmarinus officinalis* have obvious antibacterial activity; these data provide pharmacological basis for its therapeutic efficacy on infectious diseases.

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Table 1: Determination of Minimal Inhibitory Concentration (MIC) of *Rosmarinus officinalis* methanolic and aqueous extracts (mg/ml).

	Minimal Inhibitory Concentration (mg/ml)	
Bacterial strains	**Methanolic extract**	**Aqueous extract**
E. coli	1.56 ± 0.00	6.25± 0.00
K. pneumonia	3.13 ± 0.00	6.25± 0.00
P. aeruginosa	3.13± 0.00	6.25± 0.00
M. luteus	1.56± 0.00	6.25± 0.00
S. aureus	3.13± 0.00	6.25± 0.00
B. subtilis	1.56± 0.00	3.13± 0.00

Bs, Bacillus subtilis ATCC 6633, Pa, Pseudomonas aeruginosa ATCC 15442, Kb, Klebsiella pneumoniae ATCC 53153, Ec, Escherichia coli ATCC 54127, Sa, Staphylococcus aureus ATCC 6538, Ml, Micrococcus luteus ATCC 9341. Values are means±SD of three determinations. Data indicate significant difference ($P < 0.05$) with respect to positive control (Penicillin and Ampicillin).

Table 2: Inhibition by methanolic extract of *Rosmarinus officinalis* (zone size, mm)

	Different concentration of the methanolic extract (mg/ml)								Positive control	
Bacterial strains	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**Am (20 µg/ml)**	**Pe (20 µg/ml)**
E. coli	50	40	30	28	26	24	20	19	32	28
K. pneumonia	40	34	30	24	22	20	20	18	28	24
P. aeruginosa	40	26	20	20	19	18	18	17	18	18
M. luteus	60	52	34	30	28	26	24	24	33	30
S. aureus	46	34	28	26	24	24	22	20	26	24
B. subtilis	36	34	30	26	24	22	20	20	32	28

Bs, Bacillus subtilis ATCC 6633, Pa, Pseudomonas aeruginosa ATCC 15442, Kb, Klebsiella pneumoniae ATCC 53153, Ec, Escherichia coli ATCC 54127, Sa, Staphylococcus aureus ATCC 6538, Ml, Micrococcus luteus ATCC 9341, Am, Ampicillin, Pe, Penicillin. Values are means±SD of three determinations. Data indicate significant difference (P < 0.05) with respect to positive control (Penicillin and Ampicillin).

Table 3: Inhibition by aqueous extract of *Rosmarinus officinalis* (zone size, mm)

	Different concentration of the aqueous extract (mg/ml)								Positive control	
Bacterial strains	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**Am (20 µg/ml)**	**Pe (20 µg/ml)**
E. coli	26	24	20	18	18	16	16	14	32	28
K. pneumonia	26	22	20	20	18	17	14	12	28	24
P. aeruginosa	24	22	20	20	19	16	14	12	18	18
M. luteus	24	18	18	18	16	16	14	12	33	30
S. aureus	24	20	20	19	19	18	17	14	26	24
B. subtilis	30	28	26	26	24	22	20	20	32	28

Bs, Bacillus subtilis ATCC 6633, Pa, Pseudomonas aeruginosa ATCC 15442, Kb, Klebsiella pneumoniae ATCC 53153, Ec, Escherichia coli ATCC 54127, Sa, Staphylococcus aureus ATCC 6538, Ml, Micrococcus luteus ATCC 9341, Am, Ampicillin, Pe, Penicillin. Values are means±SD of three determinations. Data indicate significant difference (P < 0.05) with respect to positive control (Penicillin and Ampicillin).

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