Influence of *Rhus tripartita (Ucria) Grande* leaves on phyto-beneficial bacteria associated with its rhizosphere

Influence des feuilles de *Rhus tripartita (Ucria) Grande* sur les bactéries phyto-bénéfiques associées à sa rhizosphère

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ABSTRACT

Leaves extracts were performed using distilled water, alcohol, methanol, hexane and chloroform as solvent and diluted in concentrations of 0.001, 0.01 and 0.1 mg/mL. Meanwhile, the extraction of total flavonoids was carried out according to the standard procedure. The antimicrobial effect of the extracts was evaluated using the agar diffusion method and the determination of the minimum inhibitory concentration was carried out on a liquid medium. Alcohol, chloroform and methanol extracts were found to be the most effective on tested strains. The maximum zone inhibition was 18 mm, and the minimum zone inhibition was 7 mm. *Bacillus licheniformis* (RT 1) appears to be the most sensitive to all extracts. In contrast, *Bacillus megaterium* (RT 7) seems to be the less sensitive strain. On the other hand, total flavonoids had a significant effect on 25 % of the strains tested, espicialy *Bacillus* genus. With a broad antimicrobial spectrum, the *Rhus tripartita* leaves can be considered as a control agent for the distribution of the bacterial community in the rhizosphere. Therefore, this study showed that the plant could influence the bacterial diversity of its rhizosphere through its leaves.

RESUME

Les extraits de feuilles ont été effectués utilization de l'eau distillée, de l'alcool, du méthanol, de l'hexane et du chloroforme comme solvant et dilués à des concentrations de 0,001, 0,01 et 0,1 mg/mL. L'extraction des flavonoïdes totaux a été effectuée selon une procédure standard. L'effet antimicrobien des extraits a été évalué à l'aide de la méthode de diffusion sur gélose et la détermination de la concentration minimale inhibitrice a été effectuée sur un milieu liquide. Les extraits d'alcool, de chloroforme et de méthanol se sont avérés les plus efficaces sur les souches testées. La zone d'inhibition maximale est de 18 mm et la zone d'inhibition minimale est de 7 mm. *Bacillus licheniformis* (RT 1) semble être le plus sensible à tous les extraits. En revanche, *Bacillus megaterium* (RT 7) semble être la souche la moins sensible. En revanche, les flavonoïdes totaux ont eu un effet significatif sur 25 % des souches testées, principalement le genre *Bacillus*. Avec un large spectre antimicrobien, les feuilles de *Rhus tripartita* peuvent être considérées comme un agent de contrôle pour la distribution de la communauté bactérienne dans la rhizosphère. Cette étude donc montré que la plante pouvait influencer la diversité bactérienne de sa rhizosphère à travers ses feuilles.

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1. INTRODUCTION

Rhus tripartita (Ucria) Grande called "African sumac" is a shrub species of the botanical family of Anacardiaceae. These species are distributed in North Africa to Hoggar (North Africa), Sicily and Western Asia [1]. Therefore, many studies reported the antimicrobial potential of the shrub against a wide range of microorganisms [2-7]. On the other hand, the plant has a potential antimecrebial due to its several several phytochemicals compounds such as flavonoids [8,11]. Therefore, flavonoids constitute a large group of secondary metabolites in higher plants [12]. Moreover, several studies have demonstrated the richness of *Rhus tripartita* in flavonoid compounds [13-15].

Microorganisms play an essential role in the decomposition of organic matter, nutrient cycling, and plant productivity. In addition, soil microbes, mainly bacteria and fungi, are affected by all biochemical processes occurring in soils and play a vital role in maintaining soil productivity. Therefore, the plant strongly interacts with its biotic environment through the synthesis of secondary metabolites, most often "diffusible", often exuded secondary metabolites are sources of chemotactism allowing the selection of organisms (pathogens, mutualists or commensals) around the roots [16]. In this way, microorganisms can interact with the mutually beneficial plant; examples include the Plant Growth Promoting Rhizobacteria (PGPR) [17]. Other bacteria approximately plant roots (rhizobacteria) are able to control plant diseases caused by soil pathogens [18] called antagonists bacteria.

