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In vitro evaluation of the anti-microbial activity and the anti-oxidant activity of the flavonoids extracted from the flowers of the *Tamarix africana* Poir

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Key words: *Tamarix Africana*, Flavonoids, Antimicrobial activity, Antioxidant activity

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Abstract

The aim of this work is to determine the quantity, the quality, the antimicrobial activity and the antioxidant power of various extracts of the flavonoids obtained from the flowers of *Tamarix africana* Poir. The quantification of the extracts obtained was revealed in high yield of the flavonoids with respectively: the methanoic extracts (26.31%), the extracts of the aqueous phase (19.29%), the extracts of ethyl acetate (0.87%), the extracts of petroleum ether (0.18%). The qualitative study, using the thin-layer chromatography (TLC), showed the dominance of Flavonols, flavones, isoflavones, flavanones and 3-glycosidic Anthocyanidins. The study of microbial activity revealed an important bactericidal power for the extracts of the aqueous phase on Gram + bacteria with a disc of inhibition of 24±1mm on *Staphylococcus aureus* ATCC43300 and 20±1mm on *Staphylococcus aureus* ATCC 25923, For the antifungal activity all the extracts gave important effects on *Podosphaera leucotrichia* (apple powdery mildew), with a maximum disc inhibition of 20±1mm for the ethyl acetate extracts, on the other hand alone The ether extracts of the petrol which showed an inhibitory effect on *Penicillium* sp. The antioxidant study, expressed as a percentage of DPPH, showed a high efficiency of the various extracts; In particular that of the ethyl acetate extract which inhibits oxidation and traps the free radicals at 100%, which demonstrating the use of this plant in traditional medicine for the treatment of certain types of cancer.

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Introduction

The resistance developed by pathogenic organisms to antibiotics, the spread of many carcinogenic diseases and the excessive use of pesticides polluting ecosystems are reasons that have pushed research towards the exploitation of medicinal plants used Since antiquity using the healing power of their secondary metabolites such as flavonoids, alkaloids, terpenes, etc. (Benabdallah, 2016).

Among these plants are the *Tamarix* species from the Tamaricaceae family, of which Algeria has more than 15 species of this genus (Khabtane and Rahmoune, 2012). View the use of these plants in traditional medicine in some cases of cancer, diarrhea, hair loss, etc. (Khabtane and Rahmoune, 2010); many studies are carried out on biological activity (antibacterial effect only), neglecting the fungicidal effect, as well as the antioxidant power of the different parts of the *Tamarix* species such as (Ksouri 2009, Wang, 2009 Saıdana, 2008, Parmar *et al*., 1994 and ...)

On this vision, our work aims at: the quantitative and qualitative determination of the various extracts of the flavonoids obtained from the floral part of *Tamarix africana* Poir. The determination of the bactericidal effect and the fungicidal effect which is applied for the first in this work against a fungal species known for its detrimental effects on the production of apple trees (*Podosphaera leucotrichia*) and finally to put the accent on the antioxidative power of the extracts obtained.

To assess the antimicrobial activity we chosed five species of pathogenic bacteria that are: *Staphylococcus aureus* ATCC 43300, *Escherichia coli*, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa*, *Salmonella* sp. for the Fungi We chosed two species: *Podosphaera leucotrichia* (powdery mildew of apple) which constitutes a threat to the to the arboriculture of apple tree which characterizes the region Khenchela and *Penicillium* sp.

At the end of the in vitro determination of the antioxidant power we applied the method of Blois (Ben Mansour, 2015), (Biswas 2014), where the free radical DPPH unstable has a dark violet coloration, when it is reduced and the coloration becomes pale yellow

Materials and methods

plant material

The flowers of *Tamarix africana* Poir. are collected during the period of spring flowering from the arid zone of Babar located in the south of the region of Khenchela from east of Algeria.

Preparation of Samples

The flowers collected are cleaned and dried in the open air (during 4 days) on protected from light, then they are crushed with a mortar and sieved to obtain a powder.

The extraction of flavonoids

Protocol of extraction

The extraction was performed in the Chemistry Laboratory of the University of Khenchela. The extraction of flavonoids is carried out according to the diagram presented by Lebreton (Athamena, 2009).

