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EVALUATION OF THE ANTIOXYDANT ACTIVITY OF THE *ROSMARINUS OFFICINALIS* EXTRACTS

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Résumé

Le présent travail a pour objectif l'évaluation de l'activité anti-oxydante en tenant compte la teneur polyphénolique des extraits des feuilles du romarin par le biais de deux méthodes : la méthode de blanchissement du β-carotène et le test au DPPH, des extraits (EBr, EAcOEt et En-BuOH). L'analyse qualitative par HPLC a montré la présence de la rutine et la catéchine dans les extraits du romarin. Les résultats du test de décoloration du β-carotène ont montré une activité inhibitrice d'oxydation de l'acide linoléique pour l'ensemble des extraits du romarin, cette dernière reste significativement inférieure à celle du contrôle positif BHT ($P<0.001$). L'EAcOEt du romarin a montré l'activité la plus importante avec un taux d'inhibition égale à 79.34%. Il n'ya pas une corrélation significative ($R^2 = 0.313$) entre la teneur des polyphénols et l'activité anti-oxydante des extraits du romarin. Les résultats du test au DPPH ont montré que les extraits : En-BuOH et EAcOEt du romarin ont présenté des activités anti-radicalaires égales à 96.18%, 95.81% respectivement, qui étaient même plus élevées que celle du BHT (92.91%). La teneur des polyphénols totaux des extraits du romarin s'est corrélée significativement ($R^2 = 0.997$) avec leurs activité anti-radicalaire.

***Mots clés** : Romarin, Polyphénols, Flavonoïdes, Activité Anti-oxydante.*

Abstract

This work aims at evaluating the antioxydant activity taking count the content phenolics of the leave's rosemary extracts (EBr, EAcOEt and En-BuOH), by the means of two methods: the β-carotene bleaching method and the DPPH assay. The analysis by HPLC revealed the presence of rutin and the catechin in the rosemary extracts. The results of β -Carotene-linoleic acid assay have showed an inhibiting activity of oxidation of the linoleic acid for the whole of the extracts, the latter significantly remains lower than that of positive control BHT ($P<0.001$).The EAcOEt of rosemary showed the most important activity with a rate of inhibition equal to 79.34%. There is no significant correlation ($R^2 = 0.313$) between the total phenolic content and the antioxydant activity of rosemary extracts. The results of the DPPH assay showed that extracts: En-BuOH and EAcOEt of rosemary presented scavenging activity equal to 96.18%, 95.81% respectively, which were even higher than that of the BHT (92.91%). The total polyphenolic content of rosemary extracts was correlated significantly ($R^2 = 0.997$) with their scavenging activity.

***Key words**: Rosemary, Phenolic compounds, flavonoids, HPLC, Antioxydant Activity.*

ملخص

إن الهدف من هذه الدراسة هو تقييم النشاط المضاد للتأكسد وذلك بالأخذ بعين الاعتبار المحتوى الفينولي لمستخلصات أوراق نبتة الإكليل (المستخلص الخام، مستخلص خلات الاثيل و مستخلص البوتانول العادي)، بواسطة طريقتين: طريقة تفسخ β -carotène وطريقة النشاطية الازاحية تجاه جذر DPPH . الدراسة التحليلية بواسطة HPLC بينت وجود rutine و catéchine في مستخلصات الإكليل. نتائج طريقة تفسخ β -carotène بينت أن مستخلصات الإكليل قد ثبطت أكسدة acide linoléique تبقى هذه النتيجة أقل فاعلية مقارنة بـالمراقبة الموجبة BHT. مستخلص خلات الاثيل لنبتة الإكليل تميز بأعلى نشاط مضاد للأكسدة مع نسبة تثبيط تساوي 79.34%. لا يوجد ارتباط ذو دلالة إحصائية ($R^2=0.313$) بين المحتوى الفينولي و النشاط المضاد للتأكسد لمستخلصات الإكليل. نتائج طريقة النشاطية الازاحية تجاه جذر DPPH بينت أن مستخلصي البوتانول العادي و خلات الاثيل تميزا بنشاطية ازاحية تساوي علي التوالي : (96.18%) ، (95.81%) والتي كانت أعلى من نشاطية BHT (92.91%). يوجد ارتباط ذو دلالة إحصائية بين المحتوى الفينولي و النشاط الازاحي لمستخلصات الإكليل ($R^2 =0.997$).

