Antioxidant, antibacterial and antifungal activities of *Pergularia tomentosa L.*

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ABSTRACT

To understand the absence of *Pergularia tomentosa* L. (Apocynaceae) in the Mauritanian pharmacopoeia, we characterized the phytochemical composition, evaluated its biological and antioxidant activities. The phytochemical study shows the presence of flavonoids, tannins, saponins and steroids in the roots and aerial parts. Alkaloids, heterosides and terpenes are absent from the Mauritanian ecotype, although they are well present in *P. tomentosa* L. according to many studies. Four bacterial strains were tested: Gram positive (*Staphylococcus aureus* and *Citrobacter freundii*) and Gram negative (*Escherichia coli* and *Listeria monocytogenes*). Only the *Staphylococcus aureus* strain is sensitive to petroleum ether and ethyl acetate extracts from the roots. The diameters of the inhibition zones are 8.3 mm of petroleum ether and 7 mm of ethyl acetate. The MIC values are 400 µg/mL for petroleum ether extract and 200 µg/mL for ethyl acetate extract. The MBC shows a bacteriostatic effect. For the antifungal activity, five strains of *Candida* and one of *Saccharomyces* were tested. No sensitivity of the germs to the tested extracts is revealed. The antioxidant activity assays were determined by *in vitro* using the DPPH. The IC_{50} value shows that ethyl acetate extract is the most effective (1.9 mg/mL). The least effective is obtained with ethanolic extract (3 mg/mL for the aerial parts and 2.4 mg/mL for the roots). This efficiency remains low compared to that of ascorbic acid (IC_{50} is around 0.15 mg/mL). The absence of the species *P. tomentosa* L. native to the region of Tiris Zemmour, (Mauritania) from the Mauritanian pharmacopoeia seems to be due, according to this study, to weak phytochemical characteristics and biological and antioxidant activities.

Key words: antibacterial activity; antifungal activity; *Pergularia tomentosa* L.; Phytochemical study.

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INTRODUCTION

The species *Pergularia tomentosa* L. belongs to the family Apocynaceae (subfamily Asclepiadaceae). It comprises about 200 genera and 2500 species, generally herbaceous or shrubby, specific to temperate and subtropical regions (Al-Mekhlafi and Masoud 2017). *P. tomentosa* L. is very present in traditional medicine. In Asia, this species is used in the treatment of tumors (Al-Said and Hifnawy, 1989), rheumatism (Vincett , 1977), asthma, skin diseases (Ads et al., 2021; Fatma Haddaji et al., 2021; Hamed et al., 2006), helminthiasis, allergies and constipation (Hosseini et al., 2019; Rayyan et al., 2018). In Africa, *P. tomentosa* L. is widely used in the treatment of helminthiasis and dysentery (Burkill, 1985; Hammiche and Maiza, 2006) *angina tinea* and dermatosis (Ould El Hadj et al., 2003) hypoglycaemia (Abdellah et al., 2014) and toothache (Yebouk et al., 2020). Studies have shown that the biological and therapeutic activities of *P. tomentosa* L. are due to the presence of biologically active molecules such as cardenolides (Ahmed A et al., 2000; Hosseini et al., 2019; Al-Said et al., 1988), alkaloids (Acheuk and Doumandji-Mitiche, 2013) and flavonoids (Heneidak et al., 2006). Leaves and Latex contain ghalakinoside (Hosseini et al., 2019), 16-α-acetoxycalotropin, calactine and coroglaucigenin (Hifnawy et al., 2014). In Mauritania *P. tomentosa* L., commonly known "OumJouloud", is not included in the traditional practitioners' recipe (Yebouk et al., 2020). In spit it is, widely, distributed in the north of the country. This work aims to understand the absence of *P. tomentosa* L. from the pharmacopoeia in Mauritania. Our approach consists in characterizing the secondary metabolites present in the plant in order to evaluate their antibacterial, antifungal and antioxidant properties.

MATERIALS AND METHODS

Plant material

The plant material of *P. tomentosa* L was collected in April 2018 in two localities: Zouerate (Tiris Zemmour, Mauritania, N 13°38’41.00’’; O12°26’41.94’’) and Oued El Kebch (Tiris Zemmour, Mauritania, N 21°14’42.68’’; O 13°8’34.79’’). The identification of the species was authenticated by referring to the base of the numeric Herbarium of “École Normale Supérieure de Nouakchott-Mauritanie”; the code "HNM (51272)" in the numeric library.

