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Antimicrobial and Antioxidant activities of various extracts of *Hyoscyamus albus* L. and *Umbilicus rupestris* L. leaves

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Abstract: Our objectives were to investigate the antioxidant activity by the means of two methods: the β-carotene bleaching method and DPPH assay as well as testing the antimicrobial activity by the Agar-gel diffusion method and the microdilution method with liquid medium of the extracts (EEp, EChl and EMeOH) of the leaves of *H. albus* and (EEp, EChl and EMeOH) of *U. rupestris.* The quantitative analysis are showed that the highest content of total phenolic was concentrated in the butanolic extract of *H.albus* with 111 .1 ± 1.82 µg EAG/mg of extracts, in the second level the methanolic extract of *U. rupestris* with 105.7±0.37 µg EAG/mg of extracts. The content of flavonoids was determined by specrophotometry and showed the presence of these compounds in the all extracts from the two plants.

In the β-carotene bleaching test, the EMeOH of *H.albus* displayed highest antioxidant activity (76.00 %) and the EMeOH extract with a radical-scavenging activity (72.97%). On the other hand, the quantitative evaluation of the antiradical activity towards DPPH showed that the EMeOH of *U. rupestris* is the most active (IC_{50} = 35.33 ± 0.136 µg/ml) followed by the EMeOH (IC_{50} = 75.19 ± 0.211 µg/ml) of *H. albus.*

All the polar extracts of the two plants reacted positively at least on one of the bacterial strains tested. These extracts showed different activities and the CMI were determined starting from the most active extracts in gelose medium, Extract EBut of *H.albus* testified to a strong antimicrobial activity followed by the EMe of *U. rupestris.* Results of the anticandidose activity showed the inefficiency of the all extracts against the yeast *Candida albicans*.

Keywords: *Hyoscyamus albus*, *Umbilicus rupestris,* polyphenols, Antioxidant activity, Antimicrobial Activity.

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I. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years. Folk medicines of the almost civilizations of the world abound in herbal remedies. Majority of the traditional medicines used in healthcare are obtained from plants. In spite of several advancements in the field of synthetic chemistry drug and antibiotics, plants continue to be one of the major raw materials for drugs treating various ailments of humans. Clinical and pharmaceutical investigations have in fact elevated the status of medicinal plants by identifying the role of active principles present in them and elaborating on their mode of action in human and animal systems. *H.albus* is an annual and perennial plant of 20-50 cm, hairy-viscous with low poisonous odor; soft leaves, and even floral and oval-orbicular petal. In addition to hyoscine and hyoscyamine, a new compound starting insulated from leaves and stems of *H. albus* was characterized like 2,3-dimethylnonacosane by spectral studies [1]. They announced the production of sesquiterpene of the phytoallexine type in the roots of *H. albus* [2, 3,4]. *Umbilicus rupestris (Salisb.) Dandy* is a hardy perennial plant, belongs to the family of *Crassulaceae***.** It presents at the level of rocks, cliffs and the old walls, on the coasts and in mountains; only in North Africa [5]**.** Leaves of this plant are used in traditional medicine against the ignitions of skin [6], wounds [7], like cataplasm, against burns [8] disinfectant, parasiticidal, healing [9]. Infused (leaves) of this plant is used like an ophthalmic disinfectant [10]. The goal of this study is to evaluate the antimicrobial activity by two methods, the diffusion in gelose medium and the microdilution in liquid medium of the rough extracts of *H.albus and U rupestris*. This evaluation is connected to the phenolic contents of these extracts.

II. MATERIAL AND METHODS

II.1. Plants Materials and extracts' preparation

Leaves of *H.albus* and *U.rupestris* were freshly collected in spring season from Batna, Algeria, in April 2012. The taxonomic identification of the plants was confirmed by Dr.Oudjhih, Department of Agronomy, University of Batna .Plants were dried 40 days in the shade under ambient temperature until total dehydration. Dried leaves parts were blended into fine powder and stored in the dark at a dry place. 1 Kg of powdered leaves was extracted with petroleum ether three times 5 L for each time. Then, the marc was dried and extracted with chloroform three times 5 L for each time and with methanol three times 5 L for each time and the supernatants were filtered sequentially using cloth filter, cotton wool, and Whatman filter paper. The solvents were then evaporated under reduced pressure (204 mbar) and controlled temperature (30°C) using a vacuum rotary evaporator (Buchi Rotavapor).

II. Preliminary phytochemical analysis

The different extracts were then subjected to preliminary phytochemical analysis to assess the presence of various phytoconstituents.