Therefore, several parameters influence the distribution or activity of soil microorganisms. Furthermore, various secretions of micro and macromolecular metabolites [19] characterize the rhizosphere. The role of root secretions on the functioning and distribution of microbial communities has long been studied [20]. However, the studies of rhizobacteria beneficial to plants did not always take into account factors other than soil composition or root exsudation. Therefore, it has reported the plant biomass effects on soil community structure [21].:To our knowledge, no studies have been carried out on the effect of the leave plant on the bacteria of its rhizosphere. So, the present investigation carried out to show an exploration angle of *Rhus tripartita's* relationship to its rhizosphere bacterial community through the leaf extracts and total flavonoids effects.

2. MATERIALS AND METHODS

2.1.Biological material

Rhus tripartita leaves were collected in December 2018, in the Ilamane region (100 km north of Tamanrasset city, Algeria) which is located in the Ahaggar National and Cultural Park (22°49'59 "N, 5°19'59 "E). The antibacterial effect of the plant is tested on an antagonists population [22] that were related to bacteria that are associated with mechanisms of plant growth promotion from *Rhus tripartita* rhizosphere [23] : Rt 1 : *Bacillus licheniformis* ; Rt 2: *Bacillus circulans* ; Rt 3: *Pseudomonas aeruginosa* ; Rt 4: *Bacillus megaterium* ; Rt 5: *Bacillus subtilis* ; Rt 6: *Escherichia vulneris* ; Rt 7: *Bacillus megaterium* ; Rt 8: *Kocuria varians* ; Rt 9: *Bacillus subtilis* ; Rt 10: *Bacillus licheniformis* ; Rt 11: *Escherichia vulneris* ; Rt 12: *Bacillus licheniformis.*

2.2.Preparation of leaf extracts

The extracts were prepared using the following solvents: distilled water, methanol, hexane, ethanol and chloroform (Sigma, St Louis, MO, USA). 10 g of dried leaves, were grinded in mortar and homogenized with 100 mL of the respective solvents. The raw preparation was macerate overnight in the shaker at room temperature and then filtered through a filter paper. The supernatant is recovered and transferred to a spade and extracted concentrated by evaporation of the solvent at 50 °C. The resulting extract was then weighed and dissolved in a known volume of distilled water to obtain a final concentration of 0.001, 0.01 and 0.1 mg/mL.

2.3.Total flavonoid extract

Total flavonoid was extracted using the method reported previously [24]. It consists to mix 250 µL of leaf methanolic extract with 25 µL of 5 % $NaNO_2$, added with 150 µL of $AlCl_3$ (2 %). After 5 min, 0.5 mL of 1M NaOH was added to the solution and extract was resulting after 10 min of incubation.

2.4.Preparation of bacterial strains

Bacterial cultures were prepared in nutritious broth (bioMerieux sa, Lyon, France), which were incubated at 30 °C for 24-72 hours. Cultivated fresh crops dilutions were adjusted to a concentration of 10^6 CFU/mL.

2.5.In vitro antibacterial activity test

Direct diffusion method was used to evaluate the leaves antibacterial activity. This method is based on the preparation of 6 mm diameter wells on the Muller Hinton agar (bioMerieux sa, Lyon, France) previously seeded by the bacterial strains to be tested according to the protocol as described previously [25]. Then, 15 µL of leaf and flavonoid extracts were deposited in these wells. The antibacterial activity was evaluated by measuring the inhibition zone diameter, formed around the well after an incubation of 24 h at 37 °C.

2.6.Antibiotic resistance

The strains were tested for their susceptibility to Oxacillin (OX) 5 µg (Sigma Chemical Co., St. Louis, Mo.) as a control procedure, according to the Clinical and Laboratory Standards Institute (CLSI).

2.7. Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration represents the lowest concentration of a substance to inhibit bacterial growth in an incubation time of 24 h at 37 °C. The MIC was determined using the Delarras method [26] slightly modified. It consisted to dilute the extract lowest dilution which showed an antibacterial potential, according to geometric number of 2. Then, mixing 1 mL of each dilution with 1 mL of 24 hours bacterial inoculum and the result reading was performed after incubation for 24 hours at 37 °C. The MIC corresponds to the concentration of the first tube in which there is no growth visible to the naked eye compared to a control tube (without germ).

2.8.Statistical analysis

The data were subjected to statistical analysis using the Microsoft Excel 2010 program. All values of biochemical compounds and secondary metabolites are the mean ± ES (standard error of the mean) of three replicates of a single sample.