***الكلمات المفتاحية**: الإكليل، الفينولات ، الفلافونيدات ، HPLC، النشاط المضاد للتأكسد.*

Last years, there was a growing interest for the use of natural antioxidants; attention was on herbs and spices as source of antioxydants, which can be used to be protected from the effects of the oxidative stress **[1]**.

The Rosemary (*Rosmarinus Officinalis* L.) is a well known and considerably evaluated aromatic herb, largely widespread in the pharmaceutical products and traditional medicine. It belongs to the *Lamiaceae* family, is presented in the form of a shrub, under- shrub or herbaceous **[2]**, measuring approximately from 0.8 to 2 m of height **[3]**.

It is very appreciated for its aromatic, antioxydant, antimicrobial, antispasmodic, and antitumor properties **[2]**.

The chemical complexity of extracts, often a mixture of dozens of compounds with different functional groups, polarity and chemical behaviour, could lead to scattered results, depending on the test employed. Therefore, an approach with multiple assays for evaluating the antioxidant potential of extracts would be more informative and even necessary **[4]**.

In this work, two methods are used: β-carotene bleaching method and the DPPH assay to evaluate the antioxidant potential of the extracts (EBr, EAcOEt and En-BuOH) of rosemary leaves.

MATERIAL AND METHODS

Vegetable material and preparation of the extracts

The extraction of the flavonoïds is carried out according to the diagram presented by Lebreton **[5]**.

The dry leaves of the rosemary are subjected to maceration during one night at ambient temperature, in a mixture of hydro-alcoholic methanol-water (7: 3 V/V). After filtration and evaporation of the solvent, the crude extract obtained is exhausted successively by 2 solvents (ethyl acetate and n-butanol).

The series of extraction made it possible to to obtain the crude hydro-méthanolic (CE) extract, the fraction of ethyl acetate (AcOEt E), the fraction of n-butanol (n-BuOH E) and the residual aqueous fraction (Aq E).

Total phenolics compound Content

The total phenolic compound content was carried out with the colorimetric reagent Folin-Ciocalteu according to the method quoted by Wong **[6]**.

Total flavonoids Content

The aluminium trichloride ($AlCl_3$) method quoted by Djeridane **[7]** is used for quantitative determination of flavonoïds.

HPLC qualitative analysis

The qualitative analysis of the flavonoïds is carried out by a HPLC (VP SHIMADZU LIQUID CHROMATOGRAPH).

For this, 20 µl of each extract were injected on a column of the type reversed phase C18, of size equal to 125 X 4.6 mm. The mobile phase consists of three eluants: distilled water, methanol, acid acetic (50: 47 : 2.5) (V /V /V). The applied elution gradient is isocratically spread out over 10 min. The flow rate is 1 ml/min **[8]**. Detection was monitored by a UV-Vis detector at wavelength equal to 254 nm.

Antioxidant activity

β- Carotene –linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation **[9]**.

Briefly 2 mg of β - carotene were dissolved in 1 ml of chloroform. The solution of the β- carotene-chloroform was introduced into a flask containing 2 mg of linoleic acid and 200 mg of Tween 40.

After evaporation of chloroform, 100 ml of distilled water saturated with oxygen were added with vigorous shaking. From this new solution, 2.5 ml are transferred in test tubes, and 350µl of each extract (2g/l) and of the BHT are added.

The absorbance was immediately measured for the BHT at 490 nm.

Other readings are measured at various intervals of time (2h, 4h, 6h, 12h, and 48h) **[9]**.

