

THE ANTIBACTERIAL EFFECT OF TWO MEDICINAL PLANTS *INULA VISCOSA*, *ANACYCLUS VALENTINUS* (ASTERACEAE) AND THEIR SYNERGISTIC INTERACTION WITH ANTIBIOTIC DRUGS

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ABSTRACT

With the emergence of multidrug-resistant organisms, combining medicinal plants with synthetic medicines against resistant bacteria becomes necessary. In this study, Synergism between plants extracts (methanolic extract, essential oils) of *Inula viscosa* and *Anacyclus valentinus* and two commonly used antibiotics: gentamycin and oxacillin were investigated on three bacterian strains *Escherichia. coli*, *Bacillus subtilis* and *Staphylococcus aureus*. In the first time, the antibacterial effect of extracts alone was tested against 7 strains by disc diffusion and microdilution methods. The minimum inhibitory concentrations of methanolic extracts ranged between 6.25 and 50mg/ml while that of the essential oils varied between 12.5 and 100µL/mL. The inhibitory concentrations of antibiotics varied between 125 and 31.25 µg/ml. Interactions extracts /antibiotics and extracts/extracts were determined by disc diffusion agar and by checkboard. The results show that the synergistic effect of combinations plant extracts/antibiotics was more important than extracts/extracts.

Keywords: antibiotic resistant; *Inula viscosa*; *Anacyclus valentinus*; synergy.

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1. INTRODUCTION

The wide use of antibiotics in the treatment of bacterial infections has led to the appearance of resistant strains. The increase of this phenomenon threatens public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality and health care costs. As a result, the need for new antimicrobial agents becomes greater than ever. One of the strategies is to explore medicinal plants [1].

Healing potential of plants has been known for thousands of years. Plants produce a whole series of different compounds which are not of particular significance for primary metabolism, but can have a remarkable effect to other plants, microorganisms and animals. These organic compounds are defined as biologically active substances, and include polyphenols and essential oils [2]. Many plant extracts have been evaluated not only for direct antimicrobial activity, but also as resistance-modifying agents with an indirect effect against many species of bacteria, by enhancing the activity of a specific antibiotic [3]. In recent years there have been many studies about the beneficial role of bioactive plant extracts in increasing the *in vitro* efficacy of commonly used antibiotics against variety of microorganisms. The ability of plant extracts to act synergistically with antibiotics could be a new approach to solve the problem of bacterial resistance and less susceptible bacteria [2].

Inula viscosa L. Aiton (*Asteraceae*) also known as yellow fleabane or viscous elecampane is a perennial plant distributed in different regions of the Mediterranean Basin. In traditional medicine, it has many uses, including anti-inflammatory, antioxidant, anti-ulcerogenic, antipyretic, anthelmintic, antiseptic, and antifungal activities [4]. *Anacyclus valentinus* L. Aiton (*Asteraceae*) called valence anacycle is annual and perennial specie centred in North West of Africa but also found in other Mediterranean countries including Algeria [5]. This plant is known for its anti-diabetic [6] and antifungal [7] effects. It is also used in some parts of the country as a food condiment.

The purpose of the present work was to determine the antibacterial activity of the chosen medicinal plants and to investigate the synergistic effects of these plants combined with antibiotics, thereby throwing light on the potential role of plants in increasing the effectiveness of antibiotics.

2. MATERIALS AND METHODS

2.1. Sampling

Leaves and flowers of *I. viscosa* and *A. valentinus* were harvested at the specified regions shown in table 1. The choice of crop regions is based on the nature of the soil and climate required for plant growth. The sampling was done in a clean area, away from pollution impact and after the disappearance of the morning dew.

Table 1. Geographical situation of the two regions and characteristics of harvesting conditions

	Region	Geography	Altitude (m)	Date
<i>Inula viscosa</i>	Chorfa (SIG)/Mascara	North West	161	October - December
<i>Anacyclus valentinus</i>	El-Bayadh	South West	1135	October

2.2. Preparation of plant extracts and volatile oils

Plant material, divided into two groups, the first group was plant methanol extracts (*Inula viscosa*, *Anacyclus valentinus*) was ground, extracted with 80 % methanol and filtered after 48 h. The plant residue was re-extracted with addition of 80% methanol, and after 24 h it was filtered again. Combined filtrates were concentrated on a rotary evaporator at 45°C for methanol elimination, and the extracts were kept under refrigerated conditions until use. The extracts dry weight was obtained by the solvent evaporation and used to determine concentration in mg/ml [8]. The second group of plant material was volatile oils of the same plants. The aerial parts of plants (100 g with 1l of distilled water) were hydrodistilled for 3 h. using a Clevenger-type apparatus to produce essential oils according to the method by [9]. The oils were in sealed vials at low temperature (4°C).

