

## Phytochemical Screening, Antibacterial and Synergistic activity of *Dittrichia Viscosa* Extracts against Multi-Resistant Pathogenic Bacteria

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### Abstract

**Study contextual:** Faced with the global problem of antimicrobial resistance, and the use of traditional medicine for the research of antibacterial biomolecules.

**Aim:** our work focused on the valorization of a medicinal plant *Dittrichia viscosa* which has many therapeutic and culinary virtues worldwide.

**Methods:** To do this, a phytochemical screening of the leafy stems of the plant is carried out according to a set of physicochemical reactions, as well as an in vitro evaluation of the antibacterial activity, of the aqueous and ethanolic extracts against multi-resistant bacterial strains, by microdilution technique on microplates. An evaluation of the synergistic interaction between extracts and weakened antibiotics against pathogenic bacteria was also highlighted in this study.

**Results:** The tests revealed the richness of *Dittrichia viscosa* species by tannins, flavonoids, saponosides, sterols and triterpenes. As for the antibacterial effect, the MICs range from  $0.858 \pm 0.29$  to  $66.66 \pm 0.00$  mg / ml and the MBCs from  $4.300 \pm 1.01$  to  $11.610 \pm 2.31$  mg / ml is an interesting antibacterial activity. Regarding the combination of extracts with antibiotics tested, it revealed a synergistic action inducing an amplification of the antibacterial power of Penicillin, Imipenem and Erythromycin with a rate that reaches 471%.

**Conclusion:** The results of this study show that the aqueous and ethanolic extracts of *Dittrichia viscosa* have interesting and promising antibacterial activity in the pharmaceutical, food and cosmetic industries.

**Key words:** *Dittrichia viscosa*; phytochemical screening; antibacterial activity; synergistic effect

### Introduction

Bacterial resistance to antibiotics appeared with the introduction of antibiotics as a therapeutic treatment as early as the 1940s. Starting in the 2000s, several alert plans were launched to manage the use of these drugs. These actions were answered by a decrease in consumption until 2010, but which did not last long [1]. This issue of bacterial resistance is related to the massive or inadequate use of antibiotics that may even increase the risk of toxicity to the patient, reveal adverse effects, cause drug interactions and super infections, even lead to death [2].

The search for therapeutic alternatives will therefore be necessary to minimize the damage and limit the spread of this problem. Indeed, a series of recent studies have focused on the research of plant-based products, with an important antibacterial power, or also to combine them with antibiotics in order to amplify their antibacterial activities. Certainly, this study is a contribution to the fight against the emergence of multi-resistant bacterial strains and the search for neo-biomolecules for their eradication.

Our work consists of a phytochemical characterization of the *Dittrichia viscosa* plant and the evaluation of its antibacterial and synergistic activity of its aqueous and ethanolic extracts against multidrug-resistant pathogenic bacteria.

### Material and Methods

#### Plant material

*Dittrichia viscosa* called inul, is a perennial, wild and all covered with glandular hairs that release an odoriferous and sticky resin, with a smell of camphor. It is characterized by a taproot and deep of a height of about 1.50 m. This species has very branched stems of dense foliage inserted directly without petioles. Its leaves are alternate, elongate to lanceolate which become woody and dark at the base with age. Regarding the flowers, they are grouped in heads, either with petals welded in yellow tabs, or orange-yellow tubes [3].



## Harvest and preparation of the plant

The harvest of leafy stems of *D. viscosa* was done manually, in May of 2016, from the edges of Oued Rdoum to the city of Sidi Kacem (Latitude: 34.221 °, Longitude: -5.707) in Morocco.

The species has been identified at the Plant Biotechnology and Molecular Biology Laboratory of the Faculty of Science of Moulay Ismail University, Meknes [4]. This plant material was sorted and dried in the shade at room temperature. After drying, electric grinding is performed to obtain a fine powder which is used for the preparation of the extracts.

## Strains bacterial tested

The evaluation of the antibacterial activity of *D. viscosa* extracts was performed against five pathogenic bacteria. Four were clinically isolated from different pathological products of a Private Medical Analysis Laboratory, and *Listeria monocytogenes* was provided by CNRST's Laboratory of Microbiology and Molecular Biology (LMBM). Their removal and isolation were carried out in accordance with the hygiene standards in force [5] and using the appropriate selective culture media.