The relative antioxidant activity after 48 hours is calculated according to the following relation:

$$AAR = [Abs_{Sample} / Abs_{BHT}] \times 100$$

Where:
AAR: Relative antioxidant activity;
ABS_{Sample}: Absorbance of the sample after 48 hours;
ABS_{BHT}: Absorbance of the BHT after 48 hours;

DPPH assay

In the presence of the free radicals scavengers the DPPH (2.2 Diphenyl 1 picryl hydrazyl) of violet color is reduced to 2.2 Diphenyl 1 picryl hydrazine of yellow color**[10]**.

The DPPH scavenging activity was measured according to the protocol described by **[11]**. We introduce 2.5 ml of each extract (0.1mg/ml) into test tubes and 1ml of the methanolic solution of DPPH (0.3 mm), after agitation by a vortex; the tubes are placed in darkness at an ambient

temperature during 30 minutes. The absorbance was read against a blank at 517 nm.

The results can be expressed as antiradical activity where the percentage of inhibition of the DPPH radical (I %) by using the following relation:

$$\% = [1 - [Abs_{Sample} - Abs_{negative\ Control}]] \times 100$$

Where:
%: Percentage of of inhibition of the DPPH radical;
Abs_{Sample}: Absorbance of the sample;
$Abs_{negative\ Control}$: Absorbance of negative control;

Statistical study

The statistical study was carried out by the statistical software Graph Pad PRISM.

All the experiments were carried out in triple; the results are expressed on average ± SD. The results are analyzed by the one Anova way test followed by the Dunnet /Tukey test for the multiple comparisons and the determination of the significance rates. The values of $p \leq 0.05$ are considered statistically significant.

RESULTS AND DISCUSSION

Polyphenols and flavonoïds Contents

Generally, all the plants of the family of *Lamiacées* are known for their phenolic compounds **[12] [13]**. The flavonoïds represent the most important polyphenolic class, with more than 5000 compounds already described **[14]**. This is in accordance with our results presented in table.1

Table 1: Polyphenols and flavonoïds contents of rosemary extracts.

Extract	polyphénols content [(a)]	flavonoïds Content [(b)]
CE	195.45 ± 4.16	2.06 ± 1.14
AcOEt E	541.82 ± 3.15	21.39 ± 0.72
n-BuOH E	539.39 ± 5.25	19.58 ± 2.75

(a) mg gallic acid equivalents per gramme extract, (b) mg quercetin equivalents per gramme extract. The values represent the average of 3 measures ± SD.

The content in polyphenols of CE of the rosemary is so close to those found by Erkan: 162 mg GAE/g **[15]** and to those of Ho: 127 ± 3 mg GAE /g **[16]**, but enough far from those found by Tsai: 58.1 ± 0.9 mg GAE/g **[17]** and to those of Tawaha: 39.1 ± 3.6 mg GAE/g **[18]**. The shifted results undoubtedly come from:

The quantitative determination by this reagent gives a gross evaluation of all phenolic extracts compounds. It is not specific to polyphenols but many compounds can respond to the reagent yielding an apparent high phenolic rate **[18]**.

Indeed, the phenolic contents of a plant depend on a certain number of intrinsic (genetic) and extrinsic factors (climatic conditions), farming practices, maturity at harvest and storage conditions **[19] [20]**.

Ho found that the rosemary methanolic extract contains (20.1 ± 1.30 mg CE/g) **[16]**. In the same way, Tsai also found that the rosemary methanolic extract contains 60.7 ± 1.1 mg CE/g **[17]**. The contents reported by Ho and Tsai are very high compared to our results. This difference is probably explained in the difference of the standard used for the quantification of the flavonoïds.

Maisuthisakul noted that the total flavonoïds content of 28 plants ethanolic extracts is related to the content of the total phenolic compounds **[21]**.

Similarily, we found that the flavonoïdes content of the rosemary extracts correlated significantly with the polyphenols content ($R^2 = 0.969$).

HPLC Qualitative analysis

The comparison of retention times of the standards Quercetin (1.8 min) Rutin (3.4 min) and catechin (2.0 min) with those recorded in the various chromatograms (Table.1), allows probable identification of some flavonoïds in our extracts **[22]**.