2.3. Bacterial strains and Antibiotic susceptibility test

Several bacterial strains were isolated from different environments and subjected to disk diffusion method using 06 different antibiotics according to Stephen G *et al* (2012). The antibiotic discs Amoxicillin (AX, 30µg), Oxacillin (OX, 5µg), Cefazolin (CZ, 30µg), Gentamycin (CN, 10µg), penicillin (P, 30 µg), and Spiramycin (SP, 10 µg) were used and the susceptibility was determined by the inhibition zone in mm.

In the end, seven strains were selected for their antibiotic resistance according to the standardization of susceptibility in human medicine at the national level [11] and the recommendations of the Committee on Antimicrobial the French Society for Microbiology (2008): three of them from Meslam Taib Hospital, Mascara (*E. coli*, *Staphylococcus aureus*,

Enterococcus faecalis), three from wastewater (*Salmonella typhi*, *Shigella* sp., *Bacillus subtilis*) and *Clostridium* sp. from the great Sabkha Oran.

Table 2. Antibiotic resistance profile of tested bacterial strains

	P	AX	OX	CZ	SP	CN
<i>Escherichia coli</i>	R	R	R	R	R	R
<i>Salmonella typhi</i>	R	R	R	R	R	R
<i>Shigella</i> sp.	R	R	R	R	R	R
<i>Bacillus subtilis</i>	R	R	R	R	R	R
<i>Clostridium</i> sp.	R	R	R	R	R	I
<i>Staphylococcus aureus</i>	R	R	R	R	R	I
<i>Enterococcus faecalis</i>	R	R	R	R	R	R

2.4. Disk diffusion assay of medicinal plants

The essential oils and methanolic extracts were screened for antibacterial activity using the agar diffusion technique against strains selected. Filter paper discs (Whatman No.1, 6 mm diameter) containing 10 µl of each essential oils and methanolic extracts were applied to the surface of agar plates that were previously seeded by spreading of 0.1 ml from overnight culture. 80 % methanol (v/v) and DMSO impregnated disc were used to check whether they have any inhibitory effect on bacterial growth. The plates were incubated at 37°C/18H and the resulting inhibition zone was measured in mm. Moreover, this area is great, more bacterial species are sensitive [10].

2.5. Determination of minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The microtiter plate dilution method was used to determine the MIC of compounds under study. Sterile 96-well microplates were used for the assay. All wells were filled with 50 µL of Muller Hinton broth (MHB). Extracts were dissolved in DMSO and added to the first well (50 µL). Serial two-fold dilutions were made then. An over-night culture of bacteria suspended in MHB was adjusted to turbidity equal to 0.5 McFarland standards. The plates were inoculated with bacterial suspension (50 µL/well) and incubated at 37 °C for 24 h [10]. Each test included two growth controls consisting of the medium with the solvent (DMSO) and medium with bacterial suspension. Then the turbidity was measured every two hours using

micro-plate reader (TECAN brand) at 620 nm wavelength. The lowest concentration showing no culture was considered as the MIC and it's express as (mg/ml, μ l/ml).

MBC were conventionally measured by subculturing in 10 μ l of streaks without visible bacterial growth broth (from MIC) onto a trypticase soy agar or nutrient agar. The percentage of germs surviving compared to the initial inoculum was determined by comparing the number of colonies by streaking appeared after 24 hours of incubation at 37 ° C. The MBC is the lowest concentration of antibacterial agent allowing only 0.01% of survivors of the initial inoculum [10].

2.6. Tolerance to natural antibacterials tested

The calculation for each strain of the report MBC / MIC controls whether a strain has a tolerance to antibacterial agents (if the report exceeds 32) [12].

