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Antibacterial activity of two extracts from *Rubus fruticosus* L. against resistant pathogens and their antioxidant potential

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One of the most serious threats to public health across the world is the spread of antibiotics resistant pathogens. This situation obliges researchers from all over the world to focus on how they can overcome this obstacle mainly by searching or synthesizing new molecules to neutralize microbes and to overcome the phenomenon of resistance. One of the main reservoirs of potentially active molecules is the plantae kingdom. In this work, we focused on *Rubus fruticosus* L., a medicinal species which is used traditionally in Algeria to heal respiratory infections. Both of chemical composition and biological activities of essential oils and flavonoids extract were explored. GC-MS analysis of essential oil reveals the Lanceol as the most abundant component (20.22%). HPLC applied on flavonoids extract shows the presence of the gallic acid, gallocatechin, protocatechuic acid, catechin, caffeic acid, rutin, ellagic acid and myricetin. Tests of antioxidant capacity using the DPPH reveals an inhibiting effect which is more important with the flavonoid extract. Otherwise, resistant strains to conventional antibiotics seem to be sensitive to the flavonoids of this species with MICs varying from 2.36 to 75.54 mg/ml. This study indicates that *R. fruticosus* L. has an important antimicrobial effect, which justifies its potential use in infectious diseases. For many reasons, this species remains poorly studied, further refined studies on its pure secondary metabolites are needed in the perspective of identifying new antimicrobial molecules from this plant.

Key words: *Rubus fruticosus* L., essential oil, flavonoids, antimicrobial activity, resistant respiratory pathogens, antioxidant activity.

INTRODUCTION

Respiratory infections are considered as a major risk for human health, beside emerging respiratory infections such as severe acute respiratory syndrome (SARS), avian influenza and Hantavirus pulmonary syndrome,

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some respiratory infections are considered as eradicated or nearly, reappear with serious consequences: every year more than 8 million cases of severe respiratory infections are reported causing 3.9 million death (Sansonetti and Orth, 2006).

Despite the fact that researchers developed an important arsenal to face these infections and others, emerging resistances to antibiotics come to complicate the situation, this resistance implies various mechanisms such as modifying or synthesizing new targets, enzymatic modification, increasing efflux of antibiotics or others, the result is an under activity or non-activity of an important number of antibiotics used nowadays.

Many solutions were suggested to face this situation, and one of the most promising options is trying to extract from the plants new molecules that can be safe and effective in healing such diseases, the number of medicinal plants are estimated to be between 40000 and 70000 which are very diverse source of bioactive molecules and about 80% of world population is currently using these plants (Ramawat, 2009).

The interest in using these plants by population and also in research and academic world is increasing; new journals and reference books are published to enable researchers to find evidence based knowledge. *Rubus fruticosus* L. (Rosaceae) is a shrub well known for its fruit, called blackberries which are marketed all over the world for its delicious taste and high nutritive value. The shrub may be original from Armenia and is nowadays spreading in Europe, Asia, Oceania, northern and southern America and northern Africa (Hummer and Janick, 2007; Swanston-Flatt et al., 1990; Zia-Ul-Haq et al., 2014).

This plant is understory vegetation, can reach 3 m in length and have spiny stems. It flourishes at the end of spring and the fruit ripens in autumn (Hummer and Janick, 2007). According to some authors, this plant can be a good remedy for bronchitis and respiratory infections (Blumenthal et al., 1998).

However, no studies are reported on the Algerian *R. fruticosus* L. Two studies in Iran and Pakistan were reported by the antimicrobial activity of this species, two others were interested with the antioxidant activity.

The purpose of this study was to highlight the antimicrobial activity of this plant on both reference strains and strains isolated from patients with respiratory infections and in the same time, the study shows the antioxidant properties of its flavonoids extract and essential oil.

MATERIALS AND METHODS

Biological material

The harvest of the aerial part of *R. fruticosus* was accomplished in spring in the area of Tizi Ouzou, in the north east of Algeria, about 80 km of Algiers, the capital, between 36° 43′ 00″ North and 4° 03′ 00″ East. To extract flavonoids, collected parts were dried in open air and sheltered from light, then transformed into powder using an electrical crusher; this powder was maintained in tightly closed glass flasks.

