PHENOLIC COMPOSITION, ANTIMICROBIAL ACTIVITY OF *Rosmarinus officinalis*

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Résumé

L'objectif du travail est l'évaluation de l'activité antimicrobienne des extraits du romarin (EBr, EAcOEt et En-BuOH) sur huit souches bactériennes et trois fongiques par diffusion en milieu gélosé. Les dosages quantitatifs des polyphénols totaux au Folin-Ciocalteu ont révélé la richesse du romarin en polyphénols (195.45 ± 4.16 mg EAG/g d'EBr). L'analyse qualitative par HPLC a révélé la présence de la rutine et la catéchine dans les extraits du romarin. Les extraits du romarin ont présenté une activité antibactérienne sur la totalité des souches et l'extrait d'acétate d'éthyle (EAcOEt) s'est révélé le plus actif surtout contre la bactérie multi-résistante *Entérobacter* sp (BLSE+CHN). Seule l'inhibition de la souche *Klebseila pneumoniae* a présenté une corrélation très significative entre le taux des polyphénols de l'EBr du romarin et l'activité antibactérienne (R^2 =0.992). Les extraits du romarin se sont révélés inactifs vis-à-vis les souches fongiques.

Mots clés : *Rosmarinus officinalis, Polyphénols, Activité antimicrobienne.*

Abstract

This work aims at evaluating the antimicrobial activity of rosemary extracts (EBr, EAcOEt and En-BuOH) on eight strains of bacteria and three fungal strains by agar diffusion method. The quantification of total polyphenols using the Folin-Ciocalteu method and of the flavonoids revealed the richness of the rosemary in polyphenols (195.45 ± 4.16 mg EAG/g of EBr). The analysis by HPLC revealed the presence of rutin and the catechin in the extracts rosemary. The results revealed that the extracts of rosemary are showed antibacterial activity against the whole tested Bacterial strains and The rosemary EAcOEt has been the most active extract and it has revealed an interesting antibacterial activity against the multi-resistant strain *Entérobacter* sp (ESBL+HLC). Only inhibition of *Klebseila pneumonia* strain have shown very significatif correlation between polyphenolic content of EBr of rosemary and antibacterial activity (R^2 =0.992). The results of the antifungal activity showed the inefficiency of all the extracts against the fungal strains.

Keywords*: Rosmarinus officinalis, phenolic compounds, Antimicrobrial activity.*

ملخص

إن الهدف من هذه الدراسة هو اختبار التأثيرالنشاط المضاد للميكروبات ضد 8 سلالات بكتيرية ATCC *2 souches* و*d'Enterobacter* sp, *Serratia* sp, 3 سلالات فطرية عن طريق الانتشار على وسط صلب، لمستخلصات (المستخلص الخام، مستخلص خلات الاثيل و مستخلص البوتانول العادي) لأوراق نبتة الإكليل. قمنا أولا بإجراء تقدير كمي للفينولات و كذلك الفلافونيدات على أساس أنها أهم قسم من العائلة الفينولية ودراسة تحليلية للفلافونيدات بواسطة كروماتوغرافيا السائل العالي الأداء(HPLC). التقدير الكمي للفينولات بواسطة طريقة Folin-Ciocalteu بينت غنى الإكليل بالفينولات (195.45 ± 4.16 mg EAG/g d'EBr) الدراسة التحليلية بواسطة HPLC بينت وجود catéchine و rutine في كل في مستخلصات الإكليل.

مستخلص خلات الاثيل لنبتة الإكليل كان الأكثر فعالية على مجموع البكتيريات المختبرة واظهر نشاط مهما مضادا للبكتيريا ضد السلالة المقاومة (BLSE+CHN) *Enterobacter* sp. تثبيط السلالة *Klebseila pneumonia* هو *الوحيد الذي* اظهر ارتباط دو دلالة إحصائية بين المحتوى الفينولي لمستخلص الإكليل الخام و النشاط المضاد للبكتيريا (R^2 =0.992)نتائج النشاط المضاد للفطريات بينت عدم فعالية كل المستخلصات ضد السلالات الفطرية.