2.7. Synergy testing

Three strains were selected for this essay (*E. coli*, *Staphylococcus aureus* and *Bacillus subtilis*). An over-night culture of bacteria was adjusted to turbidity equal to 0.5 McFarland standards and inoculated on the surface of Mueller-Hinton agar plates. Subsequently, the antibiotic disk was placed on the surface of each inoculated plate and then added 20 μ l of plant extract (at a concentration of 50 mg/ml for methanolic extract and 250 μ l/ml for essential oils), to identify synergies effect between the plant extract and antibiotics. While to identify synergies between the two plant extracts, 20 μ l of *I. viscosa* extract and 20 μ l of *A. valentinus* extract were mixed and put together on a filter paper disk. Disks of plant extracts and antibiotics alone were used to compare effects. The plates were left for 15 minutes to dry at 4°C, and then incubated at 37° C for 24 h. The diameters of clearing zones were measured [13].

2.8. The checkerboard essay

The checkerboard method was used for the determination of synergy between the antibiotics and natural antimicrobials [10]. For each strain, the MIC of the extracts and the antibiotic used in combination were measured by crossing different concentration ranges of an agent with each other. The preparation of inoculums, sowing, incubations and readings were similar to those described for the CMI measures. Using this method, one is able to recognize synergistic, additive, indifferent, or antagonistic interactions occurring with the agents being tested. It is often combined with calculation of a fractional inhibitory concentration (FIC)

index which is the lowest concentration of drugs combination permitting no visible growth of the test organisms. The FIC is calculated and interpreted as follows:

$$\Sigma \text{ FIC} = \text{FIC of agent A} + \text{FIC of agent B} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}} + \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$$

Synergy is defined as $\Sigma \text{ FIC} \leq 0.5$, Indifference is defined as $0.5 < \Sigma \text{ FIC} \leq 4$, Antagonism is defined as $\Sigma \text{ FIC} > 4$, Additive when $0.5 < \Sigma \text{ FIC} \leq 1$.

2.9. Statistical analysis

Results obtained were subjected to statistical analysis using one way analysis of variance. All data were the average of three experiments.

3. RESULTS AND DISCUSSIONS

3.1. Characteristics of methanolic extracts and essential oils

Methanolic extracts of the two plants have a dark color and a strong odor, with a viscous aspect of AVME. The latter registered a higher yield (17.82%) than the IVME (12.46%). Essential oils from the aerial parts of the two plants have a liquid appearance, yellow color with strong and characteristic odors. In this case, the yield of essential oil of *A. valentinus* (0.63%) was lower than *I. viscosa* (1.49%). This may be due to the climatic conditions of the plant. The yield depends on the geographical origin of the plant, the season of harvest, method and conditions of the extraction. It is only relative [14].

3.2. Determination of minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The results obtained from the analysis of antibacterial activity exhibited by studied plant extracts are summarized in Table 3. Among of antibacterial agents used, methanolic extracts and essential oils of *A. valentinus* showed the highest activity against strains tested with MIC ranged between 3.125 – 50 mg/ml and 31.25 – 125 μ l/ml respectively. *Inula viscosa* is more active against Gram + bacteria which is shown by [15, 16]. Comparing to the study of Chaouki Selles *et al* (2013) applied on *A. Pyrethrum*, our plant is more active.

The biological activity of *plant extracts* against tested bacteria could be attributed to the presence of biologically active components such as flavonoïds; phenolic acids and terpenoïds[17].

Table 3. The minimum inhibitory concentrations (MICs) of plant extracts and the antibiotics used

	Methanolic Extracts (ME) mg/ml		Essential Oils (EO) µl/ml		Antibiotics (ATB) µg/ml	
	IVME	AVME	IVEO	AVEO	OX	CN
<i>E. coli</i>	25	3.125	125	31.25		250
<i>S. typhi</i>	50	12.50	125	31.25		125
<i>Shigella sp.</i>	25	12.50	125	62.50		125
<i>B. subtilis</i>	25	6.25	62.50	31.25		62.50
<i>Clostridium sp.</i>	25	12.50	125	62.50		62.50
<i>St. aureus</i>	12.50	12.50	62.50	31.25	250	
<i>En. faecalis</i>	6.25	6.25	62.50	62.50		125

Table 4. The minimum bactericidal concentrations (MBCs) and the report MBC/MIC of plant extracts

	MBC (mg/ml, µl/ml)				REPORT MBC/MIC			
	Methanolic Extracts (ME)		Essential Oils (EO)		Methanolic Extracts (ME)		Essential Oils (EO)	
	IVME	AVME	IVME	AVME	IVME	AVME	IVEO	AVEO
<i>E. coli</i>	50	25	125	250	2	8	1	8
<i>S. typhi</i>	50	25	250	125	1	2	2	4
<i>Shigella sp.</i>	50	25	250	125	2	2	2	2
<i>B. subtilis</i>	50	50	250	62.50	2	8	4	2
<i>Clostridium sp.</i>	50	50	250	125	2	4	2	2
<i>St. aureus</i>	50	50	250	125	4	4	4	4
<i>En. faecalis</i>	25	50	250	250	4	8	4	4