The tested strains was either reference strains or isolated from patients diagnosed with respiratory infections and hospitalized in the department of infectious diseases in the hospital university of Tizi Ouzou, the identification was effected using biochemical galleries Api 20E, Api 20NE, or specific tests.

Phytochemical screening

Phytochemical screening represents a set of colorimetric methods that can lead to detect the presence or lack of secondary metabolites and should be realized on plant powder or infusion. We searched mainly alkaloids, anthocyanins, senosids, leuco-anthocyanins, total tannins, gallic tannins, catechin tannins, alkaloids, flavonoids, saponosids, irridoids, quinones, coumarins and mucilage (Harborne, 1998; Raaman, 2006).

Extractions

The extraction of essential oil was effected by hydro distillation, to accomplish this, 100 g of fresh matter was soaked in a recipient of 1l filled with 600 ml of distilled water, the whole system was boiled for 3 h. Collected vapors were condensed by transiting a refrigerator and collected. Organic state was recovered by adding few milliliters of diethyl ether and the obtained oil was kept in a temperature between 0 and 4°C.

The extraction of flavonoids was realized according to Bruneton (1999) protocol. This extraction is based on the difference of solubility degrees of flavonoids in various organic solvents. This protocol includes two main steps: first, methanol is used to solubilize flavonoids and then washings using petroleum ether, diethyl ether, ethyl acetate and butanol are realized. After using butanol, the extract contains the most polar flavonoids.

Characterization of the extracts

GC-MS of essential oils

Analysis of the chemical composition of the essential oil was carried out by chromatography and gas chromatography coupled with mass spectrometry, the apparatus was a GC Perkin Elmer 600, MS Perkin Elmer 600C, a column Rtx-VMS menu (60m long with a diameter of 250 µm). The carrier gas was helium with a flow rate of 1ml/min. 0.2 µl oil was used to analyze injection using a special syringe.

The temperature was 70°C for 1 min, ramp 3to 160°C, 1 min ramp 2°C/min to 230°C for 5 min. The injector temperature was 230°C.

Dosage of flavonoids using a spectrophotometry method

To dose flavonoids, we used the aluminum chloride colorimetric method (Bahorun et al., 1996). Absorbance was read by a spectrophotometer (Optizen 2120 UV) at 430 nm. To determine the concentration of flavonoids in the extract, a calibration range was established using quercetin (1-25 µg/ml). The results of dosage are expressed in equivalent micrograms of quercetin for each gram of the extract.

High performance liquid chromatography

HPLC was used to achieve the quantitative analysis of flavonoids.

Column of silica was used as stationary phase (C18 reverse phase), this column measures 125 by 4.6 mm. A mixture of water/methanol/acetic acid (50:47:2.5) was used as a mobile phase, in isocratic system with a flow of 1 ml/min (Amarowicz et al., 2005). Extracts and standards were both analyzed with concentration of 0.5 mg/ml. The used volume was 20 µl. Detection was achieved by a UV-Visible detector at 254 nm.

Biological activities

Antimicrobial activity

A steers machine which is a multiple seeding instrument was used to facilitate the study of the antimicrobial activity of essential oil and this is according to the recommendations of the French Society of Microbiology. After diluting essential oil in mediums: Mueller Hinton for bacteria or Sabouraud for fungi, they were let to solidify. Bacterial suspensions of 0.5 Mc Farland was put using spots. For assessing the flavonoic extract activity, plates of Mueller Hinton agar for bacteria and Sabouraud agar for fungi were inoculated by swabbing of standardized microbial suspension (0.5 Mc Farland), according to NCCLS recommendations (NCCLS, 2006), after that, on the agar, we placed discs of 6 mm diameter containing 10 µL of extract with different concentrations.

As positive control, discs containing antibiotics was used and placed in the center of the plate. After incubating for 24 h at 37°C for bacteria and 48 h for fungi, we determined the antimicrobial activity of both extracts by measuring the MICs. Every test was performed in triplicate.