الكلمات المفتاحية : *الإكليل، الفينولات، ، النشاط المضاد للميكروبات .*

In recent years, there has been a great interest for the discovery of new antimicrobial agents, due to an alarming increase in the rate of infections with micro-organisms resistant to antibiotics. One of the common approaches to the search for biologically active substances is the systematic screening of micro-organisms or plants, which are a source of many useful therapeutic agents. The antimicrobial activity of oils and extracts of plants have formed, in particular, the basis for many applications, including pharmaceutical, medicine, natural therapy and food conservation **[1]**. The plant matter contains a large number of molecules that have multiple interests used in the industry, food, cosmetology and dermopharmacy. Among these compounds, we can find coumarins, alkaloids, phenolic acids, tannins, lignans, terpenes and flavonoids **[2]**.

The rosemary (*Rosmarinus officinalis L.*) is the object of recent research in the fields of pharmaceuticals, cosmetics and food industry. It is an aromatic grass which is presented in the form of shrub, under sapling or herbaceous that belongs to the family of *Labiees* **[3]**, measuring approximately 0.8 to 2m in height **[4]**. The leaves are closely linear lanceolate, brittle and tough. The flowers of a pale blue, stained inwardly with purple are arranged in short dense clusters flourish throughout most of the year. The rosemary is very appreciated for its aromatic properties, anti-oxidant, antimicrobial, antispasmodic, emmenagogues and anti-tumor, widely used in the pharmaceutical products and in traditional medicine **[3]**.

The aim of this work is to evaluate the antimicrobial activity by the method of diffusion in an agar medium of crude extract and their fractions (EAcOEt, En-BuOH) of the medicinal plant, the rosemary. This assessment is linked to the phenolic content of these extracts.

MATERIAL AND METHODS

Plant Material and preparation of extracts

The extraction of flavonoids is carried out according to the diagram presented by Lebreton (1967) as amended by Boutard (1972), Gonnet (1973) and Jay (1975).

The rosemary dry leaves are left to macerate overnight at ambient temperature, in a water-alcohol mixture of methanol-water (7:3 V/V). After filtration, the solvent is removed from the filtrate by rotary evaporation. The crude extract obtained is subjected to a liquid-liquid extraction successively by 2 solvents (ethyl acetate and the n- butanol).The series of extraction enabled us to obtain four fractions; the crude extract hydro-methanolic (EBr), the fraction of ethyl acetate (EAcOEt), the fraction of the n-butanol (En-B OH) and the aqueous fraction (AqE) residual.

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 [5].** 200µl of each extract were added to 1ml of 1:10 diluted Folin–Ciocalteu reagent. The solutions were mixed and incubated for 4 minutes. After incubation, 800 µl of a solution of sodium carbonate Na_2CO_3 (75 g /l) have been added. The final mixture is incubated for 2 hours in the dark at ambient temperature. The absorbance was measured by a spectrophotometer at 765 nm. The content in polyphenols is expressed in milligram of gallic acid equivalents per gram of extract (µg EAG/mg).

Total flavonoids Content

The method of aluminum trichloride ($AlCl_3$) **[6]** is used to quantify the flavonoids in our extracts. 1 ml of each extract was added to an equal volume of a solution of $AlCl_3$ (2 %). The mixture was vigorously agitated and the absorbance at 430 nm was read after 10 minutes of incubation. The results are expressed in milligrams of quercetin equivalent per gram of extract (µg EQ/mg).

Qualitative analysis by HPLC

The analysis is carried out by an HPLC (VP SHIMADZU LIQUID CHROMATOGRAPH). 20 µl of each extract were injected on a column of type reverse phase C18, of equal size to 125 x 4.6 mm. The mobile phase consists of three eluents: distilled water, methanol, acetic acid (50: 47: 2.5) (V /V /V). The elution gradient applied is of an isocratic type spread over 10 min. The flow rate is

1 ml/min [**7**]. The detection was performed by a UV-Vis detector at a wavelength equal 254 nm
The flavonoids in each extract analysis have been identified by the comparison of the retention times obtained by those witnesses.

Antimicrobial Activity

The antibacterial activity of the extracts was determined by the agar diffusion method standardized by (NCLLS) [**8**]. Eight Bacterial strains have been tested:

Escherichia coli ATCC 25922,
Pseudomonas aeruginosa ATCC 27853,
Staphylococcus aureus ATCC 25923,
Enterobacter sp (HLC+ ESBL),
Enterobacter sp,
Serratia sp,
Klebsiella pneumoniae sp (ESBL),
Streptococcus sp.