3. RESULTS

The evaluation of the antimicrobial activity of *Rhus tripartita* leaves extracts was determined by the presence or absence of the inhibition zone. The extracts antibacterial activity was evaluated against 12 antagonists' phytobeneficial bacterial strains from *Rhus tripartita* rhizosphere. The extracts showed distinct inhibitory effects compared to the strains tested. Rt 1 *: Bacillus licheniformis* appears to be the most sensitive with inhibitory zones of aqueous extract (12 mm), alcoholic extract (9±0.23 mm), chloroformic extract (13±0.46 mm), methanolic extract (10±0.84 mm), hexanoic extract (15±0.23 mm) followed-up by Rt 8 : *Kocuria varians* and Rt 11 : *Escherichia vulneris* (tab. 1). In contrast, Rt 7: *Bacillus megaterium* seems to be the less sensitive strain with maximum inhibitory zone of 9 mm (Tab. 1). On the other hand, all tested strains were resistant to oxacitin (5 µg/L) as control procedure (tab. 2).

Meanwhile, chloroformic extract was found to have the broadest spectrum of activity (7-18 mm) but only acted on eight strains of a total of twelve. Moreover, methanol extract indicated an antimicrobial effect on all tested strains. However, the extracts have approximately similar effects on both Gram-negative and positive bacteria (tab. 1). Therefore, It appears that the antimicrobial activity of hexaoic extracts was the less effective (Tab. 1). Indeed, aqueous and alcoholic extracts have been able to act on eight out of 12 tested strains.

In addition, the leaf extracts MIC showed promising results, with an effect that varied between 1.25 µL/mL and 1667 µL/mL (tab. 3). However, it should be noted that the chloroformic extract was displayd narrow MIC levels (1.67-166.7 µL/mL). Conventionally, Rt 1*: Bacillus licheniformis* was showed sensitivity at the lowest range of MIC (12.5-16.67 µL/mL).The pure total flavonoids extract showed a significant activity against 25 % of the strains tested (Fig. 1). As demonstrated in the table 4, the Gram-positive bacteria of *Bacillus* genera were the most sensitive to the pure compounds with MICs in the range of 150 and 650 µL/mL.

Table 1: Antibacterial activity of *Rhus tripartita* leaves extracts on PGPR antagonists' strains, expressed by diameter inhibition zones (mm)

Extracts	Concentrations	PGPR antagonists strains											
		Rt 1	Rt 2	Rt 3	Rt 4	Rt 5	Rt 6	Rt 7	Rt 8	Rt 9	Rt 10	Rt 11	Rt 12
Aqueous	0.1 g/L	12	0	13±0.46	10±0.46	8±0.23	0	0	9±0.58	10	0	12±0.84	13±0.46
	0.01 g/L	0	0	10±0.46	0	7	0	0	7±0.58	0	0	12	10±0.84
	0.001 g/L	0	0	0	0	7	0	0	7	0	0	0	0
Alcoholic	0.1 g/L	9±0.23	0	0	11±0.46	8±0.23	11±0.46	0	7±0.58	0	8±0.46	12±0.84	14±0.46
	0.01 g/L	7±0.46	0	0	0	6±0.46	0	0	7	0	6±0.23	8±0.23	0
	0.001 g/L	0	0	0	0	0	0	0	0	0	0	0	0
Chloroformic	0.1 g/L	13±0.46	10±0.23	0	11±0.56	7	7±0.84	0	18±0.46	0	12	16	13
	0.01 g/L	0	6±0.46	0	8±0.46	6±0.46	6±0.71	0	13±0.46	0	0	0	0
	0.001 g/L	0	0	0	0	0	0	0	7±0.84	0	0	0	0
Methanolic	0.1 g/L	10±0.84	14±0.69	9±0.44	16±0.23	7±0.78	13±2.12	9±0.46	9±0.46	10±0.84	16±0.46	12±0.46	13±0.46
	0.01 g/L	9	9±0.46	7±0.46	8	0	7±0.78	6±0.46	7	0	0	10	0
	0.001 g/L	0	0	0	0	0	6±1.08	0	0	0	0	0	0
Hexanoic	0.1 g/L	15±0.23	0	0	0	13±0.46	0	0	10±0.46	0	0	12±0.46	0
	0.01 g/L	8±0.46	0	0	0	9	0	0	7	0	0	9±0.46	0
	0.001 g/L	6±0.23	0	0	0	0	0	0	0	0	0	0	0

*0: No antibacterial activity

Table 2: Antibiogram of strains tested by disc diffusion method

	Rt 1	Rt 2	Rt 3	Rt 4	Rt 5	Rt 6	Rt 7	Rt 8	Rt 9	Rt 10	Rt 11	Rt 12
Oxacitin (5 µL)	R	R	R	R	R	R	R	R	R	R	R	R

