

IN VITRO ANTIBACTERIAL ACTIVITY OF THE METHANOLIC AND AQUEOUS EXTRACTS OF *ANACYCLUS PYRETHRUM* USED IN MOROCCAN TRADITIONAL MEDICINE

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ABSTRACT

The antibacterial activity against six standard bacterial strains evaluated with the disc diffusion assay and the MTT assay to determinate the Minimum Inhibitory Concentration (MIC). The result of the antimicrobial activity indicated by zone of inhibition of growth ranged from 13.0 to 22.2mm. The antibacterial activity demonstrated that all extracts have an inhibitory activity against bacteria with the lowest MIC at 3.125mg/ml. The extract contains some major bioactive compounds that inhibit the growth of microorganisms thereby proving very effective as alternative source of antibiotics. Our results support the ethno-pharmacological uses of this plant in folk medicine and could provide useful data for the utilization of this extracts in pharmaceutical, cosmetic and food industries.

Keywords: *Anacyclus perythrum*, Root extract, Medicinal plants, Antibacterial activity, Minimum inhibitory concentration

INTRODUCTION

All over the world, infectious disease is the number one cause of death accounting for approximately one-half of all the deaths in tropical countries. This perhaps may be attributed to the increasing incidence of multiple resistances in human pathogenic microorganisms in recent years, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases¹. Medicinal plants have been used for centuries as remedies for human diseases as they contain components of therapeutic value²⁻⁴. Very few antimicrobial studies have focused on in vivo models^{5, 6}. Recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine. However, there have been some efforts to mimic the traditional use of plant material as noted by the addition of aqueous extracts in screening assays⁷⁻¹². Several methods are currently available to detect the antibacterial activity of plant extracts using different principles to assess bacterial growth or its inhibition. Some reports have focused on the antimicrobial screening together with other pharmacological investigations including toxicity^{13, 14}. In Morocco, there is more than 42,000 species of plants; divided into 150 families and 940 genus used in traditional medicine^{2, 15}. *Anacyclus Perythrum* (AP) locally known as "Aud el-attas", 'Akkar Karha' and 'Agargarha' is a species commonly used in Moroccan traditional medicine. The powder of the root is well known as sternutatory, diaphoretic and used for many ailments. Showing the root is considered to be sialagogue, and to relieve toothache. In liver diseases is recommended, mixed with olive oil it is used in rheumatism, sciatic, colds, neuralgic, and paralysis¹⁶. In addition, the AP is also used as cardiotonic to treat typhus fever¹⁷. The chemical analysis of the roots shows that they contain three fatty acids, one sterol and ten unsaturated amides, more specifically: pellitorine, anacycline, phenylethylamide, enetriyne alcohol, inulin, polyacetylenic amides I-IV, and sesamin. The plant contains also tannins, gum and essential volatile oil. Pyrethrine, an alkaloid, yielding pyrethric acid, is stated to be one of the active principles³². However the antibacterial activity has not yet been studied. Thus, the aim of this study is to evaluate the acute toxicity and the antibacterial activity of the methanolic and aqueous extracts of *Anacyclus perythrum* against different bacterial strains which may be involved in such diseases, using a preliminary bioassay screening and to quantify the minimum inhibition concentration (MIC) using colorimetric method¹⁸.

MATERIALS AND METHODS

Plant material

Anacyclus perythrum, from *Asteraceae* family, was collected in 28 March 2009, 5km east of Morocco with collection number 77765. The plant was identified by a botanist M. Fennane from the Scientific

Institute of Rabat (Morocco), where a voucher specimen has been deposited with number 3949.

Extraction procedure

The roots were air dried for 20 days, and then washed free from soil, powdered and stored in airtight containers at room temperature until extraction. 1kg of the powdered roots was extracted with methanol through a maceration process within a period of 48h. The dark residues were obtained after the evaporation of the solvent under reduced pressure at 40°C; the percentage of extracts yield was 12% (w/w). Simultaneously, water extract was prepared by adding 1.5L of distilled water to 1Kg of powdered plants roots and macerated within a period of 48h. It was then filtered and concentrated the extract under reduced pressure and percentage of extracts yield was 10% (w/w) for plant material.

Acute toxicity

The toxicological study was conducted according to the method of OECD Guideline 423. Swiss mice (20-30g) were kept at 23°C of temperature, 50% humidity, on a 12h light/dark cycle and were fed *ad libitum* with standard feed and water in the course of the study. The animals were obtained from the elevage of animal centre of Faculty of Medicine and Pharmacy of Mohammed-V Souissi University, Rabat. The extracts of plant were administered by oral route in a single dose. The mice of both sexes received the initial dose of methanolic and aqueous extracts of AP that was chosen 2000mg/kg of body weight. The control group received only the water. The animals must be individually observed, the symptoms and weight variation was recorded during the first 30min and regularly during the first 24h after treatment and daily for 14 days. Care and treatment of the mice were in compliance with the guidelines of the guide for the care and use of laboratory animals (commission on life science, national research council, 1996).

