

ASSESSMENT OF BIOLOGICAL ACTIVITIES OF RESIN EXTRACTED FROM TUNISIAN PINE FORESTS

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Abstract

The pine resin is a natural product highly demanded in the chemical industry. The pine resin tapping is a non-existing activity in Tunisia. Extraction and commercialization of this product can improve the livelihood of Tunisian families living within the forest areas and provide additional income for more one million-forest populations. The aim of the current study was to promote this activity on the field and to identify the best practices of resin tapping taking into account the national context of Tunisian pine forests. Besides, the present paper evaluates the antimicrobial and antioxidant activity of three pine species *Pinus pinaster*, *pinia* and *halepensis*. These observational studies and experimental trials were carried out in the forests of Tabarka in the North western of Tunisia. The borehole method was employed using a drilling machine adapted to pine resin tapping. Our results suggest that maritime pine was the major resin production, followed by Aleppo pine and Stone pine with 50, 30, and 20% resin yield, respectively. The antioxidant activity of the gum resin has been tested *In vitro* by the DPPH test. The strongest anti-free radical capacity was recorded in *Pinus pinia* with the highest activity ($IC_{50} = 15 \pm 0.59$ mg/mL), while *Pinus halepensis* is characterized by a moderate antioxidant capacity ($IC_{50} = 17 \pm 0.11$ mg/mL). The antibacterial capacity of the gum resin was studied *In vitro* using the well diffusion method on the four bacterial strains *Bacillus*, *Escherichia coli*, *Listeria* and *Salmonella*. Variance analysis showed that the resin of *Pinus pinia* was the most effective in bacterial inhibition with an average inhibition zone diameter (2.13 cm) against *Pinus halepensis* and *Pinus pinaster* with 1.60cm and 1.46 cm respectively. Pine resin showed an interesting biological activity and can be used as antioxidant and antibacterial applications.

Key words: *Pinus pinaster*, *Pinus halepensis*, *Pinus pinia*, Tapping, Resin, Antioxidant capacity, Antimicrobial activity, Tunisia.

Introduction

Resin is a natural product that has multiple applications and is very demanded within several industries of coatings, adhesives, printing inks, insecticides and disinfectants (Rodrigues-Correã *et al.*, 2013; Sharma *et al.*, 2018; Salim *et al.*, 2019). Besides, the resin is the raw material that through different industrial processes is converted into gum resin, turpentine, and their derivatives (Hadiyane *et al.*, 2015). Turpentine, or gum turpentine, is a volatile liquid that is extracted during the distillation of the natural resin that comes from pine resin trees. This volatile fraction is widely employed in making essences, perfumes, and solvents (Ghanmi *et al.*, 2007). Moreover, Rosin is a solid material obtained from pines resin and other conifers. This product is an important ingredient in printing inks, soap, and wax (Fiebach & Grimm, 2000).

Launching pine tapping activity in Tunisia can reduce the rosin and turpentine imports. On average Tunisia imports 19 tonnes (T) of rosin and 40T of turpentine every year over the last ten years (2001-2019) (Trade Map, 2020). In dollars, this represents 62 000 \$ of rosin and 149 000 \$ of turpentine every year over the same period. The data may not be rigorously accurate, but it can be noted that the need for turpentine is greater than that for rosin.

Due to its richness in resin, the Mediterranean population usually used pine species by tapping technique until 1980 when the activity began to disappear (Palma *et al.*, 2016; Soliño *et al.*, 2018). In 2010, the total world production of pine resin reached the amount of 1.114.000

tons, more than 90% of the pine resin production in the world is concentrated in three countries: China, Brazil and Indonesia (Cunningham, 2012). The three pine species (*Pinus halepensis* Mill, *Pinus pinia* L., and *Pinus pinaster* Aiton) are fast-growing conifers, mainly found in the coastal areas of the Mediterranean region (López-Tirado & Hidalgo, 2016).