R : resistant ; **Rt 1** : *Bacillus licheniformis* ;**Rt 2**: *Bacillus circulans* ;**Rt 3**: *Pseudomonas aeruginosa* ;**Rt 4**: *Bacillus megaterium* ; **Rt 5**: *Bacillus subtilis* ; **Rt 6**: *Escherichia vulneris* ; **Rt 7**: *Bacillus megaterium* ; **Rt 8**: K*ocuria varians* ; **Rt 9***: Bacillus subtilis* ;**Rt 10**: *Bacillus licheniformis* ;**Rt 11**: *Escherichia vulneris* ;**Rt 12**: *Bacillus licheniformis*

Table 3: Minimale inhibitrice concentration values recorded by each extract (µg/mL)

Code	Strains	Aqueous extract	Alcoholic extract	Hexanoic extract	Chloroformic extract	Methanolic extract
Rt 1	*Bacillus licheniformis*	12.50	12.5	125	12.5	16.67
Rt 2	*Bacillus circulans*	1250	125	1250	16.67	25
Rt 3	*Pseudomonas aeruginosa*	166.7	250	1250	125	1.25
Rt 4	*Bacillus megaterium*	1250	125	166.7	2.5	12.5
Rt 5	*Bacillus subtilis*	2.5	125	2.5	125	1.25
Rt 6	*Escherichia vulneris*	1250	125	1667	125	250
Rt 7	*Bacillus megaterium*	1250	250	1250	125	166.7
Rt 8	*kocuria varians*	1.25	1.67	16.67	1.25	1.67
Rt 9	*Bacillus subtilis*	125	250	1667	16.67	125
Rt 10	*Bacillus licheniformis*	1250	250	1667	125	125
Rt 11	*Escherichia vulneris*	1.25	1.25	12.5	1.67	1.67
Rt 12	*Bacillus licheniformis*	12.5	125	1250	166.7	125

Table 4: Antibacterial activity of Total flavonoids (extracted from *Rhus tripartitus* leaves) on PGPR antagonists strains, expressed by diameter inhibition zones (mm) and Minimale Inhibitrice Concentration values recorded (µg/mL)

Total flavonoids	Rt 1	Rt 2	Rt 3	Rt 4	Rt 5	Rt 6	Rt 7	Rt 8	Rt 9	Rt 10	Rt 11	Rt 12
Inhibition Zone	6	12	6	6	7	6	12	6	13	6	6	6
MIC (µL/mL)	-	240	-	-	650	-	240	-	150	-	-	-

Rt 1 : *Bacillus licheniformis ;***Rt 2**: *Bacillus circulans ;***Rt 3**: *Pseudomonas aeruginosa ;***Rt 4**: *Bacillus megaterium* ; **Rt 5**: *Bacillus subtilis ;* **Rt 6**: *Escherichia vulneris ;* **Rt 7**: *Bacillus megaterium* ; **Rt 8**: *kocuria varians* ; **Rt 9***: Bacillus subtilis ;***Rt 10**: *Bacillus licheniformis ;***Rt 11**: *Escherichia vulneris ;***Rt 12**: *Bacillus licheniformis*

*Diameter of well (6 mm) is included

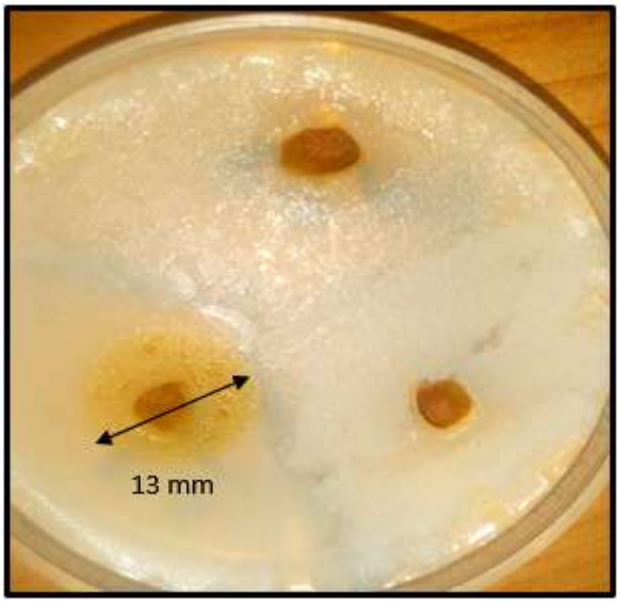

Figure 1: Antibacterial effect of total flavonoids extracted from *Rhus tripartita* against Rt 9: *Bacillus subtilis* strain using the direct diffusion method on Muller-Hinton agar

4. DISCUSSION

The focus of the present study was to establish the biological activities of organic, aquous and flavonoid extracts of *R.tripartita* leaves on phytobeneficial bacteria of its rhizosphere by comparing their antimicrobial properties on antagonists PGPR associated to plant rhizosphere.