The strains tested are:

- Cocci and bacillus Gram (+): Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes*.
- Bacilles Gram (-) : *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Salmonelle sp.*

## Preparation methods of the extracts of the plant

Two types of extract were prepared from the powdered leafy stems of *D. viscosa*, aqueous and ethanolic extract.

### • Aqueous extract

One hundred grams of vegetable of the plant are boiled for 15 minutes in one liter of distilled water. This decoction of 10% cooled was filtered successively on hydrophilic cotton and on Watman paper N° 1. The filtrate obtained was then put in the oven at 55 °C until complete desiccation. The dilution of the residue in water is made to obtain defined concentrations expressed in mg / ml.

### • Ethanolic extract

The ethanolic extract is obtained with magnetic stirring at room temperature by maceration of 100 g of the vegetable powder in a volume of 300 ml of ethanol for 24 hours. Then, the extract is filtered using the hydrophilic cotton and then Wattman paper N° 1. The filtrate collected is dried in an oven at 50 °C for the removal of ethanol and obtaining a dry residue expressed in mg. From this residue different concentrations are prepared by its dilution in dimethyl sulfoxide (DMSO), and they are expressed in mg / ml.

## Phytochemical Screening

It is a technique based on a set of physicochemical reactions, allowing determining the different chemical groups contained in a plant organ. This study is essential to understand the activities and effects of plants.

The realization of this phytochemical study was established by reactions in solution in test tubes according to the protocol described by Diallo [6], whose main phytochemical groups highlighted are: alkaloids, polyphenols (flavonoids, anthocyanins, and tannins), saponosides, steroids, sterols and triterpenes.

## Study of antibacterial activity

### • Preparation of the inoculum

The inoculum is prepared from a bacterial culture of 18-24 hours, grown on agar medium and incubated at 37 °C, taking several colonies of the same morphology and suspending them in saline with a sterile loop or a cotton swab. To have an inoculum with a density equivalent to Mc Farland Standard 0.5 [5].

### • Determination of the minimum inhibitory concentration (MIC)

MIC is the minimum concentration of the extract that inhibits the growth of 90% of the bacterial population after incubation for 18 to 24 hours at 37 °C. The determination of the MICs of the plant extracts with respect to the bacterial strains is carried out according to the microtitration technique on microplates described by Ennacerie and his collaborators [7, 8, 9]. After incubation, the bacterial viability indicator, [2, 3, 5] -triphenyl-2H-tetrazolium chloride (TTC), is added to each well. Wells where bacterial growth has occurred show a pink color.

The growth controls are prepared in isolated wells containing the culture medium and the bacterial strains tested, without adding the extract. The number of repetitions is three times for each of the tests performed.

### • Determination of the minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the lowest concentration of substance that leaves at most 0.01% of surviving germs. It is determined by following the protocol cited by Yao and his collaborators [10, 11]. Each experiment is performed three times in three successive experiments.

Referring to the CMB / MIC ratio, the antibacterial effect can be judged; if the ratio CMB/MIC < 4 the effect is bactericidal, and if CMB / MIC > 4 the effect is bacteriostatic [12, 13].

## Synergistic antibacterial interactions between extracts and antibiotics: Disk diffusion method

This technique consists in evaluating the effect of the combination of extracts and antibiotics to which the tested strains are resistant. For each strain two antibiotics to which they are resistant are tested. The test is carried out on a solid medium according to the protocol described by Ennacerie and his collaborators [9,14,15].

Regarding the negative test, it is presented by a disk impregnated with distilled water or DMSO according to the type of aqueous or ethanolic extract.

The interaction of the antibiotic and the extract can produce four main types of effects:

- **Indifference:** the activity of the extract has no influence on the activity of the antibiotic;
- **Addition:** the effect of the association is equal to the sum of the effects produced by each of the agents taken separately;
- **Synergy:** the effect of the association is greater than the sum of the effects produced by each of the agents taken separately;
- **Antagonism:** the effect of the combination is less than the sum of the effects produced by each of the antibiotics taken separately [15].

## Results and Discussion

### Phytochemical Screening

The preliminary evaluation of the phytochemical composition of the leafy stems of the *D. viscosa* plant made it possible to obtain the results summarized in **Table 1**.