II.1. Determination of total phenolic compounds, total flavonoids and tannins

II.1.1. Determination of total phenolic compounds

Contents of polyphenols in the extracts are estimated and carried out according to the process describes by Marinova et *al.* [11]. The phenolic contents were given with the method

of Folin-Ciocalteu [12]. The extract diluted ten times was left; react with 1 ml of the reagent of Folin-Ciocalteu, and then the mixture was neutralized with Na_2CO_3. The mixture was after incubated at ambient temperature during 90 min. Lastly, absorbance was measured with the spectrophotometer assistance at 750 *nm*. T with total polyphenols in our extracts, was calculated starting from a linear calibration curve (y=ax+b*)* established with precise concentrations of gallic acid (0-200 *µg/ml)*, like standard of reference, under the same conditions as the sample. The total phenolic contents were expressed in *µg* equivalent of gallic acid (GAE)/mg of sample.

II.1.2. Determination of total flavonoids compounds

The total flavonoïds were estimated by using the method of Ordonez and *al.* [13]. 0,5 ml of ethanolic solution of $AlCl_3$ of 2% were added to 0,5 ml of each extract. After 1 H of incubation at ambient temperature, the absorbance is measured at 420 nm. The extract was evaluated with a final concentration of 0, 1 mg/ml. Content of flavonoïds is expressed of microgram of equivalent of quercetin per milligram of extract (*µg Eq AQ/mg)* starting from the calibration curve established by quercetin (0-50 *µg/ml)*.

II.1.3. Determination of condensed tannins compounds

The proportioning of condensed tannins in extracts of *H.albus* and *U.rupestris* is carried out according to the method of Heimler et *al.* [14]. The principle of this proportioning is based on the aldehydic fixing of the vanillin grouping on carbon 6 of cycle A of the catechin to form a red chromophoric complex which absorbs at 500 *nm* (fig. 2) [15]**.** For 400µl of each sample or standard, we add 3 ml of a vanillin solution (4% in methanol), and 1,5 ml of concentrated HCl. The mixture is incubated during 15 min and the absorbance is read with 500 nm. Concentrations of the condensed tannins are deduced starting from the ranges of calibration established with the catechin (0-300 µg/ml) and are expressed in microgram of catechin equivalent per milligram of extract (µg ECT/mg).

II.2. Antioxidant activity

II.2.1.Evaluation of antioxidant activity by *Beta-carotene bleaching test*

The antioxidant capacity of each of the sample extracts was estimated by the β-carotene bleaching method following the procedure described by Velioglu *et al*. [16] with modifications. A mixture of 60 mg of β-carotene was dissolved in 20 ml of chloroform and mixed with 1,0 g of the linoleic acid and 20 ml of Tween® 60. Chloroform was eliminated by rotary evaporation at 40 °C. After evaporation, the mixture was immediately added to 25 ml of hydrogen peroxide to form an emulsion. 25 ml of this emulsion were transferred in tubes containing 1,0 ml from the extracts and the mixture was then mixed gently. 1 ml of the mixture was introduced with the pipette and mixed with 5 ml of ethanol with 95% at 0 °C. Absorbances were read at 450 nm all the 20 min throughout the 160 minute with a Spectrophotometer. The procedure above was made by using tocopherol as a standard (positive control). A negative control without extract was prepared. The relative antioxidant activity of extracts (AAR) is calculated according to the following equation:

$$\mathbf{AAR\% = [Abs_{160h}\ (sample)/Abs_{160h}\ (\alpha\text{- tocophétrol})] \times 100}$$

II.2.2. Determination of antioxidant activity using the free radical scavenging activity (DPPH)

The method described by Tepe *et al.* [17] was used with slight modification, the different extracts and controls (α-tocopherol "antioxidants reference"). DPPH solution was prepared by dissolving 3 mg of DPPH in 100 ml of methanol. Samples and controls are added to 2 ml of DPPH solution after incubation for 30 min, absorbances were measured at 517 nm against the blank. The antioxidant activity is estimated using the following equation:

% Antioxidant activity = [(Abs control- Abs sample) / Abs control] x 100

II.3. Antimicrobial activity

Germs tested to detect the antimicrobial activity of the extracts were three strains of reference: *S. aureus* ATCC 25923, *E.coli* ATCC 25922, *P. aeruginosa* ATCC 27853. Four clinical strains: *S.aureus, E coli P. aeruginosa, P. mirabilis .* We used only one type of yeast, Candida *albicans.* They were all provided by the laboratory of Bacteriology from the university hospital center of BATNA.