In Tunisia, pine forests occupy 400.000 Ha, which represents more than 65% of the forest area (Anon., 2010). They are the most important forest species in Tunisia due to their value in ecology, biology, and economy. In Tunisia, Aleppo pine and pinon pine forests are mainly known for their high nutritional nuts, maritime pine is used for the stabilisation of dunes and the production of plant nursery substrates (Mutke *et al.*, 2012). Their softwood has a low quality essentially due to knots frequency and resin secretion, but generally used in pulpwood and locally used for construction and furniture (Polge, 1992).

Forest areas are subject to significant use by the forest population. This population is estimated at one million inhabitants which represent about 10% of the total Tunisian population (Anon., 2015). Several investigations showed a great relationship between population and forest resources particularly in Maghreb countries (Gauquelin *et al.*, 2018). Comparing with other countries, Paudyal (2008) mentioned that in Nepal for example, natural resin extraction constituted the major income rate. In addition, the increases in the price and demand for the product have enabled the conservation of forests and the development

of populations as in Spain and Portugal (Palma *et al.*, 2012, Rodríguez-García *et al.*, 2015).

As in Europe, the exploitation of Tunisian resin disappeared from 1980 (Zas *et al.*, 2020b). currently, the main weakness in the Tunisian forest sector is the lack of knowledge and appreciation of the importance of resin tapping.

The purpose of this study is to generate new ability and the best practices of resin tapping techniques in pine forests and to evaluate the resin antioxidant and antibacterial capacities.

Experimental section

Environment study and plant materials: The study was realized in the forests of Tabarka in north-western of Tunisia (Fig. 1). The region of the study belongs to the upper humid to lower humid Mediterranean climate. The mean of rainfall is 1000mm/year. The minimum and the maximum temperatures were 4°C and 32°C respectively.

Sampling and resin extraction method: The work was carried out on the three most common pine species in Tunisia *Pinus halepensis* Mill, *Pinus pinea* L., and *Pinus pinaster* Aiton. A total of 45 healthy trees (15 trees/species) free of defects (biotic and abiotic alterations), were selected in the middle of the forest stand. The forest stand is accessible. The land is flat, and the density is around 400 stems per hectare. To compare the resin yield from each species, trees were chosen having the same characteristics. The age and diameter at breast height (DBH) of the studied trees oscillate between 55 and 60 years and 50 to 60 cm respectively. However, the height exceeds the 10m.

The tapping method was chosen according to Hodges & Williams (1993), Hodges (2000) and Barranx *et al.*,

(2002) in which the resin was collected in a closed recipient to obtain a clear product rich with turpentine. Tapping in the close environment allows collecting high-quality resin and more turpentine than tapping in an open environment by limiting the evaporative losses of the essences (Barranx *et al.*, 2002). The resin tapping was performed using a screwdriver with a whole saw to obtain a perfect round whole with the diameter of the elbow 40mm and 02cm of the depth.

The collection of resin was performed using a plastic container inserted in the whole. To extend the duration and the quantity of resin flow, an activated gemming technique was used by applying an activator based on clay and sodium chloride.

Antioxidant activity: The antioxidant capacity of pine gum resin was evaluated using the free radical scavenger DPPH method by following colour change (after reduction) by spectrophotometry method previously described by Kirby & Schmidt (1997). A liquid solution was prepared using 100 mg of gum resin dissolved in 1.0 mL of ethanol before the test. Then, 1.0 mL of DPPH solution was mixed with 1.0 mL of gum resin sample solutions at different concentrations. Then, the solutions were incubated for 30 min in at ambient temperature. The radical scavenging activity (RSA) was determined by measuring the decrease of the absorbance at 517 nm with a spectrophotometer. In addition, 1.0 mL of ethanol was added to 1.0 mL of the ethanolic solution of DPPH and used as a control solution. The reference antioxidant molecule was prepared with gallic acid. Finally, the RSA was determined using the following formula:

$$\text{RSA (\%)} = [(A_1 - A_2)/A_1] \times 100.$$

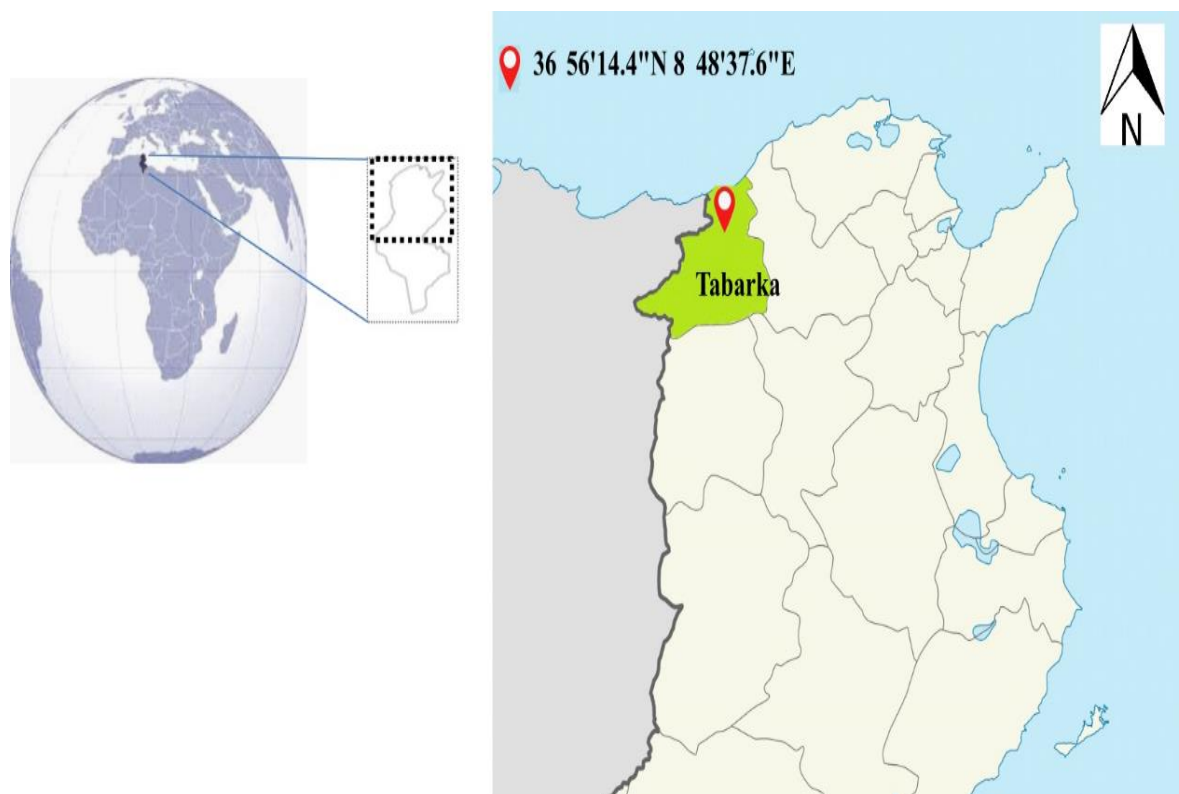


Fig. 1. Area of the study.

A_1 and A_2 were identified as the absorbances of the control and the test samples, respectively. The inhibitory concentration 50 (IC_{50}) value was determined as the concentration (in mg/mL) of the compound required to scavenge 50% of the DPPH radical. All samples were analysed in triplicates.

Antibacterial capacity assessment: To assess antibacterial activity, a collection of bacterial strains was chosen. The bacterial strains used were characterized by their pathogenic effect of contaminating foodstuffs.

Antibacterial activities of resin were inspected against four bacteria strains: two Gram negative; *Escherichia coli* and *Salmonella enterica* and two Gram positive; *Listeria monocytogenes* and *Bacillus subtilis*. The strains were provided by the Sfax biotechnology centre (Tunisia).

The antibacterial capacity of resin was mainly performed by well method previously described by Tagg & Mc Given (1971).

Firstly, the bacterial strains were multiplied in a culture medium made from Nutrient Broth (NB) and incubated at 37°C during 24h.

The pre-cultures of the strains composed of 20 mL of liquid NB and 100 μ L of the bacterial strain, the mixture is incubated at 37°C overnight in a water bath with shaking. To prepare the solid medium, 12.5 g of powder NB and 7.5 g of agar were dissolved in 0.5 L of distilled water. Once the medium is well stirred, it is autoclaved at 121°C for 1 h. Finally, 20 mL of the mixture transferred to each Petri dish (diameter = 90 mm).