Sampling and Extraction

The whole plant was harvested, dusted and separated into roots and aerial parts. It was dried in the shade at room temperature and ground to powder using an IKA M20 universal grinder (Janke & Kunkel). Four solvents ranging from non-polar to polar were used: Petroleum ether, Ethyl acetate, Ethanol and water. 80g of vegetable powder was extracted in 300 mL of Petroleum ether using a Soxhlet apparatus (Harborne, 1976). The residue was re-extracted in 300 mL of Ethyl acetate and Ethanol respectively. The filtrate was concentrated using a Rotavap (Hahnshin, hahnvapor, HS-2005S) under vacuum. The extraction time was 2 h for the aerial part and 1 h for the roots. The duration has been fixed by preliminary tests. The aqueous extract (Aq) was obtained by an infusion of 80g of the vegetable powder in 300mL of hot water for 1 h, in order to be closer to the formula of traditional practitioners. For biological activity, the extracts were dissolved in DMSO to a concentration of 100 mg/mL before use in antibacterial and antifungal tests (Abudunia et al., 2017).

Calculation of Yield

The yield (expressed as a percentage of the sample) is the quantity of extract obtained from the vegetable powder. It was determined by the ratio of the weight of the dry extract after evaporation to the weight of the dry vegetable matter powder used for extraction, multiplied by 100 (Gnahoué et al., 2015).

$$Rd = \frac{(m \times 100)}{M}$$

- Rd: Extraction efficiency in percent
- m: mass in grams of dry extract
- M: mass in grams of powder)

Phytochemical characterization

The different phytochemical compounds have been identified by methods described by the literature (Paris and Moyse , 1971; Trease and Evans , 1987; Tuo et al., 2015). The presence of chemical compounds in the plant is demonstrated by staining and/or precipitation (Bekro et al., 2008; Longanga Otshudi et al., 2000; Tona et al., 1998).

Detection of flavonoids (Tuo et al., 2015)

In the test tubes, a volume of 2 mL of the extract was added to some magnesium strip and 1 mL of concentrated Chlorhydric acid (HCl 37%). The observation of a pink or red coloration

indicates the presence of flavonoids.

Tannin detection (Tuo et al., 2015)

2 mL of distilled water and a few drops of $FeCl_3$ (1%) were added to 1 mL of the extract. A blue, blue-blackish or black coloration indicates the presence of gallic-type tannins and a green or dark green coloration shows the presence of catechic-type tannins.

Detection of steroids (Tuo et al., 2015)

To detect the presence of steroids, 5 drops of sulphuric acid ($H_2SO_4$98%) are added to 1mL of extract. A red coloration designates the presence of this chemical group.

Detection of alkaloids (Tuo et al., 2015)

For the detection of alkaloids, two types of qualitative tests were carried out:

Test 1: In this test, two drops of Bouchardat's reagent are added to 1 mL of petroleum Ether, ethyl acetate or ethanolic extracts. A brown precipitation is a mark of the presence of the alkaloids.

Test 2: In this test, the presence of alkaloids was detected by two methods: Appearance of a yellowish-white precipitate: the test was carried out by adding 1 mL of H_2SO_4 filtrate to 5 drops of Mayer's reagent. Appearance of a brown precipitate: the test was carried out by adding 5 drops of Wagner's reagent to 1 mL of filtrate.

Detection of sterols and triterpenes (Trease and Evans , 1987; Tuo et al., 2015)

The presence of this group was characterized by the appearance of a fleeting violet color turning green. It consists of adding 2mL of ethanolic extract to an Erlenmeyer flask. The extract was evaporated to dryness and then solubilized with 2mL of anhydrous chloroform. The mixture obtained was added to 5mL of the chloroform solution, 5mL of acetic anhydrous and a few drops of concentrated H_2SO_4 (98%). After resting the characteristic color appears.