Antioxidant activity

The DPPH test

To quantify the antioxidant activity of both flavonoids extract and essential oil, we used the method of Sanchez Moreno et al. (1998). For that, we prepared various concentrations of 0.1; 0.2; 0.4; 0.6; 0.8 and 1 mg/ml using a stock solution of both extracts obtained by dissolution in methanol. 1 ml from each one of the concentrations was added to 4 ml of DPPH solution whose concentration was 0.024 mg/ml, this was the preparation for assessing the activity of essential oil. For flavonoids, we mixed 25 µl of each concentration with 975 µl of the same solution of DPPH. Ascorbic acid was prepared using the same protocol. After 30 min, we measured the variation of absorbance using UV-visible spectrophotometer (Optizen 2120 UV), driven by a computing system in the wavelength of 517 nm. To express results in percentage, we used this formula: $I\% = 100 \times \frac{A\ reference - A\ test}{A\ reference}$

$A_{reference}$ is the absorbance of the control which contains only reactive. A_{Test} is the absorbance of the extract.

Statistical analysis of the data

Results of the antioxidant activity were expressed as mean ± SD.

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening of *R. fructicosus L.* reveals abundant amount of total tannins, catechin tannins, gallic tannins, flavonoids, senosids, saponosids, coumarins (Table 1).

Table 1. Phytochemical screening of *R. fruticosus*.

Test	Abundance
Total tannins	++
Catechin tannins	++
Gallic tannins	++
Flavonoids	++
Anthocyanins	-
Leuco- anthocyanins	-
Alkaloids	-
Senosids	+
Amidon	-
Saponosids	++
Irridoids	-
Glucosids	-
Mucilages	-
Coumarins	++
Quinons	-

++: abundance; +: presence; -: absence.

Table 2. Yields in % of flavonoids extracts.

Extract	%
Diethyl ether extract	21.96
Ethyl acetat extract	12.29
Butanolic extract	35.15
Flavonoids	30.6

This plant seems to have a high potential but there is no sufficient studies on its chemical composition and biological activities.

A study on the same species shows that it contains alkaloids, flavonoids, tannins, saponins and glycosides (Rameshwar et al., 2014). We found similar results with no glycosides, but the presence of other compounds such as sénosides and coumarins.

Extractions

Using 100 g of dried matter, the yield was 0.05 ± 0.01% of essential oil. We obtained lightly viscous oil, with a pale yellow coloration and a characteristic odor. The aqueous extract has a gelatinous aspect and brownish color. The yields of extraction using different solvents (diethyl ether, ethyl acetate, butanolic and aqueous) vary between 2.99 and 63.2% (Table 2).

Blackberry has about 30% of highly polar flavonoids (weak) against more than 60% butanol extract (the most abundant extract) in the plant of the present study. Total

Table 3. Retention time of the used standards.

Retention time (min)	Compound
17	Gallic acid
24.5	Gallocatechin
26.1	Protocatechic acid
30	Catechin
34.4	Caffeic acid
40.3	Rutin
50	Ellagic acid
55.6	Myricetin

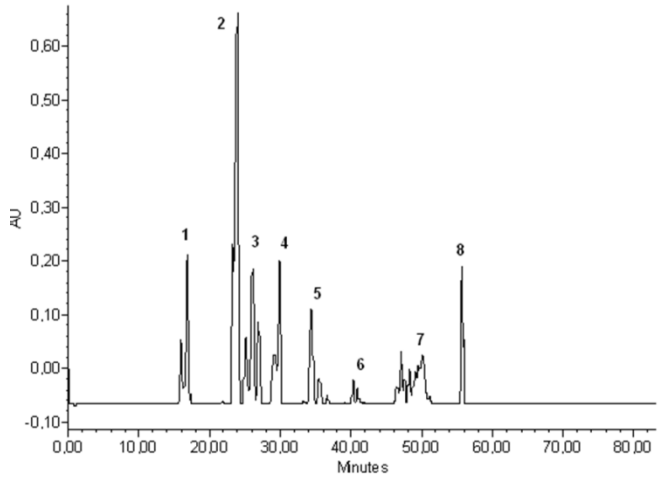

Figure 1. Chromatogram of HPLC applied to flavonoids.

polyphenols of one Rosaceae plant contain a yield of about 140 mg/100 g of the material, although this performance is quite high, but we cannot show the percentage of each phenolic extract (Benvenuti et al., 2006).

Characterization

Dosage of flavonoids

Content of flavonoids is expressed in equivalent mg of quercetin/ml of the extract of the whole plant. It was found to be 75.54 mg/ml. The content of *R. fruticosus* in flavonoids cannot be discussed because there is no published work on the chemical composition of this plant or another plant belonging to the same family, but by observing the averages of yields of extraction in many studies, 75.54% is a high yield and we it can be assumed that this extract is high in flavonoids.