II.3.1. Method of diffusion in medium gelose

Sterile filter paper discs of 9 mm of diameter, impregnated into 40 µl of each test plant extract, were placed on the surface of the inoculated agar plates. Extracts were taken with Dimethyl sulfoxide (DMSO).The antimicrobial screening was carried out with different concentrations for each extract (1g/ml, 0.5g/ml, 0.25g/ml and 0.125g/ml). Controls soaked in DMSO were only performed. Petri dishes were incubated for 24 h at 37°C. Antimicrobial activity was determined by measuring the diameter of the inhibition's zone.

II.3.2. Determination of the MIC (Minimum Inhibitory Concentration)

The MIC are determined by the standardized method of micro dilution in liquid medium [18]. The study is carried out into 96-well plate which contained a bubble Mueller- Hinton, with a bacterial inoculum of 5 UFC/ml. Samples' dilutions were distributed in cups from the strongest concentration (250 mg/ml) to the weakest one (1.95 mg/ml). Dilutions of extracts were carried out in the DMSO with 10%. Micro plaques are incubated 18h at 37°C. The MIC correspond to the first dilution where the growth is negative (not visible culture).

II.4. Statistical study

All tests were performed in duplicate or triplicate. Results are presented as mean± standard deviation of two or three independent determinations. All statistical analyses were carried out by Graphpad prism 5 using analysis of variance (ANOVA) and differences among the means were determined for significance at $p \leq 0.05$ using least significant.

III. Results and Discussion

III.1. Preliminary phytochemical analysis

The preliminary phytochemical investigations show the presence of various secondary metabolites (Table1).

Table 1. *Results of preliminary phytochemical analysis of extracts from H.albus and U.rupestris' leaves*

Plants	Extracts	Polyphenols	Flavonoïds	Tannins	Terpenoids	Alcaloïds
H. albus	EEp	++	+	-	+++	+++
	EChl	++	+	+	+++	+++
	EMeOH	++	++	+	+++	+++
U.rupestris	EEp	+++	++	++	+++	+
	EChl	++	++	+	+++	+
	EMeOH	+++	++	+	+++	+

+++ : high ; ++ : moderate ; + : mild ; - : absent

III.2. Total phenolic compounds, flavonoids content and tannins

Table 2. *Total phenolic, flavonoid and condensed tannins in various extracts of H.albus and U.rupestris' leaves*

Plants	Extracts	polyphenols[(a)]	flavonoïds[(b)]	tannins[(c)]
H. albus	EEp	55 .61 ± 1.32	14.12± 0.62	10.37 ± 0.55
	EChl	99,45 ± 2.75	18.23 ± 0.78	20.38± 0.69
	EMeOH	111 .1 ± 1.82	24.31 ± 0.62	24.87 ± 1.57
U. rupestris	EEp	23.83± 0.21	6.77± 1.24	13.27± 0.69
	EChl	95.98± 0.33	20.72± 0.37	22.93± 0.07
	EMeOH	105.7± 0.37	29.56± 1.88	25.48± 0.70

(a) µg of gallic equivalent of acid gallic by mg of extract (µg EAG/mg of extract).
(b) µg of gallic equivalent of quercetin by mg of extract (µg EAG/mg of extract).
(c) µg of gallic equivalent of catechin by mg of extract (µg EAG/mg of extract)

They proved that the methanol and the ethanol are effective solvents to extract the antioxidant phenolic compounds [19]. The examination of these results makes possible to put a significant linear correlation between the content of flavonoïds and the phenolic compounds (R^2 = 0.9987, P≤0.05), and between the content of tannins and phenolic compounds (R^2 = 0.9898, P≤0.05) in *H. albus'* extracts (fig. 1 and 2). And also to put a significant linear correlation between the content of flavonoïds and phenolic compounds (R^2 = 0.9606, P≤0.05), and between the content of tannins and phenolic compounds (R^2 = 0.9584, P≤0.05) in *U. rupestris's* extracts (fig. 3 and 4). Several factors can influence the content of phenolic compounds, recent studies showed that

extrinsic factors (such as geographical and climatic factors), genetic factors, but also the degree of maturation of the plant and the storage period have a strong influence on the polyphenol contents .The concentration of flavonoïds in the plants' extracts is according to the polarity of solvents used in the preparation of the extracts**.**