Preparation of the crude extract

For 1g of dry plant rendered in powder placed in a glass container (flask), covered with 10ml of a water-alcohol mixture (methanol/water 7/3: v/v). The whole is heated to 70°C for 5 minutes (this kills the plant tissue, preventing the oxidation or the enzymatic hydrolysis), the sample is left to macerate during a night (24 hours) at ambient temperature. The All by the suite is filtered on Whatman paper, the extraction is redone several times with the renewal of the solvent. The solvent is eliminated of the filtrate by rotary evaporation in a Rota Vapor, and the dry extract resulting is retained to +5°C.

Liquid-liquid extraction

We have implemented a series of liquid-liquid extraction in separating funnels by solvents practically non-miscible. The crude extract is mixed with distilled water boiling and left to shake in an ambient temperature. The latter is exhausted successively by 2 solvents (petroleum ether and ethyl acetate). The crude extract is initially mixed with petroleum ether, the mixture is left to settle and the organic phase higher is recovered. The extraction is repeated several times until the solvent becomes transparent.

The petroleum ether is subsequently evaporated and the resulting extract is considered as being the fraction of petroleum ether. The residual aqueous phase is subject to another liquid-liquid extraction by the ethyl acetate by following the same steps as the first extraction. The series of extraction allows to obtain four fractions; the crude extract hydro-méthanoïque, the fraction of ethyl acetate, the fraction of petroleum ether and the aqueous fraction residual. The extracts are retained until the use.

Determination of performance

The weight of each dry extract is determined by the difference between the weight of the Flask Full (after removal of the solvent by evaporation rotary) and the weight of the empty balloon.

Phytochemical characterization of extracts

Quantitative determination of flavonoids

The method of the $AlCl_3$ has been used for the determination of the total content of flavonoids extracts (Huang *et al.* 2008).

Qualitative determination of flavonoids

For the determination of the quality of flavonoids, we have applied the technique of analysis by thin-layer chromatography (TLC) with plates of silica gel G60; 0.25mm, on rigid media in glass; 20/20cm.

Two systems of solvents have been used on the two fractions of the methanolic phase: Fraction ethyl acetate and ether fraction of oil.

- Ether Oil - ethyl acetate (10:10v/v)
- Chloroform/methanol (8:12v/v)

Testing of biological effects

Evaluation of the antimicrobial activity

Antibacterial activity

The antibacterial activity of the extracts was determined by the method of dissemination in agar medium standardized by NCLLS, five bacterial strains were tested: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 (sensitive to the antibiotic), *Staphylococcus aureus* ATCC 43300 (resistant strain), *Salmonella* sp.

After the preparation of the bacterial inoculum, the extracts have been resumed with the methanol, and distilled water except for the extract of the aqueous phase with a concentration of 20mg /ml for each extract.

It was subsequently prepared the discs of paper Whatman, they are soaked in extracts (30µl for each disk), and these are deposited and sterilized using a clamp on the surface of the agar. As well witnesses imbibed in the methanol (and in the distilled water for the extract of the aqueous phase) and others in an antibiotic (here the Gentamycine Sulfate 20mg/ml) were deposited in the boxes of steeped (discs Responsible for 30µl of each solution).At the end the boxes of kneaded sown are placed in the oven for 24 hours at 37°C. The experience is repeated three (03) times for each extract and for each bacterial species.

Evaluation of the antifungal activity

The antifungal activity of the extracts was determined by the method of dissemination in Agar (Celiktas *et al.*, 2007), (Sacchetti *et al.*, 2005), and which focused on two of the following species:

- *Penicillium* sp: obtained from a colony (pre-culture) already existing in the laboratory,
- *Podosphaera leucotricha* : agent of the powdery mildew of apple tree collected from an infected tree in the region of Ensigha in Khenchela. After microscopic identification, we assessed the effectiveness of our extracts against the strains studied.

The extracts were resumed with the methanol, and distilled water except for the extract of the aqueous phase with a concentration of 20mg/ml for each extract. The disks of Whatman paper, are imbibed in the extracts (30µl for each disk), and the negative controls (methanol and distilled water) are deposited and stérilized using a clamp on the surface of the agar. The strains were incubated for a few days in normal atmosphere (temperature of the laboratory). The experience is repeated three (03) times for each extract and for each fungal strain.