The MBC values were closer than MIC values for all extracts and the report MBC/MIC ranged between 1 and 2 for most strains. It reached 8 for *E. coli* in the presence of *A. valentinus* extracts (Table 4). The action of the whole extracts is bactericidal against all strains. These results are consistent with those obtained by Laghrifi *et al.* [17] on *I. viscosa* methanolic extract.

3.3. Synergy testing

Synergistic interaction between two agents means that their joint effect is stronger than the sum of effects of the individual agents. Combinations of antibacterial agents tested for possible synergistic interactions are demonstrated on figures 1, 2. In general, the zones of inhibition of combinations ATB/EO were remarkable than ATB/ME especially for *A. Valentines* with a diameter of 14mm.

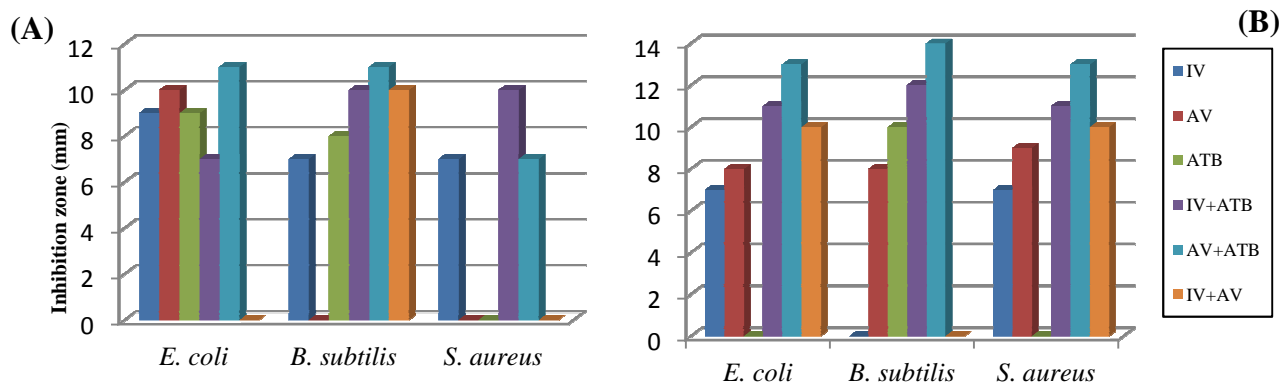


Fig.1. Inhibition diameters (mm) of antibacterial agent alone and with combination, **(A)**. Methanolic extracts, **(B)**. Essential Oils