Antibacterial Activity Test

Bacterial strains

Six bacterial strains, namely *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 15442, *Klebsiella pneumoniae* ATCC 53153, *Escherichia coli* ATCC 54127, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 9341, 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.

Disk diffusion method and Minimum inhibitory concentration

Antibacterial activity of aqueous and methanolic extracts diluted in sterile distilled water, was evaluated by paper disc diffusion method.

Disks impregnated with sterile distilled water served as negative controls, Cephaxitin and Amikacin are used as positive control^{19, 30, 31}. The viability indicator MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide, Sigma, Aldrich) and the quick microplate metho²⁰ were used for the determination of the minimum inhibitory concentration (MIC) ²¹. For the inoculum, turbidity was adjusted to 1.5 x 10⁸ CFU/ml with the reference to Mc Farland turbidometer²². Serial dilutions of extracts were dissolved in distilled water and made in a concentration range from 25mg/ml to 200mg/ml in sterile test tubes. Each well of micro-titer plate was inoculated with 20µL of the bacterial suspension (15µl of Mueller Hinton broth and 5µl of the bacterial Inoculum) and 20µl of sample of various concentrations of the extracts. The plate was incubated under optimal conditions at 37°C for 24h. 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. Optical densities were measured at 570nm by using a microplate reader. The bacterial suspension changed to blue when bacterial growth occurred. The MIC is defined as the lowest concentration of antibiotic or extract at which there is no visible growth. All tests were performed in triplicate.

Statistical analysis

The mean values and standard deviations of all replicates described in the above tests were calculated using Delta Graph (USA). The data

of the extracts were statistically analysed by ANOVA. A student's t-test was computed for the statistical significance of the results. Differences were considered significant with $p < 0.05$.

RESULTS AND DISCUSSION

Acute toxicity of *Anacyclus pyrethrum*

During the 14 days, the evolution of the weight was established. Abdominal contraction was observed by 20-25min after the oral administration of aqueous extract of *Anacyclus pyrethrum* and no mortality was recorded. Generally, no toxicity was demonstrated at the dose of 2000mg/kg and the extracts of the plant does not lead to mortality neither by oral route. Under the system of global harmonization of chemicals (GHS), this product is classified category 5, which the LD50 was higher than 2000mg/kg.

Antibacterial potential of plant extracts

The antibacterial activity of methanol and aqueous extracts of AP were assayed in vitro by a disk diffusion method (Table1) using Cephaxitin and Amikacin as positif control against Gram positive and Gram negative pathogenic bacteria. All the assayed extracts significantly inhibited the growth of the bacterial strains, the methanol extract showed the highest antibacterial activity ($P < 0.05$), the diameter of the zone inhibition ranging from 16 - 40mm (Fig.1).

Table 1: Diameters of the inhibition zones of the growth of the bacterial strains in mm induced by Antibiotics of third generation.

	Standard reference bacterial strains					
	Gram-negative organisms			Gram-positive organisms		
The antibiotics	EC	KB	BS	SA	PA	ML
Cephaxitin 30 mcg/disc	28	32	33	26	18	35
Amikacin 30 ng/disc	24	26	26	23	14	25

BS: *Bacillus subtilis* ATCC 6633, PA: *Pseudomonas aeruginosa* ATCC 15442, KB: *Klebsiella pneumoniae* CIP 53153, EC: *Escherichia coli* ATCC 54127, SA: *Staphylococcus aureus* ATCC 6538 and ML: *Micrococcus luteus* ATCC 9341.