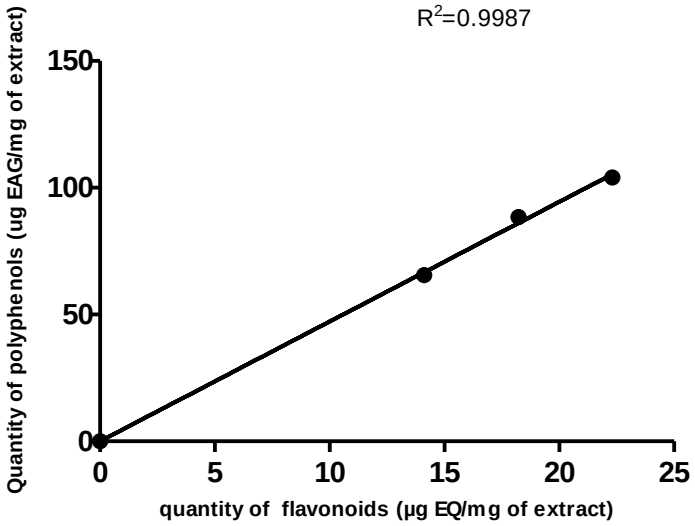

Figure 1. *Linear correlation between the amount of the total polyphenols and amount of flavonoids of different extracts of leaves of Hyoscyamus albus (p≤0,05)*

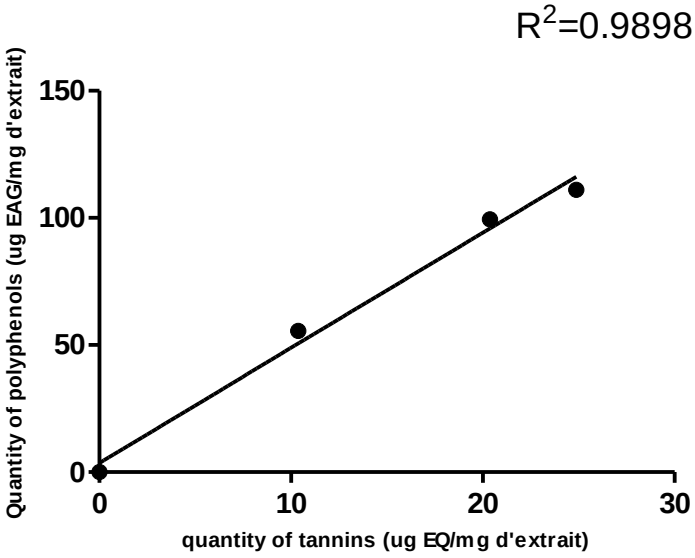

Figure 2. *Linear correlation between the amount of total polyphenols and amount of tannins of different extracts of leaves of Hyoscyamus albus (p≤0,05)*

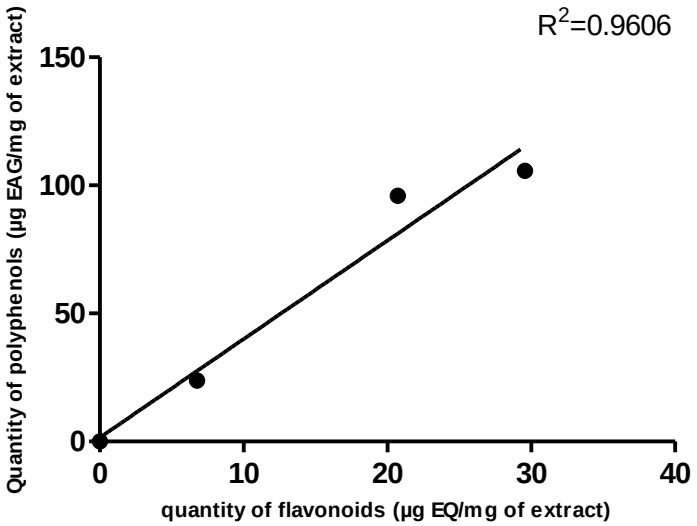

Figure 3. *Linear correlation between the amount of total polyphenols and amount of flavonoids of different extracts of leaves of U.rupestris (p≤0,05)*