Well method preparation has been described by Guven *et al.*, (2005). It is based on the solid medium diffusion technique, which consists of inoculating the bacteria at a rate of 100 μ L/dish from a prepared pre-culture. Incubated the dishes for 2 hours at room temperature. Later, 6 mm diameter wells are prepared using a sterile Pasteur pipette and filled with 50 μ L of the corresponding resin with different concentrations (pure, 0.5%, 1%, and 2%) and two control wells, one positive (the antibiotic: Gentamicin, Central Pharmacy of Tunisia) and the negative control (ethanol) and the bacterial inoculum was added. The petri dishes thus prepared are incubated at + 4°C for 3 to 4 hours to allow for diffusion of the resin present in the wells. The dishes were then incubated in an oven at 37°C for 48 hours. The antibacterial activity of the resin was measured in terms of the diameter of the zone inhibition that surrounds the wells using a caliper. All samples were analysed in triplicates.

Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA) using SAS 9.1. Duncan's multi-range tests were used and were expressed as mean \pm standard error of the mean. All statistical tests were two tailed, and a *P* value of 0.05 or less was considered significant.

Results and discussions: In this study, the resin yield, the antioxidant, and the antibacterial activities of the three

dominant pine species in Tunisia *Pinus pinaster*, *Pinus pinea* and *Pinus halepensis* were evaluated.

The resin yield was recorded by using the tapping method according to Hodges (2000) and Barranx (2002) in which the resin was collected in a closed recipient to obtain a clear product rich with turpentine. The average yield within 14 days in the three species of Maritime pine, Aleppo pine, and stone pine was about 271.83 \pm 10 mL, 168 \pm 7 mL, and 118.33 \pm 8 mL respectively which represent 49 \pm 1.8% for maritime pine, 30 \pm 1.3% for Aleppo pine, and 21 \pm 1.4% for Stone pine (Fig. 2).

Dahmane (1986), estimated the production of maritime pine in Tunisia between 1.5 kg and 1.8 kg of resin per tree per year tapped by multi-face (care) method.

The activated extraction method could contribute to the harvesting of the highest yield of maritime pine resin (Sharma *et al.*, 2018). On the other hand, the acid-free tapping method represents the lowest yield. In fact, the activated sulfuric acid tapping was a far higher yield than that obtained by the traditional process (Dahmane, 1986).

Similarly, it appears that the resin content and production was highly depending on the nature of the species studied (Zas *et al.*, 2020a). However, in the present study, Maritime pine showed the highest yield of resin compared to Aleppo pine and Stone pine as observed in Europe where Maritime pine (*Pinus pinaster*) is the main tapped species (Zas *et al.*, 2020b).

Antioxidant activity: Natural products with radical scavenging capacity are demanded for many applications especially in medicine and food. They can replace chemical antioxidant products that usually have an undesirable effect on human health (Hsouna *et al.*, 2017). Therefore, we are focused on this part of this work on the detection of the different antioxidant pine resin activity between Aleppo pine, maritime pine, and stone pine. In this work, the antioxidant activity of the resin was tested *In vitro* by the DPPH test using increasing concentrations of the resin. The DPPH test was used to measure the performance of the pine resin to scavenge the free radical DPPH by donation of hydrogen atom or an electron (Tepe *et al.*, 2005). The result was based essentially on the determination of the efficacy concentration of IC_{50} value (Fig. 3). The IC_{50} value indicated the concentration of sample that could inhibit 50 percent reduction of DPPH radical. The lowest IC_{50} value means the most active sample as antioxidant.

The results showed that the higher inhibition activity was detected in stone pine with the lower IC_{50} about 15 mg/mL. Nevertheless, Aleppo and Maritime pine have the lower inhibition activity with an activity about 17 and 16.81 mg/mL respectively.

The DPPH activity in the resin could be attributed to their high contents of phenolic compounds. However, the anti-radical activity depends on the chemical structure of phenolic compounds and the availability of hydroxyl groups (OH) (Jayaprakasha *et al.*, 2008). Furthermore, the antioxidant activity is linked to the chemical composition of pine resins (Fenglin *et al.*, 2008). It could be correlated with the majority or minority constituents or also with a synergy between them (Wang *et al.*, 2008).