Groups of compounds	Classes	<i>D. viscosa</i>
Nitrogen compounds	Alkaloids	-
	Tannins	+++
	Gallic Tannins	+++
	Catechetical Tannins	-
Polyphenolic compounds	Free Flavonoids	++
	Anthocyanins	-
	Leucoanthocyanins	-
Terpene compounds	Steroids	-
	Sterols and triterpenes	++
	Saponosides (Foam index)	100

**Table 1:** Results of characterization reactions of chemical groups in leafy stems of *D. viscosa* plants.

The saponoside characterization is carried out by determining the height of the foam formed in the tubes.

+++ : Frankly positive reaction; ++ : Positive reaction; + : Reaction moderately positive; - : Shady reaction; - : Negative reaction.

Phytochemical screening allowed us to highlight the presence of secondary metabolites in the plant tissues of the studied plant. The detection of these chemical compounds is based on tests of constituent solubilities, precipitation and turbidity reactions, and a specific color change.

In fact, *Dittrichia viscosa* leafy stems contain gallic tannins, flavonoids, saponosides, sterols and triterpenes, and they lack alkaloids, steroids, anthocyanins and leucoanthocyanins. Referring to previous studies conducted by Boumaza in Algeria, we note a similarity in results concerning the presence of tannins, flavonoids, saponosides, sterols and triterpenes, but a difference for anthocyanins that are absent in our case [16]. Other studies have identified the phytochemistry of the aerial part of the same plant, they revealed that it contains flavonoids, sesquiterpene acids and triterpenes esters [17]. According to Cohen et al. [18] *D. viscosa* leaves also contain phenolic compounds, terpenoids and sesquiterpene lactones. The difference in chemical composition is directly related to climatic and soil factors, as well as the physiological characteristics of the plant.

#### Antibacterial activity of *D. viscosa* extracts

The profile of antibiotic resistance bacteria tested (**Table 2**), revealed a high resistance level for the majority of antibiotics prescribed currently in antibiotherapy [19].

Antibiotic disc	Disc load	SARM	<i>Listeria monocytogenes</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Salmonella sp</i>
Ampicillin	10 µg	NT	R	NT	NT	R
Amoxicillin	25µg	NT	NT	R	NT	NT
Amoxicillin + Clavulanic acid	20/10µg	NT	NT	R	NT	NT
Penicillin G	6 µg	NT	S	NT	NT	NT
Ceftriaxone (C3G)	30 µg	S	NT	S	NT	S
Ceftazidime (C3G)	30 µg	NT	NT	R	R	S
Ticarcillin	75 µg	NT	NT	RN	R	NT
Imipenem	10 µg	NT	NT	NT	R	NT
Oxacillin	5 µg	R	NT	NT	NT	NT
Meropenem	30 µg	NT	S	NT	NT	NT
Gentamycin	15 µg	S	NT	S	S	S
Ciprofloxacin	5 µg	R	NT	R	R	S
Ofloxacin	5 µg	R	NT	S	S	NT
Sulphamethoxazol + Trimethoprim	1,25/23,75 µg	R	S	R	S	S
Doxycycline	30 µg	S	NT	R	NT	NT
Erythromycin	15 µg	R	S	NT	NT	NT
Streptomycin	300µg	NT	NT	NT	NT	R



<b>Colistin sulfate</b>	50µg	NT	NT	NT	NT	R
<b>Ceftriaxone</b>	30 µg	NT	NT	NT	NT	S
<b>Tetracycline</b>	30µg	NT	NT	NT	NT	S
<b>Cefamandole</b>		NT	NT	NT	NT	S
<b>Chloramphenicol</b>	30 µg	NT	NT	NT	NT	S
<b>Kanamycin</b>	30µg	NT	NT	NT	NT	S

**Table 2: Antibiotic resistance profile of tested bacteria.**SARM: *Staphylococcus aureus* resistant to methicillin.

(-): Not determined NT: Not tested

The antibacterial effect of the two aqueous and ethanolic extracts prepared from the leafy stems of *D. viscosa* on the five strains tested is reported in **Table 3**.

The results of the antibacterial activity reveal that the MICs of the two aqueous and ethanolic extracts range from  $0.858 \pm 0.29$  to  $66.66 \pm 0.00$  mg / ml with a strong activity for the alcoholic extract. The latter showed a stronger inhibition of bacterial growth against Gram-negative than Gram-positive strains. While the decoction generally has the same lower activity against both types of Gram is a MIC of  $33.33 \pm 0.00$  mg / ml,

except for *Pseudomonas aeruginosa* where the MIC is  $66.66 \pm 0.00$  mg / ml.