Evaluation of the antioxidant activity

The antioxidant activity of the four extracts was tested by the method of Blois free radical DPPH unstable has a dark violet coloration, when it is reduced, the coloration becomes pale yellow.

Solution of Extract

The 1,1-diphenyl-2-picrylhydrazyl scavenging activity (DPPH) ($C_{18}H_{12}N_5O_6$, M=394.33), is solubilized in the absolute methanol for having a solution of 100 µl. For the test, the samples have been prepared by dissolving in the absolute methanol.

We have prepared solutions in the absolute methanol and that of the aqueous phase in the distilled water to reason from 20mg/ml for each extract, which offer a solutions mothers, the dilutions for having different concentrations of the order of: 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.53mg/ml, 0.30mg/ml, 0.15mg/ml, 0.075 mg/ml. and the witnesses of antioxidants reference, here are the ascorbic acid.

Test of the DPPH

We introduced in tubes, dried and sterilized, 30µl of the solution to test, it adds 3ml of the solution to the DPPH, after agitation by a vortex, the tubes are placed in the darkness at ambient temperature for 30 minutes, for each concentration, the test is repeated 3 times.

The reading is performed by measuring the absorbance at 517nm in a spectrophotometer. For each dilution, it has prepared a White consisting of 3ml of the DPPH solution and 30µl of methanol. The percentage discoloration of the DPPH, in methanol solution determines the antioxidant activity. The results have been expressed by the average of the three measures. The percentage of the activity anti-radical is calculated according to the following equation:

Antiradical activity (%) = [(ac - at)/ AC] X100

ac: absorbance of the control.

at: absorbance of the test.

Expression of results

The antibacterial activity and antifungal have been determined by measuring with the aid of a rule the diameter of the inhibition zone. The results have been presented by the average with its standard deviation (n=3) for each case as well the histograms are achieved by the Excel.

Results and discussion

The performance of the extractions

To determine the performance of the extraction we applied the following equation:

R= Pe/Pa x 100.

Where

R: performance of the extract in percentage.

Pe: Weight of the extract in gram.

Pa: Weight of the plant in gram.

As indicated in Table 1, the best performance value was obtained with the methanolic extracts (26.31%), followed by the aqueous phase extracts (19.29%), then the ethyl acetate extracts with (0.87%), finally the petroleum ether extracts with the lowest value (0.18%). The variability in performance, in appearance and in color is mainly related to the nature of the solvent used in the extraction, to the fractionation and its polarity and to the extraction method which affects also the total content in phenol and flavonoids (Rasooli *et al.*, 2008).

Table 1. Appearance, color and performance of different extracts from the flowers of *Tamarix africana*.

Extracts	Aspect	Color	% of Performance
Méthanoïc	Paste form	Reddish brown	26,31%
Aqueous phase	Oily pate	Dark brown	19.29%
Ethyl acetate	Paste form	Orange brown	0.87%
Ether of oil	Fine powder	Crystalline Green	0.18%

The results of the quantitative determination of the Extracts

Dosage of flavonoids

The $AlCl_3$ method is applied for estimate the flavonoid contents in the extracts and the spectrophotometry allowed to quantify them in the extracts obtained, Then calibration curve is plotted using different concentrations of Quercetin, which is a flavonoid very known from the family of falvonols.

The main reason for what we choosed this class of polyphenols, lies in the fact that the flavonoids constitute the most significant class of the polyphenol; with more than 5000 compounds already described (Marfak, 2003).

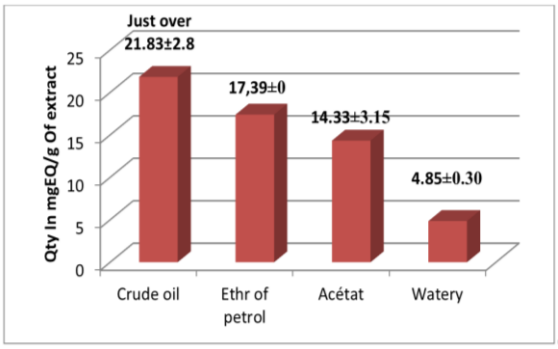

Fig. 1. The content of total flavonoids (mgEQ/GE).