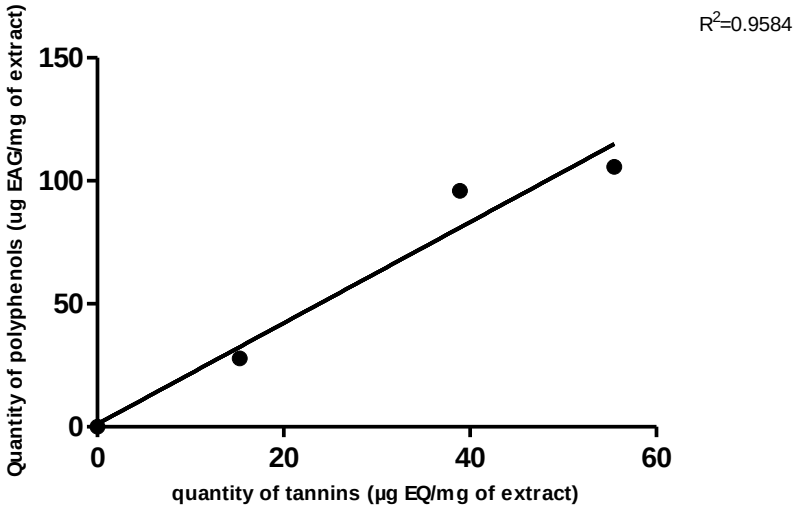

Figure 4. *Linear correlation between the amount of the total polyphenols and amount tannins of different extracts of leaves of U.rupestris (p≤0,05)*

III.3. Antioxidant activity

III.3.1. Antioxidant activity by beta-carotene bleaching test

In the system β -carotene / linoleic acid undergoes a fast discoloration in absence of an antioxidant. Free radicals of the linoleic acid formed during the production of an hydrogen atom from one of its methylene diallylic group, these free radicals attack highly the unsaturated β-carotene. Consequently, the β-carotene is oxidized and broken up partly; then it loses its characteristic of orange color, which is controlled by a spectrophotometer at 470 nm [20, 21]. In this test, the antioxidant capacity is given by measuring the inhibition of the hydroperoxides combined diene resulting from the linoleic oxidation of the acids. The β-carotene undergoes a fast discoloration in absence of an antioxidant; however the presence of an antioxidant is to minimize its oxidation. This test measures the potential of the plant to inhibit the hydroperoxide diene formation combined during the linoleic oxidation of the linoleics acids [22]. Table 3 brings back the average values of three

measurements of AAR% ± SD. These values facilitate the comparison of relative activities of the various extracts, the positive control (α-tocopherol) and the negative control.

Table 3. *Relative antioxidant activity of the rough extracts of H.albus and U rupestris' leaves and of the positive control.*

Extracts and control	**AAR %**
EEp (*H.albus*)	33.78 ± 1.28
EChl (*H.albus*)	43.24 ± 0,92
EMeOH (*H.albus*)	76.00 ± 0,92
EEp (*U. rupestris*)	63.51 ± 1.28
EChl (*U. rupestris*)	24.32 ± 0,92
EMeOH (*U. rupestris*)	72.97 ± 0,92
α-tocopherol	100 ± 0,00
Control (-)	8.10 ± 1,28

Values are an average of three measurements ± SD. Comparisons are carried out between the AAR% of the positive control (α- tocopherol) and of the rough extracts.

According to these results, it is clear that all the extracts tested of *H. albus* and *U .rupestris* inhibit in a highly significant manner ($P \leq 0,0001$) oxidation coupled of linoleic acid and β - carotene compared to the negative control which represents 100 % of the peroxidation. For the extracts of the two plants, highest inhibition was provided by EMeOH of *H. albus* (76.00 %) followed by EMeOH of *U.rupestris* (72.97 %) without significant difference between these two extracts ($P \leq 0.05$). EEp showed an intermediate inhibiting activity (63.51 %) and either for EChl (43.24 %), and in the last, it is EChl which represents the lowest inhibiting value (24.32 %). The activity of *H. albus* and *U.rupestris'* extracts remain significantly lower ($P \leq 0,05$), compared to the positive control (α- tocopherol).The antioxidant activity of polar extracts (EMeOH) could be explained by their wealth of polyphenolic substances. Many studies showed the antioxidant properties of the flavonoïds and tannins [23]. Polar extracts are especially rich in chemical water-soluble substances, their antioxidant activity is shown by this method (BCB), it can be due especially to the presence of phenolic compounds present in these extract, which is confirmed by the notable and the significant observed linear correlation ($R^2=0.9999$, $p<0.05$) between their content of polyphenols and their antioxidant capacity.The antioxidant effect of the extract can also have a biological interest because it can prevent the oxidation of lipidic components in cellular membranes and consequently vegetable extract can appear beneficial for health [21].

III.3.2. Antioxidant activity by DPPH method

The antioxidant activity of various extracts of *H. albus* and *U. rupestris'* leaves against DPPH radical was evaluated spectrophotometrically while following the reduction of this radical which is accompanied by its passage from the violet color to the yellow one at 515 nm. The IC_{50} of various extracts of *H. albus* and *U rupestris'* leaves as well as the standard were illustrated in figures 1 and 2 respectively. Results are mentioned in table 4.