High performance liquid chromatography

On the chromatogram of flavonoids of *R. fruticosus* L., we found eight peaks corresponding to eight main compounds which are: gallic acid, gallocatechin, protocatechic acid, catechin, caffeic acid, rutin, ellagic acid and myricetin. Results are shown in Table 3 and Figure 1. An analysis by HPLC made in South America by Mertz et al. (2007) showed the presence of flavonol hexoside-malonates and hydroxycinnamic acids in *R. fruticosus* extracts.

A study which was realized by the same authors using HPLC with diode array reveals the presence of cafeic acid in two neighbors species which are *Rubus glaucus* Bent and *Rubus adenotrichus* Schlech, this two plants are not rich in this component, the same component was find using HPLC in a variety of *R. fruticosus* obtained from the region of Tizi Ouzou in Algeria, that shows the presence of cafeic acid in the botanical kind, *Rubus*.

Another study has revealed, using HPLC, the presence of ellagic acid and 3,4-dihydroxy-benzoic acid in the flavonoids issues from leaves of *R. fruticosus* gathered in Bulgaria. Ellagic acid was also detected in the aerial part of the studied plant. Other molecules were identified in this study and were never identified in previous studies,

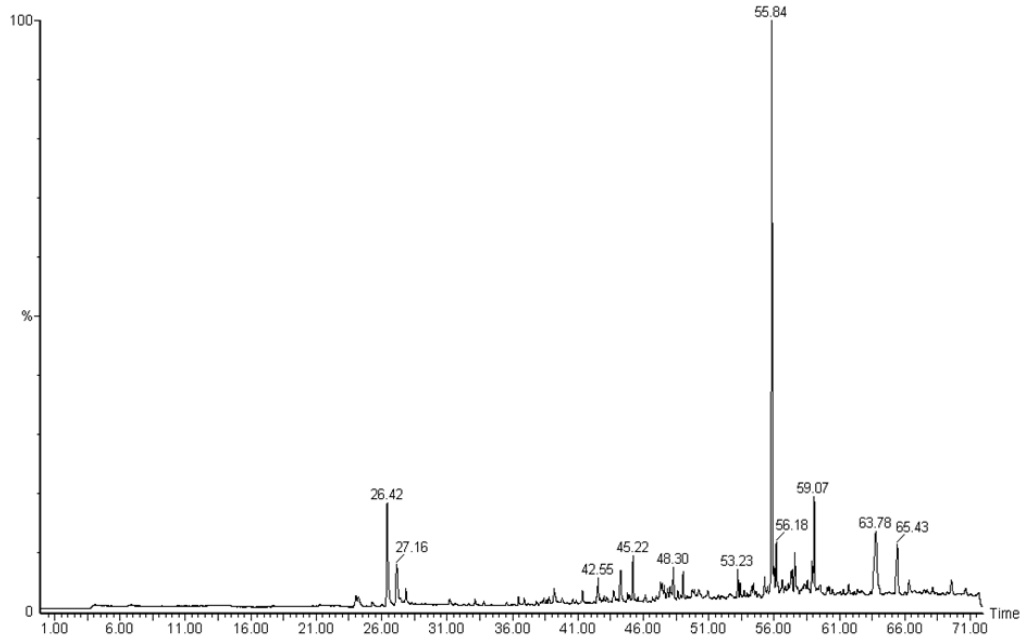

Figure 2. Chromatogram of GC-MS applied to essential oils.

Table 4. Retention time of essential oil compounds.

R_t	Name of the compound	%
26.42	Limonene	13.09
27.16	Trans-3-caren-2-ol	4.21
42.55	Caryophylene	3.13
45.22	Thymol	5.59
48.30	Veridiflorol	4.02
53.23	Epiglobulol	4.22
55.84	Lanceol	20.22
56.18	Globulol	5.15
59.07	Methyl steviol	14.12
63.78	Fenitrothion	12.85
65.43	Benzene, dodecyl	12.01
Total		98.61

this may be explained by the difference of the area of harvest (Milivojevic et al., 2011; Radovanović et al., 2013; Carlsen et al., 2003).