According to Fig.1, the levels of flavonoids, expressed in mg Quercetin Equivalent per g of extract, the results obtained ware 21.83mg, 17,39mg, 14.33mg and 4,85 mg respectively with the crude extracts, ether of petrol extracts, ethyl acetate extracts.

Then the aqueous phase extracts: this observation is supported by several jobs, including those of Moroh and Bagré, which have shown that the extracts hydro alcoholic allows a better concentration of active principles, while the content of the lowest value has been obtained with the aqueous phase extracts. However, the results obtained with the fraction of the methanolic extracts show in a general way that the content from the flavonoids of the two phases: the petroleum ether and the acetate of ethyl is well high. This distribution depends on the nature of the phenolic substances contained in each crude extracts, of their solubility and the polarity of each solvent.

The results of the qualitative determination of the extracts

Results of the thin-layer chromatography of the different extracts

The thin Layer Chromatography (TLC) of the extracts from the flowers of *Tamarix africana* Poir. is based primarily not only on the mobile phase, but also on the stationary phase (stationary phase used is the silica gel).

Several tests have been carried out for having a good separation, by using two solvent systems for the two fractions; petroleum ether and ethyl acetate: Ether Oil - ethyl acetate (10:10v/v) and Chloroform/methanol (96:4v/v) which allowed a good separation and an acceptable visibility of spots.

The identification of compounds was based on the comparison of the RF and the observed colors under UV light bulb of spots appeared on TLC. The variation of the types and rates of flavonoids have been linked to environmental conditions and the growth stage of plants.

The results are expressed, in table 2, in which the RF of the different spots emerged, according to the solvents used as well the different colors are marked in the UV. That represents the different compounds of *Tamarix*. The results obtained from this method are:

Ether Oil System - ethyl acetate (10:10 v/v)

Five compounds are the resulted respectively 2 spots for the extract petroleum ether and 3 spots for the extract Ethyl Acetate each of the two fractions; petroleum ether and ethyl acetate, Different polyphenolic compounds were separated but a large part probably belongs to the classes of the flavonols (Table 2).

Table 2. Result of the TLC in the Fraction petroleum ether and ethyl acetate Solvent System: Ether Oil - ethyl acetate (10:10 v/v) Adsorbent: silica gel.

Color under UV 365 (nm)		Rf (cm)	Flavonoid type possible
Fraction Ethyl acetate	Bright violet	0.09	Flavones
	Pale yellow	0.23	The flavonols
	Yellow	0.34	The flavonols
Ether fraction of oil	Violet	0.06	Flavones
	Yellow	0.30	The flavonols

System Chloroform/methanol (8:12 v/v)

Also five compounds resulted for the two fractions respectively petroleum ether (2spots) and ethyl acetate 3spots; the spots are viewed by the UV light; the separation allows you to have the migration of polyphenolic compounds belonging to the: Anthocyanidine

3-glycosides, and Phenols Acid and the flavonols; it also shows a considerable wealth in substances (Table 3).

Table 3. Result of the CCM in the fraction Petroleum ether Solvent System: Chloroform/methanol (96/4) Adsorbent: silica gel.

	Color under UV 365 (nm)	Rf (cm)	Flavonoid type possible
Fraction of Ethyl Acetate	Blue White Fluorescent	0.71	Flavonols, flavones, isoflavones, flavanones,
	Yellow	0.82	The flavonols
	Red	0.93	Anthocyanidine 3-glycosides
Fraction of petroleum ether	Blue White Fluorescent	0.74	Flavonols, flavones, isoflavones, flavanones,
	Red	0.89	Anthocyanidine 3-glycosides

Results of biological tests

Evaluation of antimicrobial activities

The antibacterial activity tested by the method of Diffusion in agar

The Method of Disks or method of Diffusion in agar was designed to study the antibacterial activity of natural substances. It has allowed obtaining the results mentioned in table 4.

Table 4, The diameter of the inhibition zones.