Three Fungal strains: Two yeasts: *Candida albicans*, Candida *Kefyr* and a fungus: *Aspergilus niger*.

Of microbial communities well isolated were transferred into tubes of sterile distilled water in order to have a microbial suspension having a cell density adjacent to that of Mc Farland 0.5 (10^6 CFU /ml). It was subsequently spread the entire surface of the Agar Agar (Mueller Hinton for the bacteria non-demanding; Agar Mueller Hinton, which contains 5% blood of the horse for the bacteria demanding; Sabouraud for yeasts) by the microbial suspension. For the fungus the Sabouraud agar is inoculated by the suspension.

The disks sterile impregnated of increasing concentrations of extracts resumed with the Dimethyl Sulfoxide (DMSO) to reason of 10 µl per disc **[9]** have been deposited out by sterile methods on the agar surface. The boxes have been incubated 24 h at 37 °C in normal atmosphere for the bacteria non-demanding and in an atmosphere containing 5% CO_2 for the bacteria demanding. The yeasts were incubated 48 h at 37 °C, while the fungus has been incubated 10 days at 27 °C in normal atmosphere. The antibacterial activity was expressed by measuring the diameter of the inhibition zone.

Statistical study

The statistical study has been carried out by the statistical software Graph Pad Prism. All experiments were performed in triplicate; the results are expressed in average ± SD.

RESULTS AND DISCUSSION

Content in polyphenols

Generally, all plants of the family *Lamiacee* are known for their phenolic compounds **([10], [11])**.

This is in accordance with our results presented in the Table 1

Table 1: *Polyphenols content of rosemary extracts.*

Extract	Polyphenols contents [(a)]
EBr	195.45 ± 4.16
EAcOEt	541.82 ± 3.15
En-BuOH	539.39 ± 5.25

(a) mg of equivalent of gallic acid per gram of extract.
The values represent the average of 3 measurements ± SD

The content of CE rosemary is so close to that of Erkan *et al.* **, [12]** : 162 mg GAE/g and **Ho *et al.* , [13]** : 127 ±3 mg GAE /g, but far enough to that of **Tsai *et al.* , [14]** : 58.1 ± 0.9 mg GAE/g and Tawaha *et al.* **, [15]** : 39.1 ± 3.6 mg GAE/g.

The shifted results result likely of:

- The low specificity of the Folin-Ciocalteu reagent is the primary disadvantage of the colorimetric assay. The reagent is extremely sensitive to the reduction of all the groups of hydroxyl, not only those of phenolic compounds, but also of certain sugars and proteins etc **[16]**.

- The distribution of secondary metabolites can change during the development of the plant. This may be linked to the harsh climate conditions (high temperature, solar exposure, drought, salinity), which stimulate the

biosynthesis of secondary metabolites such as polyphenols **[17]**.

Content in flavonoids

The main reason for the choice of this class of polyphones, lies in the fact that the flavonoids represent the most important polyphones class, with more than 5000 compounds already described **[16]**. The results of the flavonoids assay are represented in table 2

Table 2: *Flavonoids Content rosemary extracts*

Extract	Flavonoids Content [(b)]
EBr	2.06 ±1.14
EAcOEt	21.39 ± 0.72
En-BuOH	19.58 ± 2.75

(b) mg of equivalent of quercetin per gram of extract.
The values represent the average of 3 measurements ± SD

According to the results of Ho and his collaborators **[13]** the extract of rosemary is rich in polyphenols (127 ±3 mg EAG/g) and poor in flavonoids (20.1 ± 1.30 mg EC/g). We can say that our results confirm those of Ho.

In addition, Tsai *et al.,* **[14]** have also found that the methanolic extract of the rosemary contains 60.7 ±1.1 mg EC/g. The levels reported by Ho and Tsai are very high compared with our results; this difference can probably explained by the difference of the standard used for the assay of the flavonoids.