Detection of steroidal and triterpenic heterosides (Tuo et al., 2015)

The appearance of bluish-green and violet-green coloration reveals the presence of steroid and triterpenic heterosides, respectively. The test was performed by adding 2mL of ethanolic extract in an Erlenmeyer flask. After dry evaporation, the residue was dissolved in a mixture of acetic anhydride/chloroform 1/1: v/v and filtered. Finally, a few drops of concentrated H_2SO_4 (98%) were added to the filtrate obtained.

Detection of Saponins (Trease and Evans , 1987)

The detection of saponins was performed by adding a little water 2 mL of the aqueous extract. The solution obtained was strongly agitated. The mixture was left to stand for 20

min.

- No foam: weakly positive test.
- Foam less than 1 cm = positive test.
- Foam of 1-2 cm = significantly positive test.
- Foam more than 2 cm = very positive test.

Biological activities

Microorganisms

Six strains of yeast were used: *Saccharomyces cerevisae* (BY4741), *Candida albicans* (SC5314), *Candida tropicalis* (MY070362), *Candida krusei* (ATCC6258), *Candida glabrata* and *Candida paraplinosis* (ATCC90018). Antibacterial activity was studied on four strains (Gram positive: *Staphylococcus aureus*; *Citrobacter freundii*) and (Gram negative: *Escherichia coli* and *Listeria monocytogenes*).

Growing media

Four culture media were used: yeast extract peptone dextrose for yeast growth; for bacteria, Müeller-Hinton Agar (MHA), *Laurea Bertoni broth* (LB) and red phenol. The preparation of one liter of each culture medium requires:

- Liquid yeast extract peptone dextrose: 10g Yeast Extract (Biokar Diagnostics), 20g Bacto-peptone and 20g Glucose.
- Müeller-Hinton Agar: 38g Müeller-Hinton Agar powder (Biokar Diagnostics).
- lysogeny broth liquid: 5g yeast extract, 10g Bacto-trypton (Biokar Diagnostics) and 5g NaCl (Himedia).
- Red phenol: 0.5g meat extract, 10g Bacto-peptone, 5g NaCl, 0.02g Phenol Red and 10g Glucose (Biokar Diagnostics).

Antifungal activity

The study of the antifungal power was carried out according to the method described by (Bouchal et al., 2019). 5mL of yeast extract peptone dextrose was inoculated by the yeast. The culture was incubated overnight at a temperature of 30°C with 150 x g. From this culture, an inoculum was prepared with an OD of 0.08 to 0.1 read at a wavelength 600nm using a V-1200 spectrophotometer (Shanghai Mapada Instruments CO., LTD). The inoculum was incubated until an OD of 0.14 which reflects the exponential phase of the yeast. Extracts solubilized in dimethyl sulfoxide were added to the culture. The tubes

were incubated at a temperature of 30 °C with 150 x g. OD was measured every 2h.

Five yeast strains from laboratories of the genetics unit of the Faculty of Medicine and Pharmacy of Oujda were tested: *Saccharomyces cerevisae* (BY4741), *Candida albicans* (SC5314), *Candida tropicalis* (MY070362), *Candida krusei* (ATCC6258), *Candida glabrata* and *Candida paraplinosis* (ATCC90018)

Antibacterial activity

Antibacterial activity was determined by using agar diffusion method (Boulekbache-Makhlouf et al., 2013; De Oliveira-Júnior et al., 2018; Lesueur et al., 2007). Briefly, in sterile Petri dishes previously poured with Müeller-Hinton Agar culture medium, a volume of 0.1 mL containing 10^7 CFU/mL of the bacterium was spread on the surface of the agar. Sterile Wattman No.1 paper discs, 6 mm in diameter, were sterilely deposited on the surface of the medium. After that, these were impregnated with 10 µL of the various extracts solubilized in dimethyl sulfoxide. After 24 h incubation at 37°C, the results were read off by measuring the diameter, in mm, of the inhibition zone. The antibiotic represents the positive control, streptomycin (concentration 25mg/mL) carried out under the same conditions as the samples. Four strains from laboratory of the genetics unit of the Faculty of Medicine and Pharmacy of Oujda are tested: *S. aureus*, *E. coli*, *C. freundii* and *L. monocytogenes*.