Bacterial strains	Diameters of the zone of inhibition (mm) of each extract				
	Methanolic Extract	Acétate ethyl Extract	Ether of petrol Extract	aqueous phase Extract	Gentamycine antibiotic used
Staphylococcus aureus ATCC43300	no activity	no activity	no activity	24 ±1	37
Escherichia coli	no activity	no activity	no activity	8±0	30
Staphylococcus aureus ATCC 25923	8±0.5	no activity	8±0	20±1	25
Pseudomonas aeruginosa	no activity	no activity	no activity	10±2	27.5±1
Salmonella sp.	no activity	no activity	no activity	no activity	30

(Average diameter ±Standard deviation in mm)

-Diameter: D< 8 mm, $9 \leq D \leq 14$ mm, $15 \leq D \leq 19$ mm, and> 20 mm is considered respectively as resistant strain (-), sensitive (+), very sensitive (++), extremely sensitive (+++).

No zone of inhibition was observed around the disks to the load 30 µl/disk after the end of the incubation of bacterial cultures of *Salmonella sp*. this strain has a very high potential for resistance against the antibacterial action of these extracts.

The results obtained with the fraction of ethyl acetate show no antibacterial activity on all the strains studied. *Escherichia coli* was not sensitive (8 ±0 mm) to the extracts of the aqueous phase; and also for all extracts tested no marked sensitivity.

The aqueous phase extracts, have shown a great antibacterial Power with diameter of inhibition of (24±1mm), on *Staphylococcus aureus ATCC43300* and *Staphylococcus aureus ATCC 2592* with diameter of inhibition of (20±1 mm), representing a strains extremely sensitive against this fraction. And also against other extracts of methanolic and petroleum ether with (8±0.5 mm) and (8±0) respectively. The *Pseudomonas aeruginosa* presents a moderate sensitivity with this extracts from the residual aqueous phase. The negative controls (methanol and distilled water) showed no activity against the nine strains tested.

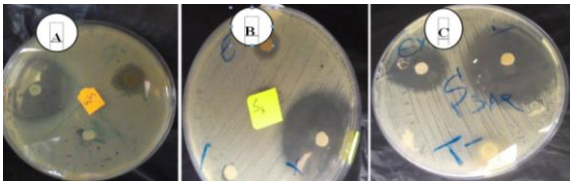

Photo 1. Showing the antibacterial effect of the methanolic extract (A) and the extract ether of oil (B), the extract of the aqueous phase (C) Against *Staphylococcus aureus* ATCC 25923.

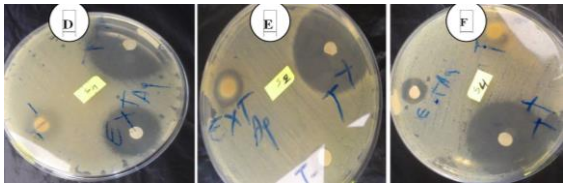

Photo 2. Showing the antibacterial effect of the extract of the aqueous phase against *Staphylococcus aureus ATCC43300* (D), *Escherichia coli* (E), *Pseudomonas aeruginosa* (F).

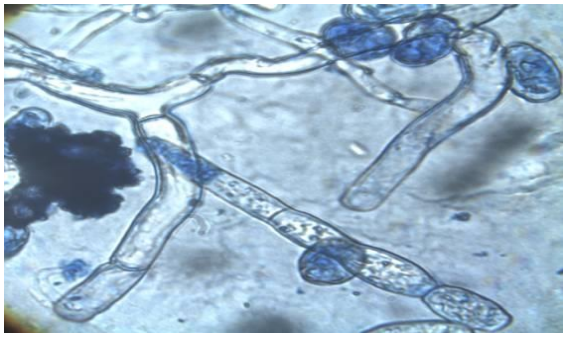

Photo 3. Coloration in the fresh state of *Podosphaera leucotrichiaa* causal agent of powdery mildew of the apple tree (optical enlargement x100).

Evaluation of the antifungal activity

Microscopic identification of the powdery mildew

The coloring in the fresh state allowed us to see the mycelia elements that present the fundamental part of the fungus, thus the spores in abundance.

The antifungal activity tested by the method of Diffusion on agar

The Disks Method or method of Diffusion in agar was applied to study the antifungal activity of flavonoids extracted from the flowers of *Tamarix africana* Poir. against Pathogenic Fungi required; the diameter of the inhibition is measured at the end of five days, or more on depending on the speed of growth of fungi. Positive results satisfactory are validated for a zone of inhibition with a greater diameter than 15 mm.The negative controls have shown negative results against the two molds. The results are as follows in table 5.