Table.2. Retention Time of the flavonoïds present in the rosemary extracts.

Retention time (min)			The probable flavonoïd
CE	AcOEtE	n-BuOHE	
1,7	0,5	0,8	-
2,0	0,8	1,7	Presence of catechin in the E
2,6	1,2	**2,0**	Presence of catechin in n-BuOHE
3,4	1,7	**3,4**	Presence of the rutin in the CE and n-BuOHE
3,8	**2,0**	3,7	Presence of catechin in the AcOEtE
4,7	2,6	4,7	-
5,2	**3,4**	5,3	Presence of rutin in the AcOEtE
6,3	3,7	7,5	-
7,6	4,6	9,4	-
8,9	5,5		
9,6	6,1		
	7,5		
	8,0		
	8,8		

The results show the presence of the catechin, the rutin and the absence of quercetin in all rosemary extracts.

Similarily, Justesen and Wojdylo who used a gradient system as a mobile phase revealed the absence of quercetin in the methanolic extracts of the rosemary **[23] [24]**.

Antioxidant activity

β-Carotene–linoleic acid assay

In this test, the inhibition of the oxidation of the linoleic acid is measured in the presence of the β-carotene, which is employed as a marker.

Change of absorbance of the β-carotene at various time intervals (Figure.1) showed that the AcOEt E of rosemary seems to be the best inhibitor of the linoleic acid oxidation.

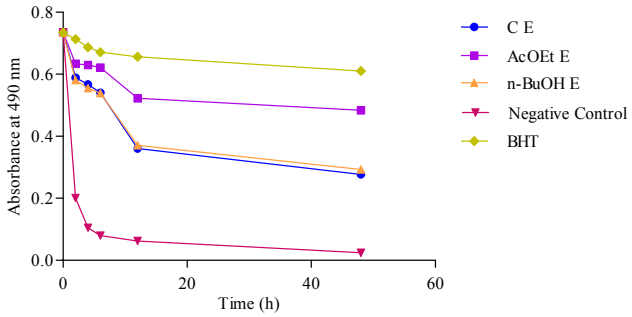

Figure 1: Absorbance Change of the β - carotene at 490 nm in the presence of the rosemary extracts, BHT and the negative control.

The results (Figure.2) indicated that the rosemary extracts as well as the BHT inhibit in a significant way ($P<0.001$) the coupled oxidation linoleic acid β-carotene compared to the negative control.

The inhibition of oxidation was important 79.34% for the AcOEt E followed respectively by n-BuOH E (48.03%) and the C E (45.41%), which did not present a significant difference ($P<0.05$). We note that the rosemary extracts showed an important inhibition, but remains significantly lower compared to the BHT ($P<0.001$).

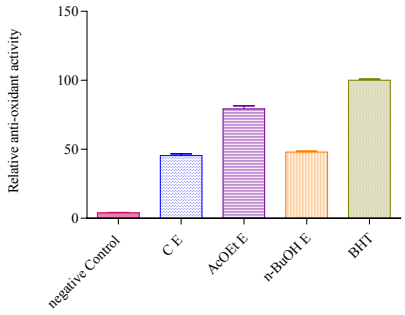

Figure 2: Relative antioxidant activity of the rosemary extracts, BHT and the negative control.
The bars with different letters indicate significantly different activities [$P<0.05$].

The two fractions AcOEt and n-BuOH are rich in flavonoïds; this suggests a link between the antioxidant activity of the two fractions and its components. This link remains conditioned by the the flavonoïds structure, particularly the substitution of hydroxy for the aromatic rings A and B and the model of substitution of the ring C, the most active flavonoïds possess from 3 to 6 groups of hydroxyl **[25]**.

We also note that the extracts proved to be modest antioxidants compared to the literature data. This result is probably due to the high specificity of the β-carotene bleaching method for the lipophilic compounds **[26]**.

There is no significant correlation ($R^2 = 0.313$) between the content of polyphenols and the rosemary extracts antioxidant activity. Dorman noted that the antioxidant activity of the extracts of the plants [oregano, rosemary, sage and thyme] is not necessarily related to high contents of phenolic compounds, but probably depends strongly on the rosmarinic acid, the principal current phenolic component in this type of *Lamiaceae* extract **[27]**.