Table 4 . *Antiradical activity of H. albus and U .rupestris' extracts*

Extracts and standards	IC_{50} (µg /ml)
EEp (*H. albus*)	-
EChl (*H. albus*)	330.19 ± 1.149
EBut (*H. albus*)	75.19 ± 0.211
EEp (*U. rupestris*)	610 .34 ±0.153
EChl (*U. rupestris*)	135.97 ± 0.584
EMeOH (*U. rupestris*)	35.33 ± 0.136
α-Tocopherol	15.08 ± 0.112

According to figures 5 and 6, we note that the extracts of *H. albus* and *U rupestris'* leaves, having an antiradical activity dependent amount the IC_{50} of each various extract, were determined. According to Kadri *et al.* [22], a lower value of the IC_{50} (concentration of the substrate which causes an inhibition of 50 % of the activity of DPPH) indicates a higher antioxidant activity. The antiradical activity of EEp of *H. albus's* leaves could not be really quantified but with the maximum tested concentrations, it does not collect more than 40 % of the DPPH. The comparison of the extracts EMeOH (*Ur)* EMeOH (*Ha)* EEp (*Ur)* EChl (*Ur)* and EChl (*Ha)* reveals that EMeOH (*U.rupestris*) represents the most active extract with an IC 50 of 35.33 µg/ml followed by EMeOH (*H.albus)* with an IC_{50} about 75.19 µg/ml while EEp represents the weakest antiradical activity (IC_{50} = 610 µg/ml). For comparison, we used α-tocopherol like a standard antioxidant; it showed an interesting antiradical activity with an IC_{50} about 15.08 µg/ml. In comparison with α-tocopherol, the tested extracts prove less active.

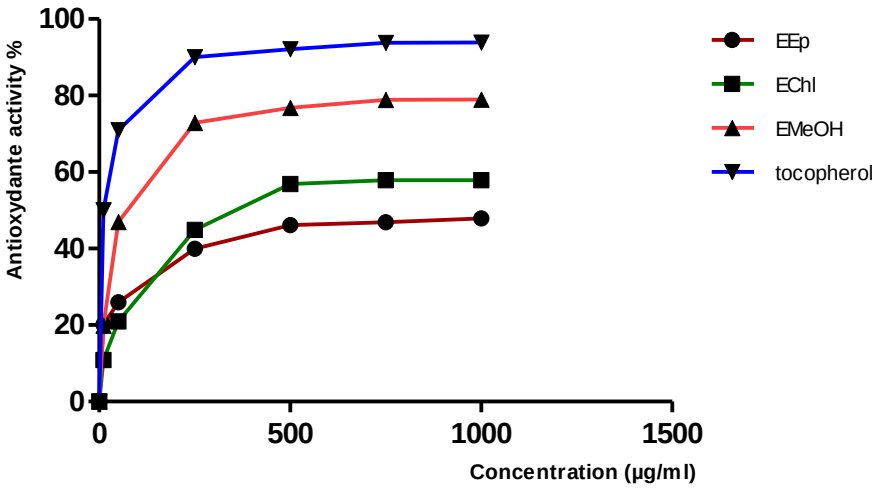

Figure 5. Antiradical activity of extracts from *H.albus's* leaves.