These findings showed that extracts made with organic solvents haven't a significant effect as compared to n aqueous extract. In contrast, it has been previously reported that organic extracts had shown a better antibacterial effect than aqueous extracts [27]. Thus, several parameters affect the effectiveness of bioactive substances, it depends on bacterial species, whether resistant or sensitive and the solvent type. It is interesting that the aqueous extract would have an antimicrobial on the majority of strains tested. Theoretically, it is assumed that leaves in the environment when they are found on the ground are certainly in contact with surface water, which probably over time can extract bioactive substances from the leaves and influence microbial diversity.

Therefore, *Rhus tripartita* extracts showed a significant broad spectrum activity against all tested microorganisms. It mentionned that the leaves had a negative effect on the development of these bacteria. Many studies have reported the antimicrobial effect of Rhus tripartita extracts against bacterial Gram negative and positive strains such as *Staphylococcus aureus* [6], *Bacillus subtilis* [4], *Escherichia coli*, *Salmonella typhimurium*, *Salmonella argenosa* [7] and *Pseudomonas aeruginosa* [5]. Furthermore, it is necessary to take into account that the tested bacterial population belongs to the group of Antagoinist PGPRs, basically beneficial for the plant health and development. On the other hand, It has been hypothesized that a general reduction in soil microbial diversity will result in reduced functional capacity of the soil [28].

In the current study, *Bacillus* was the most sensitive species to the extracts used, which was reflected in the MIC values. Furthermore, *Bacillus* genera represent a large fraction of the microbial community living in soil and the rhizosphere, especially the root systems of plants. They are part of the zymogenic flora of the soil and are found in plant endophytes or epiphytes, and the rhizosphere of various cultivated [29]. They have been studied a lot for their beneficial and protective effect on plant [30-33].

Then, this preliminary study demonstrated that the MIC exhibits real antibacterial activity. In fact, solvent nature plays a key role in the plant antimicrobial activity. However, the results found are difficult to generalize before carrying out experiments on the natural environment.

Moreover, the highest antibacterial effect of the methanol extract, may be due to its high content on flavonoids. In fact, these compounds were extracted using methanol which suggests a positive correlation between the antibacterial effect of the methanolic extract on the one hand and flavonoids extracted on the other. The term flavonoid includes the following commonly occurring polyphenols: flavanones, flavones, flavan-3-ols, flavonols and anthocyanins [34]. However, all these compounds produce different levels of antimicrobial effects. In addition, plant extracts generally contain flavonoids in glycosidic form [12], which may explain why total flavonoids had a significant effect only on 25 % of tested strains. Whereas their antimicrobial effect have been reported in some studies [35, 36]. However, it should also be noted that antimicrobial studies of flavonoids have been carried out on human or foodborne infection bacteria. On the other hand, all tested rhizobacteria in the

present work, were resistant to oxacitin. Therefore, resistant bacteria have been detected in the environment such as sediments and soils. This resistance can be attributed to the use of antibiotics for livestock entering the environment when manure is applied to fields [37]. Otherwise, the excessive use of fertilizers and pesticides in agriculture, has made it possible to promote this resistance. Moreover, Bacteria in the soil live in community, which implies that there is gene transfer between species, especially those of resistance.

5. CONCLUSION

This study was viewed a first report on the impact of *Rhus tripartita* on their benefical rhizospheric bacteria. Therefore, with a broad antimicrobial spectrum against Gram positive and negative species, *the Rhus tripartita* leaf extracts can be considered as a control agent for the distribution of the bacterial community in the rhizosphere. On the other hand, the total flavonoids have a lower effect than the leaf extracts but they push for further future investigation. Therefore, it suggested in rhizobacteria studies to take into account for the fall of dead leaves of the plants on the rhizosphere soil concerning it**.**

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