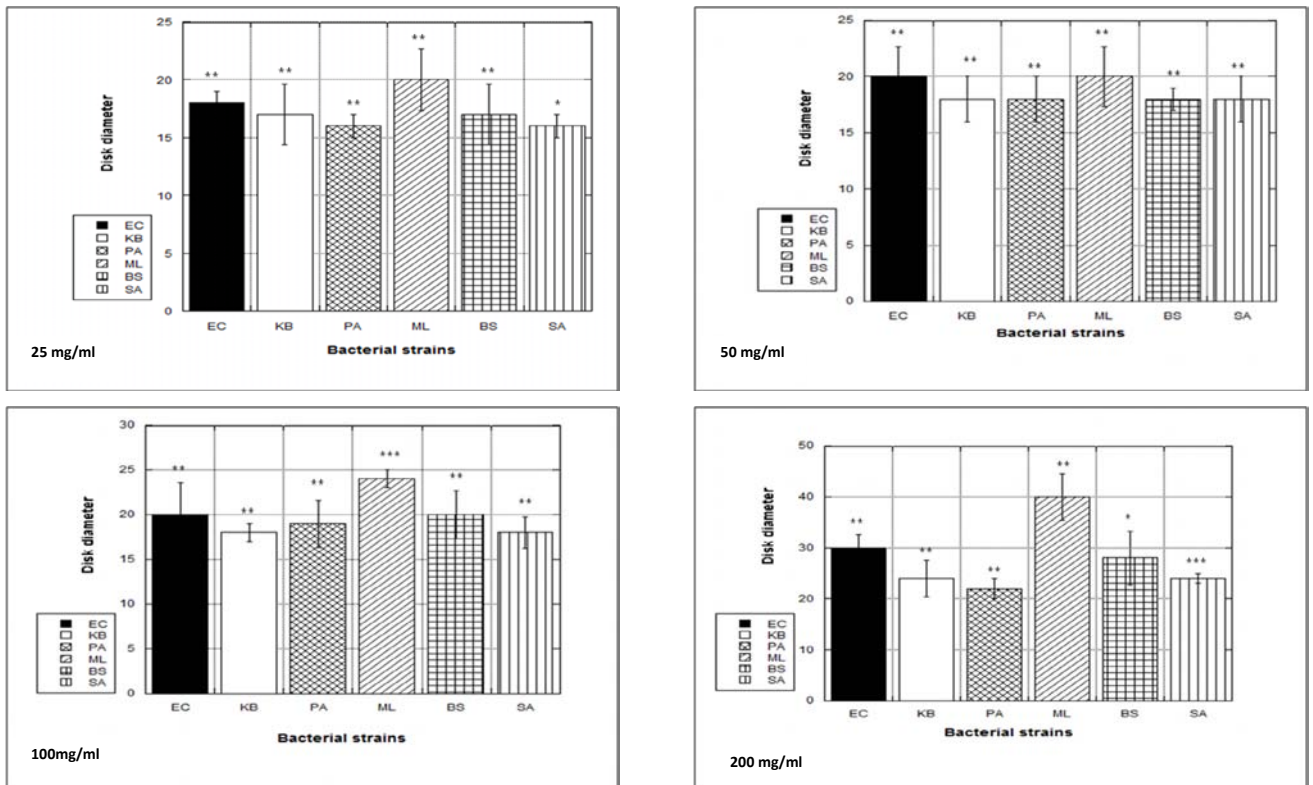


Fig. 1: Graph of the inhibition diameters in mm of methanolic extract tested on bacterial strains.

The significance was evaluated by means values ± S.E. of Student test in triplicate experiments: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