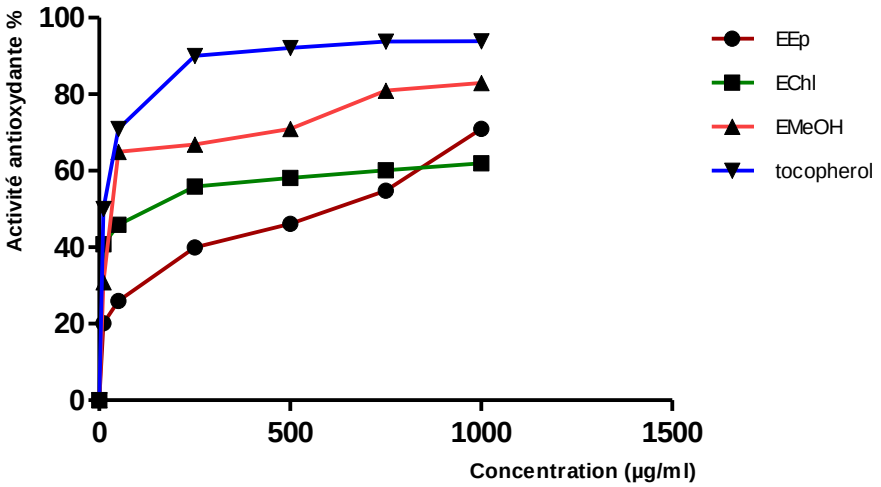

Figure 6. Antiradical activity of extracts from *U.rupestris's* leaves

Alghazeer *et al.* [24] showed that the methanolic extract of *H. albus'* leaves gives an IC_{50} of 60.4 ± 1.1 which is higher than what we noted in our study (IC_{50} = 35.33 ± 0,136 µg/ml). Phenolic compounds show the good candidates for their antioxidant activities. It suggested that the polar molecules present in the vegetable extracts contribute to the increase in the antiradical activity [25]**.** The presence of many hydroxyls, being able to react with the free radicals [26]. The antiradical activity of the extracts EMeOH is probably related to their contents out of polyphenols, flavonoïds and tannins.

III.4. Antimicrobial activity

III.4.1. Method of diffusion in solid medium

The polar extracts EMeOH of *H.albus* and *U rupestris* respectively showed an antimicrobial activity with all the microbial strains except the yeast *Candida albicans.*It appears that *S. aureus* Gram + is the most likely bacterium by comparison with the other Gram strains; this can be allotted to the difference of the structure between the bacteria Gram+ and the bacteria Gram - [27]. Our plants is very rich in alkaloids, it is for this reason, this antibacterial activity can be due to the synergistic action of polyphenols and alkaloids.They revealed that certain chemical components like tannins, flavonoïds, alkaloids are used as defense's mechanisms of many micro-organisms and the antibacterial activity of the flavonoïds is probably due to their capacity to be complexed with extracellular proteins of the bacterial cellular wall [28]**.** The antimicrobial activity of the phenolic compounds present in the plants vary according to its structure for example flavone, quercetin and naringenine are effective to inhibit the growth of *Aspergillus niger E coli P. aeruginosa S. aureus* and *Staphylococcus epidermidis* while the gallic acid can inhibit only *P. aeruginosa* [29] (Fig.7).

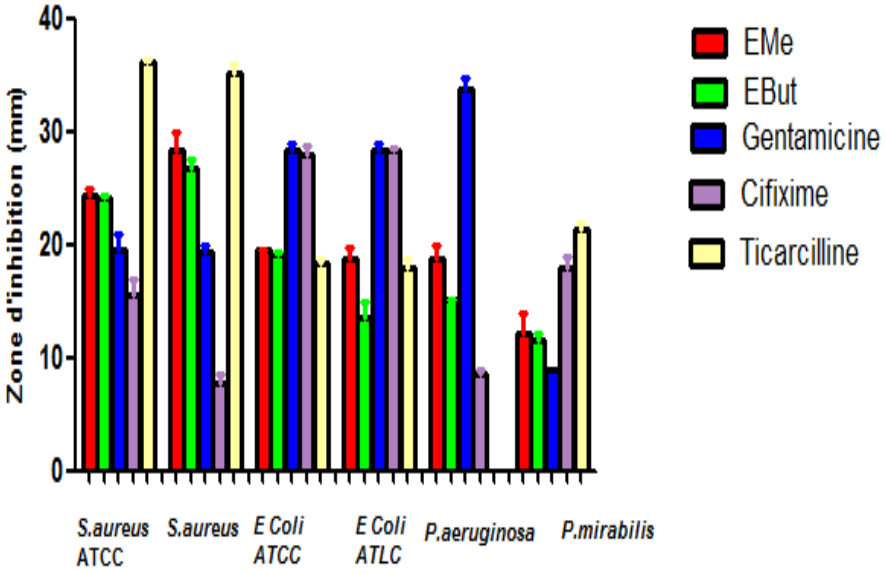

Figure 7. *In vitro Antimicrobial activity of MeOH extracts from of H. albus and U.rupestris respectively expressed in diameters of the inhibition's zones (mm).*

III.4.2. Method of microdilution in liquid medium

We report in figure 8 the minimum inhibitory concentrations (MIC) of our most active extracts noted at the time of the study in solid medium, whose diameters of inhibition are ≥12 mm. The MIC is inversely proportional to the diameter of the zone of inhibition, obtained with the method of the discs in solid medium.