Determination of the Minimum Inhibitory Concentration (MIC)

The MIC was defined as the lowest concentration of the extract capable of inhibiting any visible germ growth (Biyiti et al., 2004). The bacterium was inoculated in 5mL of lysogeny broth medium. The culture was incubated overnight at a temperature of 37°C under stirring 150 x g. A pre-culture to reach the exponential phase of the bacteria was prepared. Once its optical density was obtained, this pre-culture was used for the preparation of an inoculum of 10^6 CFU/mL of bacteria read at a wavelength of 625nm a V-1200 spectrophotometer (Shanghai Mapada Instruments CO., LTD). In test tubes, 1mL of sterile distilled water with neutral pH and 1mL of the previously prepared medium were added. 250µL of the 10^6 CFU/mL bacterial suspensions were homogenized in each tube (in the concentration range 100, 200, 400 and 600 µg/mL) previously prepared. The tubes were incubated at 37°C under stirring 150 x g for 24 h. Two tubes represent the controls: one tube contains distilled water, culture medium and inoculum. A second tube contains only distilled water and culture medium.

Determination of the Minimum Bactericide Concentration (MBC)

It corresponds to the lowest concentration of extract capable of killing 99.9% of the inoculum, i.e. less than 0.01% of survivors. It defines the bactericidal effect of a given sample (Ennadir et al., 2014). Samples were taken from the control tubes and from each of the bacteria-free tubes. They were then inoculated into Petri dishes containing the Müeller-Hinton Agar medium. Petri dishes were incubated at 37°C for 24 h.

Antioxidant activity

The antioxidant activity of *P. tomentosa* L. was carried out by the DPPH· radical scavenging method (Bougandoura and Bendimerad, 2013; Sánchez-Moreno et al., 1998). In this test, a volume of 50 µL of different extracts of petroleum ether, ethyl acetate and ethanolic were added to 1.950 mL of the methanolic solution of DPPH· (0.025g/L). At the same time, a negative control was prepared by mixing 50 µL of methanol with 1.950mL of the methanolic solution of DPPH·. The positive control was represented by a solution of a standard antioxidant; ascorbic acid whose absorbance was measured under the same conditions as the samples and for each concentration. The IC_{50} value was determined graphically.

PI. (%) $=[\frac{A0-A}{A0}]\times 100\%$

- PI: Percentage of inhibition
- A_0: The optics density of the free radical (DPPH) solution in the absence of the extract.
- A: Absorbance of the free radical (DPPH·) solution in the presence of the extract.

RESULTS

Yield

The highest yields were found in the aerial part (Table 1). However, in both roots and aerial parts, the highest yield is present in Aq extract. On the other hand, the lowest yield is given by Ethyl Acetate for both organs.

Table1. Yield of two organs (Aerial Part and Root Part) in *P. tomentosa*L. using three organic solvents (Petroleum Ether, Ethyl Acetate and Ethanol) and aqueous extract

Plant Organ / Solvent	**Petroleum Ether**	**Ethyl Acetate**	**Ethanol**	**Water**
Aerial part (%)	2.86	1.21	2.33	4.62
Root part (%)	0.31	0.2	1.17	2.71

Phytochemical characterization

The phytochemical study of *P. tomentosa* L. reveals the presence of the following secondary metabolites: flavonoids, tannins, steroids and saponins in both plant organs (Table2). Nevertheless, steroids are absent in Petroleum Ether and Ethyl Acetate extracts. The roots are distinguished from the aerial part by the absence of flavonoids in the Ethyl Acetate extract and tannins in the Petroleum Ether extract. Alkaloids, sterols and triterpenes, steroid and triterpenic heterosides are absent in the plant. Aqueous extract shows saponins and tannins as well.

Table2. Phytochemical studies of two organs (Arial part and Root part) in *P. tomentosa* L. using three organic solvents (Petroleum Ether, Ethyl Acetate and Ethanol) and the aqueous extract; +: positive test; -: negative test

Métabolites/ Sample	**Petroleum ether**		**Ethyl acetate**		**Ethanol**		**Water**	
	Aerial Part	Root Part	Aerial Part	Root Part	Aerial Part	Root Part	Aerial Part	RootPart
Flavonoids	+	-	+	-	+	+	-	-
Tannins	+	-	+	+	+	+	+	+
Saponins							+	+
Steroids	-	-	-	-	+	+		
Sterols and triterpenes	-	-	-	-	-	-		
Alkaloids	-	-	-	-	-	-		
Steroid and triterpeneheterosides					-	-		

Antibacterial activity

The antibacterial activity was studied using extracts of three organic solvents (Petroleum ether, Ethyl acetate and Ethanol) and Aqueous extract both from roots and aerial parts. Four bacterial strains were tested: *S. aureus*, *E. coli*, *C. freundii* and *L. monocytogenes*. Thus, except of S. aureus strain which is weekly sensitive to PE and EA extracts from the roots, all the other tested bacteria were resistant (Fig.1). In fact, the diameters of halos of inhibition are 8.3 mm and 7.5 mm, for PE and EA, respectively (Table3).