Maisuthisakul *et al.*, **[18]** have found that the total content of the ethanolic flavonoids extracts of 28 plants, is linked to the content of the total phenolic compounds. Similarly, we have found that the content of the flavonoids extracts of the rosemary was correlated significantly with the content of polyphenols (R^2 = 0,969).

Qualitative analysis by HPLC

The chromatograms of HPLC of the different extracts are represented below.

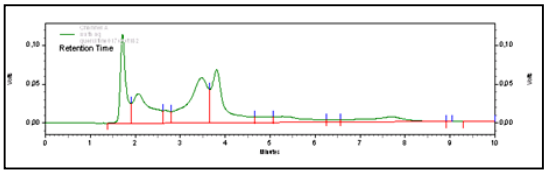

Figure 1: *Chromatogram of HPLC of EBr of rosemary registered to 254 nm*

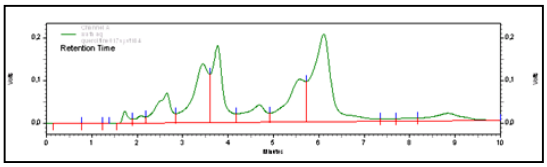

Figure 2: *Chromatogram of HPLC of EAcOEt rosemary recorded at 254 nm*

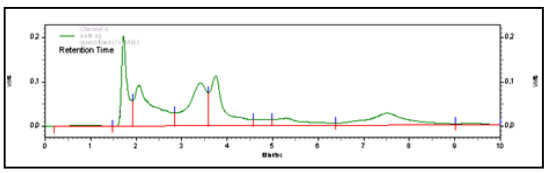

Figure 3: *Chromatogram of HPLC of En-BuOH rosemary recorded at 254 nm*

The comparison of the retention times (Table 3) of the standards with those recorded in the different chromatograms (Table 4), allows a possible identification of some flavonoids in our extracts **[19]**.

Table 3: *Retention time of the flavonoids standards.*

Retention Time (min)	The Standard flavonoid
1.8	Quercetin
3.4	Rutin
2.0	Catechin

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

Similarly Justesen *et al.,* **[20]** and Wojdylo *et al.,* **[21]** who have used as a mobile phase a gradient system have revealed the absence of quercetin in the methanolic rosemary extracts.

Table 4: *Retention time of the flavonoids present in the rosemary extracts.*

Retention Time (min)			The possible flavonoid
Ebr	**EAcOEt**	**En-BuOH**	
1.7	0.5	0.8	-
2.0	0.8	1.7	Presence of the catechin in the EBr
2.6	1.2	**2.0**	Presence of the catechin En-BuOH
3.4	1.7	**3.4**	Presence of the rutin in the Ebr and En-BuOH
3.8	**2.0**	3.7	Presence of the catechin in the EAcOEt
4.7	2.6	4.7	-
5.2	**3.4**	5.3	Presence of the rutin in the EAcOEt
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		

Antimicrobial Activity

The results presented in the tables below show that:

The rosemary extracts display important activities, which extend on the totality of the collection strains, including the EAcOEt which is the most active.

The strains *Escherichia coli* ATCC and *Klebseila pneumoniae* possess a very high resistance potential against the antibacterial action of 3 rosemary extracts.

A few zones of inhibition with moderate the EAcOEt were recorded with *Pseudomonas aeruginosa ATCC* (11.33 ± 1.15mm), the two strains of *Enterobacter* sp (13.00 ±0 .00 ; AT 15.67 ± 0.58) and *Serratia* sp (12.33 ± 1.53mm).

The bacterium *Enterobacter* sp (ESBL+HLC) is a bacterium highly resistant to antibiotics, but has proved to be very sensitive to the EAcOEt rosemary than the bacterium *Enterobacter sp* sensitive to Cefotaxim.

A higher activity with the three rosemary extracts, has been noticed in *Staphylococcus aureus* ATCC, which is sensitive to the low concentrations of extracts.

Sterptocoque sp, a bacterium Gram (+), has proved resistant to extracts tested.