GC-MS of essential oil of *R. fruticosus*

By analyzing the essential oil of *R. fruticosus* using GC-MS, we identified 11 molecules (Figure 2). The major component is the lanceol (20.22%) (Table 4). Concerning the essential oil of *R. fruticosus*, no studies were done on it or any essential oil of any species belonging to *Rubus* family.

Antimicrobial activity

Table 5, the antimicrobial activity of flavonoids extract and the essential oil on reference strains are represented, both substances have an effect on the respiratory flora. The activity of both extracts was evaluated on strains isolated from patients with respiratory infections; these patients were diagnosed with respiratory infections, ORL infections or bronchitis infections.

Clinical strains chosen to evaluate antimicrobial activity showed an important resistance to conventional antibiotics, Table 6 shows the results. By assessing the antimicrobial activity of this species against some respiratory pathogens, this plant seems to be promising but no many works was done on evaluating the effect of the extract of this species against microbes. A study evaluated the activity of the ethanolic extract of *R. fruticosus* against *Helicobacter pylori* which was resistant to nalidixic acid, this extract had a MIC of 400-450 µg/ml (Abachi et al., 2013).

Another study showed that the blackberry juice inhibits the growth of *Bacillus cereus*, *Bacillus subtilis*, *Streptococcus marcescens* and *Escherichia coli* with percentages varying from 50 to 75% (Poyrazolu and Biyik, 2010).

Flan et al. (2011) found that the methanolic extract of aerial parts of *R. fruticosus* inhibits the growth of *Mycobacterium tuberculosis* with a MIC of 1 mg/ml. Another study by Riaz et al. (2011) on methanolic extracts from various parts of the plant against eight reference bacterial strains (*Salmonella typhi*, *E. coli*,

Table 5. Results of the antimicrobial activity against reference strains.

Strain	Reference	MIC_s	
		E.O (mg/ml)	Flavonoids (mg/ml)
E. coli	ATCC 11229	R	R
Enterobacter cloacae	ATCC 13047	0.5	2.36
Staphylococcus aureus	ATCC 6538	R	4.72
Haemophilus influensiae	ATCC 10211	0.25	4.72
Streptococcus pneumoniae	ATCC 10015	R	4.72
Pseudomonas aeruginosa	ATCC 15442	0.25	R
Klebsiella pneumoniae	ATCC 13883	C.E	37.77
Candida albicans	ATCC 10231	R	37.77

R: Resistant.

Table 6. Activity of *R. fruticosus* extracts against respiratory pathogens.

Strains	Antibiotics								Essential oils (CIM_s)	Flavonoids (MIC_s) (mg/ml)
E. coli	Cip	Amo	Ceph	Tic	Trim	Cefo	Cefu	Ceft		
1	R	R	R	R	R	R	R	R	R	C.E.
2	R	R	R	R	R	R	R	R	R	C.E.
P. Aeruginosa	Fost	Imi	Levo	Amik	Cet	Cip	/	/		
1	R	R	R	R	R	R	/	/	0.5	37.77
2	R	R	R	R	R	R	/	/	0.5	18.88
P. fluorescens	Azith	Cipr	Levo	Tic	Trim	Gent	Imi	Cef		
1	R	R	R	R	R	S	S	S	0.125	09.44
2	S	R	R	R	S	R	R	R	0.125	18.88
B. Cepacia	Amox	Imi	Gent	Cefo	Col	Cefa	/	/		
1	S	S	S	S	S	R	/	/	0.25	04.72
2	S	S	S	S	S	S			0.125	04.72
H. Influensiae	Cot	Amo	Ofl	Tet	Cefo	/	/	/		
1	R	S	S	R	S	/	/	/	0.25	02.36
2									0.25	02.36
K. pneumoniae	Amo	Cef	Gent	Amp	Ami	Col	Imi	Cot		
1	R	R	R	R	R	R	S	R	0.5	18.88
2	R	R	R	R	R	R	R	R	0.25	18.88
A.baumannii	Tic	Pop	Cef	Imi	Ofl	Cip	Col	Tic + Ac		
1	R	R	R	R	R	R	S	R	C.E	37.77
2									R	75.54
E. cloacea	Azith	Cefac	Ceft	Cefur	Eryt	Peni	Tetr	Amo		
1	R	R	R	R	R	R	R	S	0.5	37.77
2									0.5	37.77
S. aureus	Vanc	Oxa	Gent	Amp						
1	S	S	S	R	/	/	/	/	R	02.36
2									R	02.36
S. Pneumoniae	Azith	Oxa	Gent	Amp	Amo	Imi	Tetr			
1	R	R	R	R	R	R	S		R	18.88
2	R	R	R	R	R	R	R		R	18.88
C. albicans	Azith	Oxa	Gent	Amp	Amo	Imi	Tetr			
1	R	R	R	R	R	R	R		R	18.88
2	R	R	R	R	R	R	R		R	37.77

R: Resistant; S: sensitive.