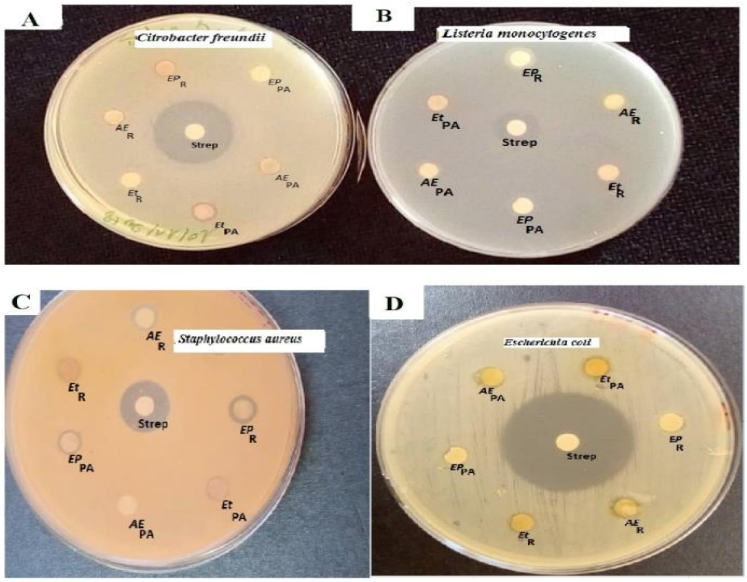

Figure 1. The activity of extract from the roots and aerial parts of *P. tomentosa* L against bacterial strains. The positive control used in this test was the antibiotic streptomycin.

Table 3. Antibiogram of the three organic extracts obtained from aerial part roots of *P. tomentosa* L

Strains	Positive control	Extract of the aerial part			Extract of the root part		
	Streptomycin	Petroleum Ether	Ethyl acetate	Ethanol	Petroleum Ether	Ethyl acetate	Ethanol
E. coli	+	-	-	-	-	-	-
S. aureus	+	-	-	-	8.3mm	7.5mm	-
C. freundii	+	-	-	-	-	-	-
*L.**monocytogene***	+	-	-	-	-	-	-

The minimum inhibitory concentrations were 400 µg/mL for the petroleum ether roots extract and 200 µg/mL for the ethyl acetate extract (Fig.2). On the other hand, the extracts remained inactive with respect to the other strains tested. However, the MBC results show a bacteriostatic effect.

Antifungal activity

Antifungal activity was evaluated toward five strains of *Candida* and *Saccharomyces cerevisae*. Our results revealed the ineffectiveness of all the extracts on the germs tested. However, the growths after 24 h were different depending on the solvents used.

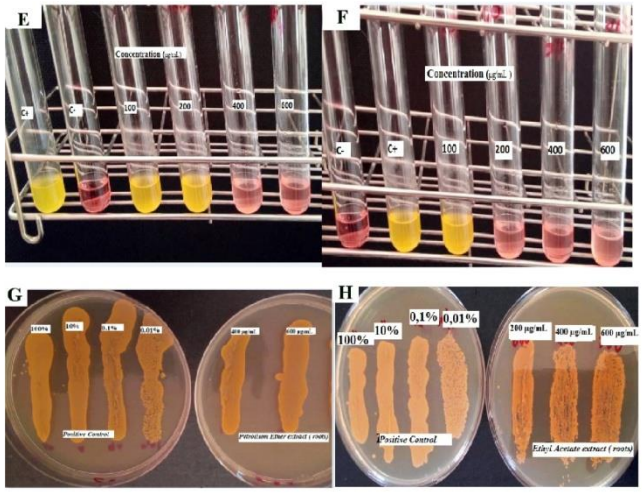

Figure 2. The determination of the MIC and MBC of the petroleum ether (fig2E, G) and ethyl acetate (fig 2F, H) extracts marked by the inhibition of any visible germ growth.