The inhibitor effects increase considerably with the concentration of extracts. The majority of extracts can retain a detectable activity, after weak dilutions. The Rosemary EAcOEt has remainded active also after the 1/16 dilution for strains *Pseudomona aeruginosa* ATCC, *Staphylococcus aureus* ATCC and *2 strains of Enterobacter sp.*

Table 5: *Diameter of the inhibition zone of the rosemary EBr.*

Bacterial strains	**Diameter of the inhibition zone * (mm)**			
	The dilutions of the EBr rosemary			
	1/2	**1/4**	**1/8**	**1/16**
Escherichia coli ATCC	-	-	-	-
Pseudomonas aeruginosa ATCC	10.00 ± 1.73	8.67 ±1.15	7.33 ±1.15	-
Staphylococcus aureus ATCC	25.33±1.15	23.67± 0.58	17.67 ± 0.58	17.33 ± 0.58
Enterobacter sp	9.00 ±1.00	8.00 ±0.00	-	-
Enterobacter sp BLSE+HLC	12.33 ± 1.15	9.66 ±0.58	9.33 ±0 .58	-
Klebseila pneumoniae BLSE	7.67 ±0.58	-	-	-
Serratia sp	7.00 ± 0.00	-	-	-
Sterptocoque sp	7.67 ±0.58	-	-	-

(*) Diameter of the inhibition zone produced around the disks by the addition of 10 µl of extract. (Diameter of the disc is included)

Table 6: *Diameter of the inhibition zone of the rosemary EAcOEt.*

Bacterial strains	**Diameter of the inhibition zone * (mm)**			
	The dilutions of the EAcOEt rosemary			
	1/2	**1/4**	**1/8**	**1/16**
Escherichia coli ATCC	8.00 ±0 .00	-	-	-
Pseudomonas aeruginosa ATCC	11.33 ± 1.15	11.33 ± 1.15	9.33 ±0.58	8.67 ±0.58
Staphylococcus aureus ATCC	28.33 ± 0.58	27.67 ±0.58	24.67 ± 1.53	23.33 ± 0.58
Enterobacter sp	13.00± .00	12.33 ± 0.58	9.33 ±2.31	9.33 ±0.58
Enterobacter sp ESBL+HLC	15.67 ± 0.58	16 ±2.00	13.67 ± 0.58	11.00 ± 2.64
Klebseila pneumoniae ESBL	-	-	-	-
Serratia sp	12.33 ± 1.53	12.33 ± 0.58	9.67 ±1.53	7.33 ±1.15
Sterptocoque sp	8.67 ±1.15	8.00 ±0 .00	-	-

(*) Diameter of the inhibition zone produced around the disks by the addition of 10 µl of extract. (Diameter of the disc is included)

Table 7: *Diameter of the inhibition zone of the En-BuOH of rosemary.*

Bacterial strains	**Diameter of the inhibition zone * (mm)**			
	The dilutions of the En-BuOH rosemary			
	1/2	**1/4**	**1/8**	**1/16**
Escherichia coli ATCC	-	-	-	-
Pseudomonas aeruginosa ATCC	-	-	-	-
Staphylococcus aureus ATCC	24.00 ± 0.00	24.00 ± .00	19.67 ± .58	18.33 ±0.58
Enterobacter sp	-	-	-	-
Enterobacter sp also ESBL+HLC	8.33 ±0.58	-	-	-
Klebseila pneumoniae ESBL	-	-	-	-
Serratia sp	-	-	-	-
Sterptocoque sp	9.67 ±0.58	-	-	-

(*) Diameter of the inhibition zone produced around the disks by the addition of 10 µl of extract. (Diameter of the disc is included).

The results reveal variable answers in function of the strains, of the concentration, type of the tested extract and that the sensitivity or resistance to antibiotics has no relation with that of the extracts.

Several works have highlighted the great sensitivity of the bacteria Gram (+) compared to the Gram (-) **([17]**; **[22]**; **[23]**; **[24]; [25])**, this can be attributed to the difference in the outer layers of bacteria Gram (-) and Gram (+).

The bacteria Gram (-), independently of the cells membrane, possess an additional layer: the outer membrane, which is composed of phospholipids, proteins and lipopolysaccharides. This membrane is impermeable to most molecules. Nevertheless, the presence of porins in this layer allows the free diffusion of molecules with a molecular mass below 600 Da. However, the inhibition of the Gram (-) bacteria growth has been reported, particularly in combination with the factors that can disturb the integrity of the cell and/or permeability of the membrane, such as low values of pH and increased concentrations in NaCl **[26]**.