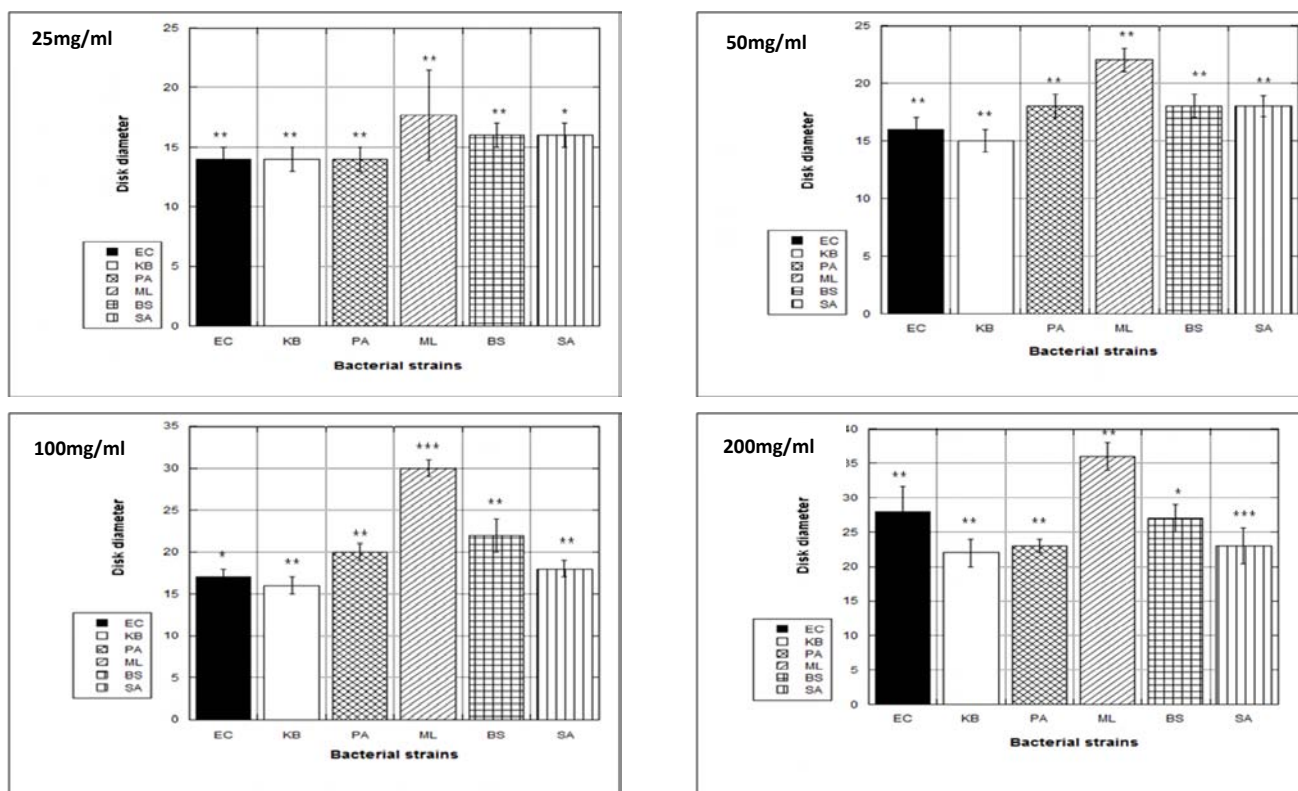


Fig. 2: Graph of the inhibition diameters in mm of aqueous extract tested on bacterial strains.

The significance was evaluated by means values \pm S.E. of Student test in triplicate experiments: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

According to the results, methanolic and aqueous extracts (Figure 2) were found to be active against all pathogenic bacteria and the best results were observed with methanolic extract. The strongest antibacterial activity was seen against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Micrococcus luteus* with a MIC value of 3.125mg/ml followed by MIC 6.25mg/ml while *Klebsiella pneumoniae* methanolic extracts and *Pseudomonas aeruginosa* aqueous extracts of AP. Overall, the reduction in bacterial viability was dose dependent and it was higher ($p < 0.05$) (Table 2).

Most of the researchers have evaluated first the antibacterial spectra of plant extracts; secondly they have determined the chemical composition of the extracts, and thirdly they have retroactively attributed such activity to such molecule²³⁻²⁷. In this study the extracts of AP showed higher antibacterial potential against different bacterial strains, this antibacterial activity is may be related to their secondary metabolites; alkaloids, phenolics and terpenoids reported in this plant (Guide to Medicinal Plants in North Africa).

Table 2: The minimum inhibitory concentration (MIC) of the extracts of the plant tested against different bacteria. Data represent the MICs (mg/mL).

Extracts	Standard reference bacterial strains			Gram-positive organisms		
	Gram-negative organisms			SA	ML	BS
AE. AP	EC	KB	PA	3.125	3.125	3.125
ME. A P	3.125	6.25	3.125	3.125	3.125	3.125

BS: *Bacillus subtilis* ATCC 6633, PA: *Pseudomonas aeruginosa* ATCC 15442, KB: *Klebsiella pneumoniae* CIP 53153, EC: *Escherichia coli* ATCC 54127, SA: *Staphylococcus aureus* ATCC 6538 and ML: *Micrococcus luteus* ATCC 9341, AE: aqueous extract; ME: methanolic extract; AP: *Anacyclus perythrum*.

Gram-negative bacteria which are responsible for a large number of infectious diseases have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial compounds²⁸. All extracts (methanolic and aqueous extracts of AP) showed antibacterial activity against both Gram-positive and Gram-negative bacteria. This gives an indication of the presence of promising antibacterial compounds. The selection of bacterial strains was based on their pathogenicity lead to infectious diseases, poor sanitary conditions, unclean unsafe drinking water and inappropriate food storage conditions are common in poor families that frequently suffer from diarrhea and vomiting. In most cases, they treat themselves by using local herbs²⁹. Our results against these bacterial strains suggest the efficacy of plant claimed by traditional healers. This plant may be a source which could yield

drug that could improve the treatment of infections caused by this organism.

CONCLUSION

The data indicate that the extracts of the *Anacyclus pyrethrum* species inhibit efficaciously some resistant bacterial strains which make serious sanitary problems worldwide and confirm that there is a good correlation between the reported traditional uses of the plant for infectious diseases. This indicates that this plant may be useful for developing alternative compounds to treat infections caused by these antibiotic resistant pathogens. According to these results, it is possible to conclude that *Anacyclus pyrethrum* had a strong and a broad spectrum of antibacterial activity. To the best of our knowledge, this is the first study to provide data that the methanolic

and aqueous extracts of *Anacyclus pyrethrum* evaluated against a wide range of bacteria.

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