Antioxidant activity

The antioxidant activity of *P. tomentosa* L. was evaluated by the DPPH method. The highest OD was observed with ethyl acetate extract. The lowest absorbance was found in the ethanol extract of the roots, while that of the aerial part occupies an intermediate position (fig. 3). The trend curves show highly significant correlation coefficients (>96).

The inhibition capacity of *P. tomentosa* L. extracts of the DPPH· radical was greater than 90% at concentrations of 4.5 mg/mL of ethanol root extract and 5 mg/mL of ethanol aerial part extract. For ethyl acetate extracts, the inhibition power exceeds 90% at concentrations of

4mg/mL. That of ascorbic acid was 0.35mg/mL. The values obtained by the extracts were not very significant compared to those of ascorbic acid.

The results of IC_{50} show that ethyl acetate extract is the most effective (1.9mg/mL) (Fig.3). The least effective was obtained with the ethanol extract of the order of 3mg/mL for the aerial part and 2.4 mg/mL for the roots. However, this efficacy is lower than that of ascorbic acid, which has an IC_{50} value of 0.15 mg/mL.

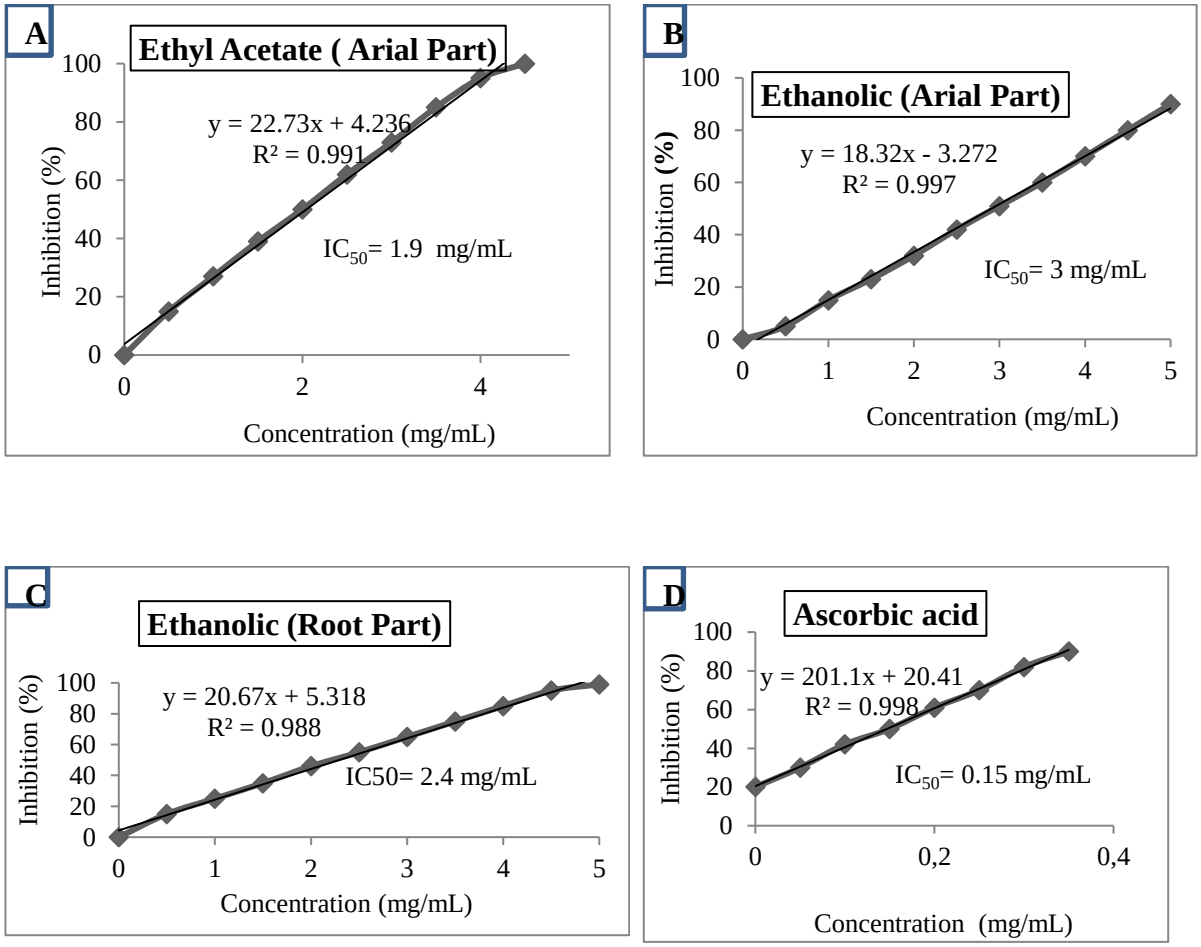

Figure 3. The IC_{50} values of the different extracts expressing the capacity of antioxidants to trap 50% mole of DPPH˙.

DISCUSSION

The ecotype of *Pergularia tomentosa* L. used in this study shows a higher yield (1.21-4.62%) than four Algerian ecotypes. However, it remains lower than those of other plants such as

Dialium dinklagei 18%, *Newbouldia leavis* 18.5% and *Cnestis ferrugineavahl* 17.95% (Tuo et al., 2015). This drop in yield compared to other species and the limited area of its expansion suggest its substitution in Mauritania by another species of the same phytotherapeutic value. The biological and therapeutic activity of a plant is generally due to the presence of biologically active molecules. Sterols are active ingredients used in the treatment of hypercholesterol (Luu and Ourisson, 1989). The Mauritanian ecotype is characterized by the absence of this molecule. Other authors have shown the presence of this principle in the same species (Shinkafi, 2014). Alkaloids are widely sought in medicinal plants for their pharmacological activities: as depressants (morphine), stimulants (caffeine), paralysing substances (curare) and anticancer drugs (vinblastine and vincristine) (Wink, 1999). The ecotype used in this study differs from the others by the absence of this molecule. Our bibliographical research has shown the presence of alkaloids in *P. tomentosa* L. (Hassan and Oumar, 2007). Triterpenes and heterosides are also absent in our study although they are well cited in other studies on *P. tomentosa* L. (Babaamer et al., 2012).