Regarding MBC, **Table 3** shows that decoction 10% does not cause any lethal effect on the five strains tested. However, the ethanolic extract has MBCs ranging from  $4.300 \pm 1.01$  to  $11.610 \pm 2.31$  mg / ml. Based on the MBC / MIC ratio, it is clear that the alcoholic extract of *D. viscosa* has a bactericidal effect against both Gram-positive germs, MRSA and *Listeria monocytogenes*, moderately bactericidal against *Salmonella* sp and *Pseudomonas aeruginosa* and a bacteriostatic effect against *Klebsiella pneumoniae*.

Extracts		Gram -			Gram +	
		<i>Klebsiella pneumoniae</i>	<i>Salmonelle sp</i>	<i>Pseudomonas aeruginosa</i>	SARM	<i>Listeria monocytogenes</i>
Ethanolic extract	MIC	1,425 ±0,40	2,423 ±0,55	0,858±0,29	1,425±0,43	3,096±0,71
	MBC	11,610±2,31	10,2±1,16	4,300±1,01	4,583±0,41	9,16±0,12
	MBC/MIC	>4	≥4	≥4	<4	<4
Decocted 10%	MIC	33.33±0,00	33,33±0,00	66,66±0,00	33.33±0,00	33.33±0,00
	MBC	-	-	-	-	-
	MBC/MIC	-	-	-	-	-

(-) : Indeterminate  
**MRSA: Methicillin-resistant *Staphylococcus aureus*.**

**Table 3: Antibacterial Activity Evaluated by Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Expressed in mg / ml and the CMB/MIC of Extracted Leaf Stems of *D. viscosa***

The comparison of these results with those of other studies shows a correlation in results with those found by Rhimi and his collaborators [20] whose effect of the ethanolic extract was stronger against gram-type bacteria negative. The sensitivity of the latter is related to the characteristics of their membranes and their permeability to bioactive molecules.

Regarding the effect of the extracts of this plant, based on the MBC / CMI ratio, we notice a difference in the bactericidal power between *D. viscosa* of the province of Sidi Kacem and that collected by Laghrifi and his collaborators around the city of Fez to Ain Taoujtat in Morocco [21]. They revealed a bactericidal effect against *Klebsiella pneumoniae*. This difference between the results that it is between the two distinct extracts of the same plant, or the same extract of the same species of two different

regions, is explained firstly, by the difference in the chemical composition of the extracts which depends on the solvent used and the extraction technique. Secondly, by the chemical composition and the concentration of substances with antibacterial activity which depend on the edaphic and climatic factors as well as the drying technique. It is also reported that despite the low content of active molecules or their low levels, their synergy may be responsible for a strong antibacterial activity.

#### Synergistic antibacterial interactions between extracts and antibiotics

The results of the combination of decoction 10% and ethanolic extract of *D. viscosa* with nine antibiotics against the five strains tested are summarized in **Table 4**.



		Diameters of the inhibition zones (mm)		
		Combined extract with antibiotics		
Bacterial Strain	Antibiotic Code and Critical Diameter	Antibiotic Alone	Ethanol Extract	Decocted 10%
SARM	(Extract alone)		23	7
	E<18	8±0.00	6	40
	CN <18	17.33±0.88	15	6
<i>Listeria monocytogenes</i>	(Extract alone)		19.5	8.5
	E<25	7±0.00	30	14
	P<13	7±0.00	40	13
<i>Klebsiella pneumoniae</i>	(Extract alone)		37	7.8
	CRO<23	16±3.66	7	6
	C<20	11.5±0.5	0	10
<i>Pseudomonas aeruginosa</i>	(Extract alone)		25	6
	AK<15	14.5±0.44	15	14
	IMP<17	12±0.00	38	24
<i>Salmonelle sp</i>	(Extract alone 1)		35	7.5
	CTX<23	10±0.00	8	10
	CT<15	13±0.66	10	13

**Table 4:** Combination effect extracted from *D. viscosa* / antibiotic against five bacterial strains.

MRSA: Methicillin-resistant *Staphylococcus aureus*

P : Pénicilline (10 Unités), CN : Gentamicine (10µg), E : Erythromycine (15µg), CRO : Ceftriaxone (30µg), C : Chloramphénicol (30µg), IMP : Imipénème (10µg), CTX : Cefotaxime (30µg), CT : Colistine (50µg), AK : Amikacine (30µg)

Of the 20 combinations, 15 showed an antagonistic effect by reduction of inhibition diameter. While five combinations allowed the amplification of the antibacterial power of antibiotics from a percentage of 75 to 471%. This synergistic effect is recorded for three antibiotics, Penicillin and Imipenem from the beta-lactam family and Erythromycin from the Macrolide family against three resistant strains MRSA, *Listeria monocytogenes* and *Pseudomonas aeruginosa*.