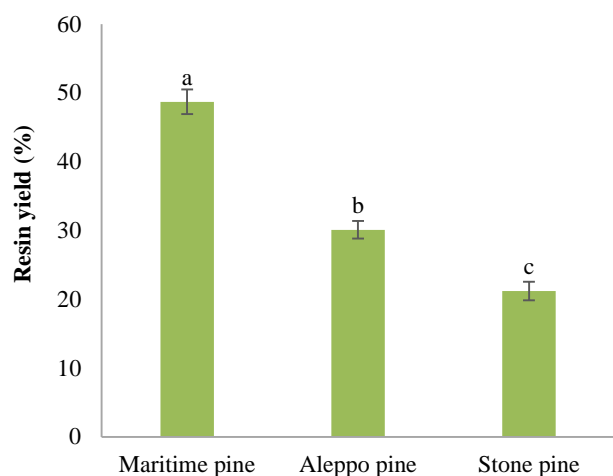


Fig. 2. Resin yield percentage from different pine species (means with the same letter are not significantly different according to Duncan's multi-range test at the level below 0.05).

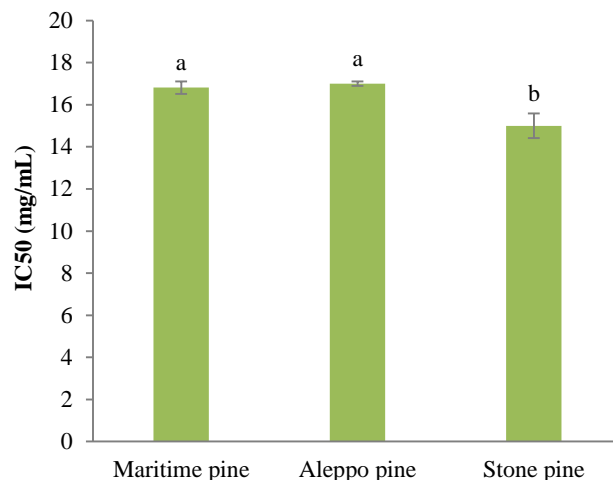


Fig. 3. Interspecific change in IC₅₀ (means with the same letter are not significantly different according to Duncan's multi-range test at the level below 0.05).

Table 1. Antibacterial effect of pine gum resin against bacterial strains.

Pine species	Bacterial strains	Inhibition-zone diameter [cm] ^[†]				
		C ₀	C ₁	C ₂	C ₃	G
<i>Pinus pinaster</i>	<i>Bacillus s.</i>	1.96 ± 0.15 ^a	1.43 ± 0.15 ^a	1.53 ± 0.2 ^a	1.6 ± 0.2 ^a	3 ± 0.62 ^a
	<i>Listeria m.</i>	1.8 ± 0.26 ^a	1.2 ± 0.11 ^b	1.5 ± 0.25 ^a	0.93 ± 0.3 ^b	3 ± 0.62 ^a
	<i>Escherichia c.</i>	1.7 ± 0.26 ^a	1.5 ± 0.11 ^a	1.36 ± 0.4 ^a	1 ± 0.32 ^b	3 ± 0.62 ^a
	<i>Salmonella e.</i>	1 ± 0.35 ^b	1.1 ± 0.11 ^b	1.28 ± 0.3 ^a	1.53 ± 0.2 ^a	3 ± 0.62 ^a
<i>Pinus halepensis</i>	<i>Bacillus s.</i>	1.36 ± 0.11 ^b	1.23 ± 0.25 ^b	1.23 ± 0.25 ^a	1.4 ± 0.36 ^a	3.36 ± 0.15 ^a
	<i>Listeria m.</i>	1.96 ± 0.89 ^a	1.53 ± 0.2 ^a	1.33 ± 0.58 ^a	0.93 ± 0.05 ^b	3.36 ± 0.15 ^a
	<i>Escherichia c.</i>	1.6 ± 0.69 ^b	1.4 ± 0.25 ^a	1.3 ± 0.15 ^a	1.1 ± 0.36 ^a	3 ± 0.62 ^a
	<i>Salmonella e.</i>	1.46 ± 0.2 ^b	1.13 ± 0.4 ^b	1.13 ± 0.1 ^a	0.8 ± 0.11 ^b	3.4 ± 0.13 ^a
<i>Pinus pinea</i>	<i>Bacillus s.</i>	1.23 ± 0.5 ^b	1.45 ± 0.2 ^b	1.5 ± 0.3 ^a	1.34 ± 0.3 ^a	3.4 ± 0.13 ^a
	<i>Listeria m.</i>	3.06 ± 0.5 ^a	1.3 ± 0.15 ^b	1.26 ± 0.6 ^b	1 ± 0.43 ^b	3.36 ± 0.15 ^a
	<i>Escherichia c.</i>	3.13 ± 0.4 ^a	2.03 ± 0.2 ^a	1.6 ± 0.26 ^a	1.4 ± 0.11 ^a	3.36 ± 0.15 ^a
	<i>Salmonella e.</i>	1.1 ± 0.75 ^b	1.13 ± 0.17 ^b	1.3 ± 0.4 ^b	1.7 ± 0.3 ^a	3.36 ± 0.15 ^a
Bacterial effects	**	**	**	**	**	**
Concentrations effects	*	*	*	*	*	*