The hypersensitivity of the strain *Staphylococcus aureus* ATCC can be explained by the probability of the sensitivity of bacteria Gram (+) to external environmental changes, such as temperature, pH and the natural extracts due to the absence of the outer membrane **[27]**. Some studies show no selective antimicrobial activity towards the bacteria Gram (+) or Gram (-) **[28]**. The resistance of the strain *Sterptocoque* sp can be attributed to the ability of the antibacterial agent to diffuse uniformly in the agar **[22]**.

The inhibition zone increases significantly with the concentration of the extracts, a fact also noticed by Dordevic and his collaborators**, [29]**. The disk load affects the antimicrobial activity, Rasooli and his collaborators, **[30]** have noted that the inhibition of the growth of *Aspergillus parasiticus* is strong when the disk is more responsible in essential oils of *Rosmarinus officinalis* and *Trachyspermum Copticum*. The method used for the evaluation of the antibacterial activity also affects the results Natarajan *et al.***[31]** and Fazeli *et al.,* **[32]** have found that the method of dissemination from wells on agar is more suitable for studying the activities of aqueous extracts and organic of the *Euphorbia fusiformis* and Hydro-ethanolics of *Rhus coriaria and Zataria multifora*, than the method of agar diffusion. Polyphenols, such as tannins and flavonoids like epigallocatechol, the catechin, the myricetin, quercetin, **[24]** and luteolin **[33]** are important antibacterial substance.

The HPLC has revealed the presence of the catechin in all extracts of rosemary, which may explain the antibacterial activity of the extracts of this plant.

We have found that there is not a correlation between the content of rosemary phenolic extracts and antibacterial activity. The values of the correlation coefficient R^2 calculated were between $R^2 = 0{,}442$ and $R^2 = 0.001$ ($P<0.05$) and decrease in the following order: *Sterptocoque* sp;

- *Escherichia coli* ATCC;
- *Pseudomonas aeruginosa* ATCC;
- *Staphylococcus aureus* ATCC,
- *Enterobacter sp*
- *Enterobacter sp* ESBL+HLC.

Similar results ($R^2 = 0.00$) were obtained by Turkmen *et al.,* **[23]** during the evaluation of the antibacterial activity of the extracts of tea.

Only the inhibition of the strain *Klebseila pneumoniae* which presented a correlation very significantly, between the rate of polyphenols of the EBr of the rosemary and the antibacterial activity with a coefficient $R^2 = 0{,}992$. This result is consistent with the first systematic study prepared by Shan *et al.,* **[24].** In fact this study shows a report highly positive between antibacterial activity and the rate of the polyphenols of a large number of extracts (46) of spices and herbs, the values of the coefficients of correlation R^2 were between 0.93 and 0.73.

The antimicrobial activity depends not only on the presence of phenolic compounds, but also on the presence of various secondary metabolites **[34]**, on the location and on the number of hydroxyl groups **[17]**.

Although, ethanol and methanol were the best solvents than other ones by extracting the phenolic compounds, because of their polarity and of their good solubility for these compounds, the results have proven that ethanol was the best solvent to extract the phenolic compounds, followed by methanol and finally by water **[35]** which could explain the difference mentioned below.

We found a difference by comparing the inhibition zone of *Escherichia coli* ATCC (0.00 mm) obtained by the rosemary EBr with that (16.62 mm) obtained by the ethanolic extract tested by **Zhang *et al.*,[36].**

The differences found can be attributed to several reasons such as inherent factors, methods of extraction **([8]; [23]; [37]),** preparation of the extract, solvent used, the sensitivity of the bacteria **[38],** and finally the part of the plant used **[31]**.

No inhibition zone was observed around disks impregnated of the different extracts. In the light of these results, we can conclude that the extracts from the two plants do not contain antifungal agent.

CONCLUSION

Through this work, we want to show that the plants constitute a very interesting reservoir for research in the future. An extension of this work in the future is desirable to study the components present in the rosemary EAcOEt and to assess their antibacterial activities.

A search of the antibacterial agent responsible for the inhibition of the*Enterobacter* sp (ESBL+HLC) is also necessary and seems of great importance.

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