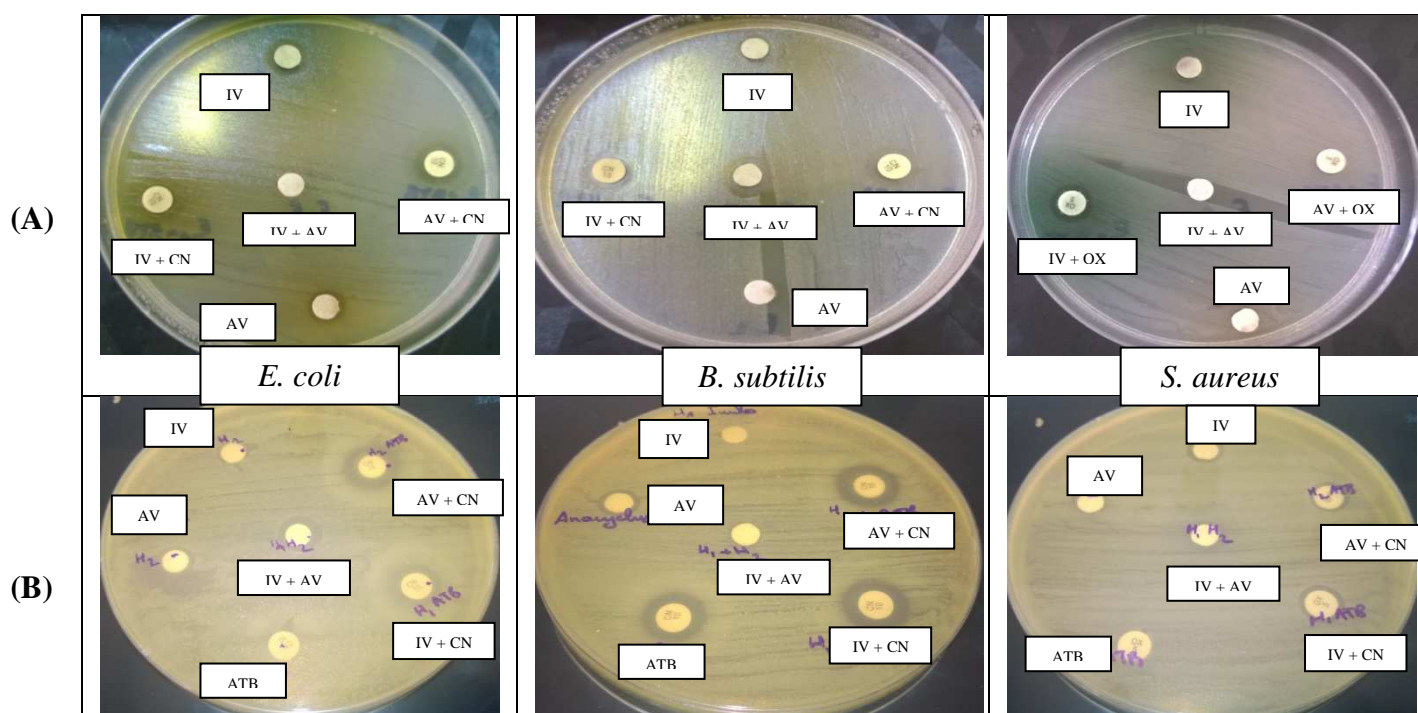


Fig.2. Inhibition zones (mm) of antibacterial agent alone and with combination on plate agar, **(A)**. Methanolic extracts, **(B)**. Essential Oils

Based on the FIC calculations (Table 5), the combinations of compounds involving plant extracts and antibiotics showed synergistic and additive/indifference effects. Therefore, an antagonism between plant extracts was demonstrated, except essential oils combinations which showed a synergistic effect.

Table 5. Fractional inhibitory concentrations (FIC Index) of different combination of plant extracts and the antibiotics

	METHANOLIC EXTRACTS			ESSENTIAL OILS		
	<i>IVME</i>	<i>AVME</i>	<i>IVME</i>	<i>IVEO</i>	<i>AVEO</i>	<i>IVEO</i>
	+ <i>ATB</i>	+ <i>ATB</i>	+ <i>AVME</i>	+ <i>ATB</i>	+ <i>ATB</i>	+ <i>AVEO</i>
<i>E. coli</i>	0.15 Synergy	0.08 Synergy	6 Antagonism	0.06 Synergy	0.067 Synergy	0.09 Synergy
<i>B. subtilis</i>	0.30 Synergy	0.36 Synergy	2.5 Indifference	0.09 Synergy	0.09 Synergy	8.24 Antagonism
<i>S. aureus</i>	0.6 Additive	1.125 Indifference	9 Antagonism	1.006 Additive	2.007 Indifference	0.09 Synergy

These beneficial interactions can be explained by the fact that the compounds contained in plant extracts are believed to disturb permeability of the cytoplasm membrane and there by facilitate the influx of antibiotics [18-19]. The results presented in this report highlight the potential of *I. viscosa* and *A. Valentinus* extracts to have a direct and indirect antibacterial activity as a source of antibiotic resistance modifying compounds.

In fact, several studies [3, 20, 21] showed that there are varied interactions between plant extracts and antibiotics. Therefore, our study was the first approach testing the synergistic effect of the plants used (*I. viscosa* and *A. valentinus*) and the interaction combinations of extract/extracts.

In conclusion, this work confirms the antibacterial activity of methanolic extracts and essential oils of *I. viscosa* and *A. valentinus*. It shows their potential use as agents which enhance antibiotic activity. This indicates that these plants may be useful for developing alternative compounds to treat bacterial infections. The use of extracts as antimicrobial agents shows allow risk of increasing resistance to their action, because they are complex mixtures, making microbial adaptability very difficult and it is also reported to have minimal side effects.

Further study can be made to isolate the pure compounds responsible for the activity from the extracts.

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