^[†]Values as mean ± S.D. of triplicate experiments. Means having different superscript in columns are significantly different ($p < 0.01$) and ($p < 0.05$). *: significant at an error threshold $\alpha = 5\%$; **: significant at an error threshold $\alpha = 1\%$. C₀: Pure gum resin; C₁: 2% of resin in ethanol; C₂: 1% of resin in ethanol; C₃: 0.5% of resin in ethanol; G: Gentamicin antibiotic reference.

Antibacterial activity: The analysis variance of antibacterial performance (Table 1) of the pine resin showed that the gum resin of *Pinus pinea* was the most effective in bacterial inhibition with an average inhibition diameter (2.13 cm) close to that of the reference antibiotic Gentamicin (3.37cm). *Pinus pinaster* and *Pinus halepensis* showed lower inhibition with 1.46 cm and 1.60cm, respectively.

The *Listeria* strain and the *Bacillus* strain (Gram+) were more sensitive than the *Echerichia coli* and *Salmonella* (Gram-) strains under the action of the resin tested. The activity of tested resin was probably due to the antibacterial compounds of the pine resin. In fact, resin is mainly composed of abietic acid, palustric, neoabietic acid, and the dehydroabietic acid, and some non-abietane diterpenoid, such as acid and isopimarik acid (Gonzalez, 2014). Resin demonstrated its

antibacterial effects, mainly against Gram-positive bacteria. Antibacterial activity seems to result from a combination of several modes of action (Lis-Balchin & Deans, 1996), implying different resin compounds. Antibacterial capacity is due to diterpenoids compounds which can damage microbial cell membranes due to their amphiphilic nature (Tillah *et al.*, 2017).

Conclusion

The main weakness in the Tunisian forest sector is the absence of skilled labour, the lack of knowledge and appreciation of the importance of resin tapping; moreover, the knowledge of resin extraction is very limited or even non-existent with outdated published studies. In this study, the identification of the best yield performances in some pine species was investigated.

Maritime pine could be the major resin production, followed by Aleppo pine and Stone pine with 50, 30 and 20% resin yield, respectively.

Furthermore, the antioxidant and the antibacterial capacity of the resin from different pine species were studied. These results could be considered as a first step in the research of the microbial and antioxidant activity of the three pine species and have suggested that *Pinus pinea* would be a potential source as a natural antibacterial applied in the pharmaceutical and food industries. However, Stone pine showed the highest antioxidant capacity.

Additional studies and methods are needed to provide more details about the mode action on the bacterial strains. In this context, this work would be more improved by the research of other molecules at the origin of this antimicrobial property of resin extraction using gas chromatography. Furthermore, it would be necessary to improve this work by evaluating the antioxidant activity *In vitro* against other free radicals generated by enzymatic systems. The isolation and identification of phenolic compounds by more efficient methods (HPLC) and the use of other methods of evaluation of the antioxidant potential of the resin according to their principles will be interesting.

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