Table 5. Diameter of the zones of inhibition of Mold E: extract, (average diameter ± standard deviation in (mm).

The fungal strains	Diameters of the zone of inhibition (mm) of each extract			
	E. Methanolic	E. Acétate of ethyl	E. Ether of petrol	E. of the aqueous phase
Leucotrichia podosphaera	20±0.0	20±1	10±0.5	15±0.0
Penicillium spp.	5±1.0	No activity	20±	No activity

All methanolic fractions as well the extracts of the aqueous phase showed a large inhibitory power against *Podosphaera leucotrichia*, agent of the powdery mildew, in particular the germ has presented an extreme sensitivity against the methanolic extracts and that of the ethyl acetate extracts, on the other hand the ether of oil extracts has no effect noticed on this strain (low inhibition). *Penicillin* sp. has shown a moderate resistance against the ethyl acetate extracts and the aqueous phase extracts. On the other hand, the highest diameter is that of the ether of oil extracts with an inhibition of 20mm.

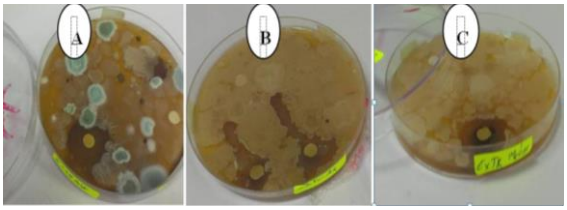

Photo 4. Showing the antifungal effect of the extract of acetate of ethyl (A) the extract of the aqueous phase (B), and the extract of crude extract (C) against *Leucotrichia Podosphaera*.

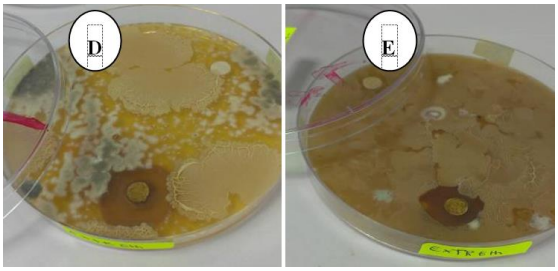

Photo 5. Showing the antifungal effect of the extract of petroleum ether *against Penicillium* sp. (D) *and Podosphaera leucotrichia (*E).

Table 6. Represents the percentage of reduction of radical DPPH.

Concentrations		The percentage of reduction of radical DPPH of each fraction			
Concentration Initial mg/ml	Concentration in the reaction mixture mg/ml	Ether of oil	Ethyl acetate	Methanolic	Extract from the aqueous phase
10	0.1	95.36 ± 0.11	95.83 ± 0.23	93.9 ± 0.47	95.42 ± 0.10
5	0.05	95.02 ± 0.11	95.70 ± 0.40	93.74 ± 0.09	95.04 ± 0.18
2.5	0.025	60.67 ± 0.26	95.27 ± 0.24	80.48 ± 5.16	93.08 ± 0.81
1.25	0.0125	43.67 ± 5.63	94.37 ± 0.12	44.79 ± 0.94	78.13 ± 0.65
0.60	0.006	23.09 ± 1.53	94.15 ± 0.09	26.29 ± 3.03	31.77 ± 0.62
0.31	0.0031	14.98 ± 0.16	75.49± 4.70	14.01 ± 0.67	18.97 ± 1.78
0.15	0.0015	/	71.55 ± 0.86	10.5 ± 1.03	12.24 ± 2.81
0.075	0.00075	/	63.54 ± 0.00	7.02 ± 0.09	12.61 ± 2.41

Evaluation of the antioxidant activity

The results was expressed as a percentage of the activity antiradical or in percentage of DPPH remaining or can also be expressed using the parameter IC 50, which is defined as the concentration of the substrate that cause a loss of 50% of the activity of DPPH (Ishtiaq *et al* 2014). In the evaluation of the antioxidant activity we have used the Ascorbic Acid as a reference (Fig.2 and Fig. 3).