The analysis of these results shows that the two types of extract can improve the antibacterial power of antibiotics during their association. However, the explanation of this amplifying effect requires in-depth studies that aim to elucidate the detailed mode of action of the active ingredients of each extract on pathogenic bacteria. However, this component is still poorly illustrated.

Referring to the results of other research, that of Side Larbi and his collaborators [22] treating extracts of *D. viscosa*, they found a synergistic effect of 77% in the case of the association of the methanolic extract of this species harvested from Algeria, with Gentamicin against *E. coli*.

This synergism between antibiotics and extracts is generally related to the destabilization of the bacterial wall. Indeed, it can be assumed that the components of the extracts facilitate the penetration of antibiotic molecules and thus their access to their intracellular targets.

More particularly, according to Esimone et al. [23] polyphenols coupled with β-lactams can enhance antibacterial activity by disrupting cell membrane transpeptidation. As for the combination of imipenem with extracts, it promotes membrane permeability and makes it easier for antibiotic molecules to reach their intracellular target while helping stop bacterial growth. For Macrolides, their association with biologic agents

can inhibit bacterial overgrowth by reversible binding to the bacterial ribosome 50s subunit, which can prevent transpeptidation and translocation reactions, resulting in synthesis of RNA-dependent proteins [24,25].

Taking into account the results of the antibacterial activity of *D. viscosa* extracts against *P. aeruginosa* resistant to imipenem, it was noted that the aqueous extract was not effective for the inhibition of its growth compared to the ethanol extract and whose explanation can be attributed to the texture of the wall of this bacterium which is provided with an outer membrane rich in phospholipid and forming a barrier impermeable to hydrophobic molecules [26]. In addition, its resistance to imipenem is due to a loss of porin D2 causing a decrease in its permeability, and a production of chromosomal céphalosporinase [27]. The combination of the two extracts with imipenem has produced a restoration of the sensitivity of this strain by an improvement of more than 100% of the antibacterial power of this antibiotic. The inhibitory effect of the growth of this bacterium is most likely due to the creation of porosity and an increase in cell permeability in favor of antibiotic entry. As it can be explained by the breaking of other mechanisms of resistance of this germ. In addition, this surprising result in restoring the weakened efficacy of imipenem on *P. aeruginosa*, an opportunistic and multidrug-resistant pathogen that is constantly evolving, is a promising and promising addition to antibiotic therapy that is currently therapeutic impasse for certain bacterial strains.

*L. monocytogenes*, also revealed this synergistic effect with the two antibiotics erythromycin and penicillin whose maximum amplification is evaluated at 471%. The wall of this strain is characterized by a low content of phospholipids hence the ease of contact of the phospholipid

bilayer of the bacterial membrane with the hydrophobic compounds. This factor may be responsible for enzymatic system failure, or ion leakage and loss of vital intracellular components [28], hence the amplification of the growth inhibitory power of the antibiotic.

Evaluating the effect of extract / antibiotic combinations is a method that can provide therapeutic and socioeconomic solutions. It is an opportunity for valorization and recovery of the effectiveness of antibiotics weakened by the acquisition of multiple resistances. It is also a contribution to reducing the cost of infection medication, which is generally expensive, and the fight against incurable infectious diseases.

The interest in results is not limited to antibiotic therapy, but it can be of considerable importance in the pharmaceutical, food and cosmetic industries.

## Conclusion

It can be concluded that the two extracts of the *D. viscosa* medicinal plant are rich in active compounds as they have interesting activities on multi-resistant pathogenic bacteria. As well as their combinations with antibiotics of the family Macrolides and beta-lactams amplified their antibacterial effect.

So these extracts could be a source of bioactive molecules that can be identified and isolated for use as antibiotic adjuvants against these pathogens.

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