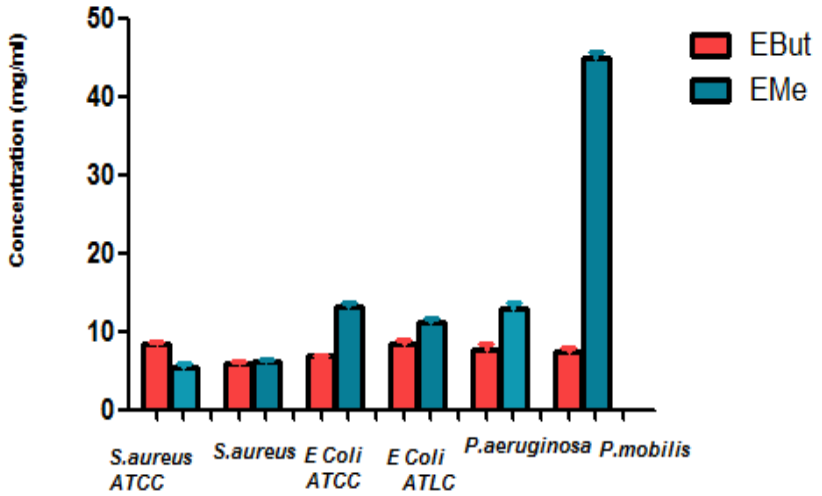

Figure 8. *In vitro* antimicrobial activity of methanolic extracts of *H. albus* and *U.rupestris* respectively, plants tested expressed in minimum inhibitory concentration (MIC) (mg /ml) against the bacteria tested.

These results show that the butanolic extract of *H. albus* has an effect on all the following bacterial strains *S. aureus* ATCC 25923, *S. aureus, E coli* ATCC 25922, *E coli, P. aeruginosa* ATCC 27853, *P. mirabilis* with values of MIC respectively: 8.30 mg/ml, 6.00 mg/ml, 6.93 mg/ml, 8.32 mg/ml, 7.63 mg/ml, 7.53 mg/ml with the same effect without significant difference (P≥0.05). The EMe of *U rupestris* have an effect on all the following bacterial strains *S. aureus* ATCC 25923 *S. aureus E coli* ATCC 25922 *E coli P. aeruginosa* ATCC 27853 *P. mirabilis* with values of MIC respectively:

5.43 mg/ml, 6.18 mg / ml respectively without significant difference P≥0.05) while the latter remain significantly lower than the MIC of the bacteria *E coli* ATCC 5922 , *E coli, P. aeruginosa* ATCC 27853 *P. mirabilis* (bacteria with Gram -) this returns to the difference between the plasmic membrane. The demonstration of the antibacterial activity against the bacteria Gram + and Gram - can be the sign of the presence of an active ingredient with broad spectrum and that will be an immense advantage in the fight of pathogenic which are so frequent lately [30]. Alghazeer et *al.* [24] showed that the methanolic extract of *H. albus* has a lower value of MIC (0,156 mg/ml) on the Gram + strains (*B subtilis* and *S. aureus*, this value is inferior that what we found. They also proved that the alcaloidic extract of *H. albus* showed a remarkable activity on *S. aureus* *E coli* and *S. typhi* with values of CMI 0,156, 0,313 and 0,313 mg / ml respectively. It is extremely probable that the toxicity of this plants is mainly related to the high concentration of alkaloids in their extracts [24] .They revealed that alcaloïdic extracts of *H. albus* are effective against mushrooms in particular of *Trichoderma spp, Sclerotium spp, Aspergillus niger* and still present an antiviral activity [31]. The method of diffusion starting from the wells of gelose is advantaged to study the activity of the aqueous and organic extracts than the method of diffusion in gelose directely [32].

IV. CONCLUSION

Aptitude of our rough extracts to inhibit *in vitro* the peroxidation of the lipids was evaluated by the test of bleaching of the β-carotene, usually employed to consider the antioxidant activity of the substances in the emulsions. The antioxidant activity expressed like values of AAR% decreased in the order α-tocopherol ≥ EMeOH (*H.albus*) ≥ EMeOH (*U.rupestris*) ≥ EEp (*U.rupestris*) ≥ EChl (*H.albus*) ≥EEp (*H.albus*) ≥ EChl (*U.rupestris*) and with method of reduction of free radical 1,1-diphenyl-2-picryl-hydrasyl (DPPH),the polar extracts of the two plants EMeOH of *H.albus* and *U.rupestris* respectively have strong capacities of neutralization of free radical DPPH (IC50 =75.19 ± 0.211 µg /ml et IC50 = 35.33 ± 0.136 µg /ml) respectively. The extracts EMeOH of *H.albus* and *U.rupestris* respectively testified a strong activity with respect to the bacteria tested, but they did not show any activity on the yeast *Candida albicans*. Our preliminary results show that all the polar extracts of the two plants tested have antimicrobial activities *in vitro*.

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