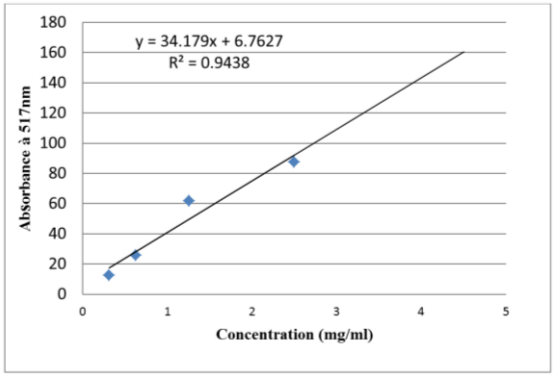

Fig. 2. The calibration curve of the ascorbic acid.

The results showed that the most active antioxydant is the ethyl acetate extracts with an IC 50 in order of 0.009mg/ml, it can be explained by the fact that the substances contained in this fraction respond directly and very quickly with free radicals of DPPH.

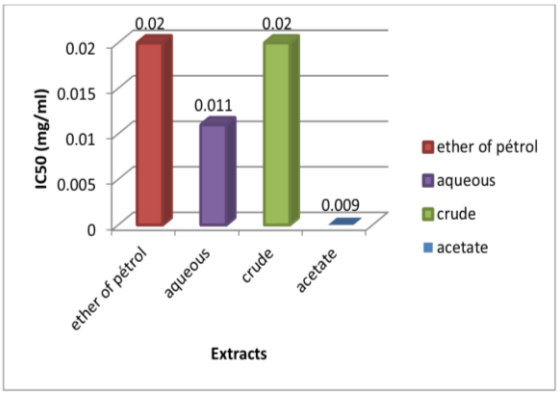

Fig. 3. Histogram of the different IC50 Extracts studied.

The Ethyl acetate is often used as a solvent extraction with selectivity in the extraction of phenolic compounds of low molecular weight (Markowicz Bastos *et al*., 2007). However the antiradical capacity of other extracts is less low than the fraction of acetate, the extracts of the aqueous phase with IC 50 in order of 0.011mg/ml, who has shown as an excellent power of neutralization of the radical of DPPH; also we find that there is no significant difference between the activity of both extracts of petroleum ether and the crude extract with an IC 50 in order of 0.02mg/ml (table 6). We Note that the trappers more effective of the free radical DPPH are those with the lowest value of IC 50 (Bonina *et al*., 1996).

The activities measured to assess the capacity of our extracts to inhibit the free radicals. The flavonoid compounds contained in the flowers of *Tamarix africana* Poir. are endowed of an important power antioxidant which showed a better inhibition 95.83% from the antioxidant activity more significant (IC50 = 0.009mg/ml), which could be exploited for the research of new molecules antioxidant, solutions for the multiple consequences of oxidative stress, and even useful against diseases induced by free radicals such as cancer, atherosclerosis and the aging of the tissues.

We can explain the highest results obtained of the antibacterial activity and the important antioxidant power of the flavonoids extracted from the flowers of *Tamarix afrcana* Poir. In comparison with other similar studies by the effect of the environment, because more the environment conditions becomes arid more the active molecules became concentrated.

Conclusions

The study concludes that the flavonoids extracts from the flowers of *Tamarix afrcana* Poir. has an important antimicrobial activity, especially on Gram + bacteria, also we had tested, for the first time in this work, the antifungal activity of these extracts on two strains of fungi where the results showed an acceptable effect; in particular the powdery mildew of apple tree which has expressed an extreme sensitivity against all of the fractions studyed in principle; the crude extracts and ethyl acetate extracts. These results have shown that the plant of *Tamarix* is capable of preventing the fungal contamination of species potentially phytopathogéne

The antioxidant activity of the different flavonoid extracts of flowers of *Tamarix afrcana* Poir. evaluated by the method of reduction of free radical DPPH, and the results obtained were very satisfied, with an IC 50 value of 0.009mg/ml using the acetate extracts; showing an interesting antioxidant power induced by the flavonoids of this plant.

The whole of these results obtained in vitro constitutes a first step in the search for substances of natural origin biologically active, a study in vivo is desirable, for a more in depth on the antioxidant and antimicrobial activities of the flavonoid extracts from the *Tamarix*.

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