Streptococcus aureus, *Micrococcus luteus*, *Proteus mirabilis*, *Bacillus subtilis*, *Citrobacteri sp*., *Pseudomonas aeruginosa*), revealed that all the extracts inhibit the growth of bacteria with different MICs.

All these studies showed that different extracts of *R. fruticosus*, especially phenols, have a big potential to inhibit the growth of bacteria or fungus.

Antioxidant activity

DPPH test

Free radical scavenging was appreciated using DPPH discoloration test that revealed a high antioxidant activity of the flavonoic extract of *R. fruticosus.* This test revealed a weak scavenging of free radical activity for the essential oil, the EC_{50} was 9.24 ± 0.48 mg/ml. This value is 2.68 ± 0.88mg/ml for flavonoids. In Figure 3, these results are compared with ascorbic acid as a standard.

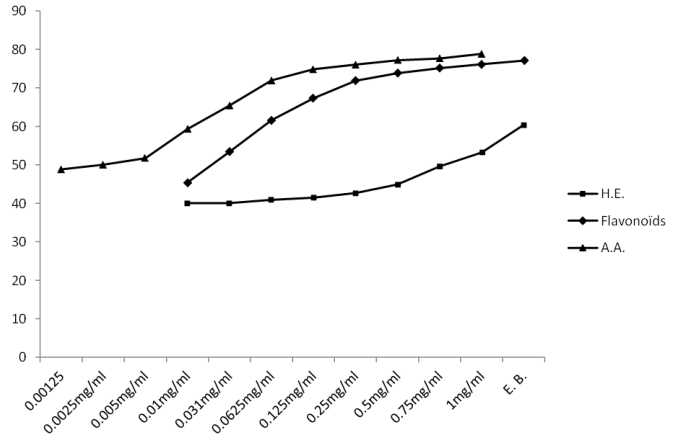

Figure 3. Antioxidant activity using the DPPH test.

The antioxidant activity of flavonoids of *R. fruticosus* is closer to the values of reference which is ascorbic acid (IC_{50} was found 1.46 ± 0.01mg/ml).

A study showed that IC50 varies from 15.2 to 76.5 µg/ml for different extracts including n-hexane, dichloromethane, chloroform, ethyl acetate, methanol. Blackberries are a rich source of natural antioxidants as they contain high levels of phenols and flavonols and are therefore well reputed scavengers and inhibitors of free radicals (Ivanovic et al., 2014).

The study of both biological activities: antimicrobial and antioxidant of the extract of flavonoids and essential oil of *R. fruticosus* reveals that the essential oil has a weak biological activity while flavonoids seem to have strong biological effects.

Conclusion

By assessing the antimicrobial and antioxidant activities of the flavonoic extract and the essential oil of Algerian *R. fruticosus* L., we can assume that this species and particularly its flavonoic extract have a remarkable inhibitory action against resistant respiratory pathogens.

More advanced studies are needed to investigate the possibility of developing formulations for pharmaceutical products from this extracts.

Conflict of interests

The authors did not declare any conflict of interest.

Abbreviations: C.E, Crude extract; **EC_{50},** efficient concentration 50; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **MIC,** minimal inhibitory concentration; **R_t,** retention time; **HPLC,** high performance liquid chromatography; **GC-MS,** gas chromatography-mass spectrophotometry; **AMP,** ampicilin; **IMI,** imipenem; **GENT,** gentamicin; **AZITH,** azithrimicin; **CIP,** ciproflaxin; **VAN,** vancomycin; **OXA,** oxacilin; **TET,** tetraciclin; **ERYT,** erythromicin; **PENI,** penicilin; **AMO,** amoxicilin; **CEF,** cefazolin; **LEV,** levoflaxin.

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