The exact attribution of the antioxidant capacity to a compound, or a small group of components in a plant extract is a difficult task, since the effective activity depends on several factors, such as the concentration, the isomeric forms and the synergistic interaction with other components **[28]**, periods of harvest **[29]**, extraction mode **[30]** and the solvents polarity **[31]**.

DPPH assay

In this test the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants **[32]**.

Our results expressed as a percentage of the anti-radical activity (Figure .3), reveal that all the extracts tested as well as the BHT taken as a reference are scavengers.

The rosemary n-BuOH E presented the highest anti-radical activity (96.18%), followed by the AcOEt E (95.81%) and in the last place the C E (80.50%). These two latter do not present a significant difference in their activity ($P<0.05$).

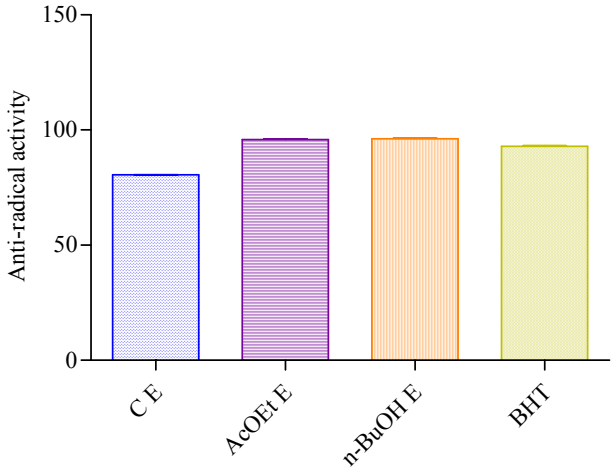

Figure.3. Anti-radical activity of the rosemary extracts and the BHT.

The bars with different letters indicate significantly activities different ($P<0.05$).

The results of the anti-radical activity of the rosemary extracts are in accordance with those obtained by Almela **[28]**. The latter noted that the methanolic extracts of the rosemary resulting from various matters (wild plants, drip-irrigated plants, by-product resulting from the distillation of aromatic essential oil) showed an anti-radical activity, of which that of the extracts resulting from the wild plants is almost identical to that of the δ- tocopherol and higher than that of the BHT.

We also note that the n-BuOH E and AcOEt E of the rosemary presented a higher activity than that of the positive control (BHT). This activity could be related to their high content in polyphenols which reported to be potent hydrogen donators to the DPPH radical, because of their ideal chemistry structural **[33]**.

The content of total polyphenols of the rosemary extracts was significantly correlated ($R^2 = 0.997$) with their anti-radical activity. These results corroborate with the results already mentioned **[6]; [18]; [7]**.

Turkmen noted that the extracts of black tea with a higher antioxidant activity also possess higher polyphenol content **[33]**.

The others minor phenolic compounds should not be neglected, because the synergy between the various chemicals should be taken into account in the evaluation of the biological activity **[34]**.

On an other side, the phenolic fraction does not incorporate all antioxidants and the synergistic interactions between antioxidants in a mixture makes that the antioxidant activity depends not only on the concentration, but also on the structure and the nature of antioxidants **[19]**.

The results of Fellah, showed that the anti-radical activity of the of *Cynara cardunculus* methanolic extracts was organo-dependent, the seed extracts showed the best inhibiting activity of the DPPH, followed-up by the leaves and the flowers **[19]**.

This hierarchy observed in the antioxidant activity was also noted in our results, which reveal a better antioxidant activity of all the extracts in the DPPH test than the test of β-carotene bleaching. The DPPH test is simple, very fast and independent of the polarity of samples **[35]** which can explain this hierarchy.

CONCLUSION

The coefficient of correlation between the content of the rosemary extracts of polyphenols and the anti-radical activity was strongly significant, indicating that 99% of this activity is due to the contribution of the phenolic compounds.

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