The antibacterial activity shows that only *Staphylococcus aureus* strain is sensitive to extracts of *P. tomentosa* L. Our results are similar to some studies at the literary texts (Al-Mekhlafi and Masoud , 2017; Rayyan et al., 2018). This kind of bacteria causes food poisoning (Schmid et al., 2009). In general, the antibacterial activity of plant extracts depends on their chemical composition. The polyphenols present in plant extracts could be at the origin of this activity. Nevertheless, this inhibition of the bacterium remains weak and bacteriostatic.

The evaluation of antifungal activity shows that all *P. tomentosa*L. extracts are ineffective against the strains tested. These results contrast with some works (Bekheet et al., 2011; Jdey et al., 2017; Lahmar et al., 2017), which showed that aqueous and multi-organic extracts of leaves, fruits, stems and roots have antifungal activity. Even more, one of these investigations (Lahmar et al., 2017) showed a strong ability of *P. tomentosa* L extracts to reduce the DPPH$^{\cdot}$ radical. However, our results reveal a low capacity to reduce this radical. The IC_{50} values are 8 to 20 times higher than those of ascorbic acid. This activity appears to be due to the presence of free hydroxyl chemical groups. The results of other researchers working on the same species and in other regions of the world (Al-Mekhlafi and Masoud , 2017; Yakubu et al., 2015)have shown, contrary to our results, a very significant antioxidant activity in extracts of *P. tomentosa* L. explaining its use by phytotherapists in the treatment of cancer.

CONCLUSION

The absence of the species *P. tomentosa* L. native to the region of Tiris Zemmour, (Mauritania) from the Mauritanian pharmacopoeia seems to be due, according to this study, to weak phytochemical characteristics and biological and antioxidant activities.

The results of this work allow us to conclude that:

- This ecotype of *Pergularia tomentosa* L. is characterized by a low yield of extracts and the absence of secondary metabolites such as alkaloids, sterols, heterosides and terpenes, which are well researched in the traditional pharmacopoeia.
- There is a very limited biological activity, as well as a not very significant antioxidant power compared to other ecotypes.

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