

## ANTIBACTERIAL ACTIVITY OF ALGERIAN SUN-DRIED RAISINS EXTRACTS AGAINST ISOLATES OF EXTENDED-SPECTRUM BETA-LACTAMASE ESBL-PRODUCING *ENTEROBACTERIACEAE*

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### Abstract

**Description of the subject:** Until as recently as the early 20<sup>th</sup> century, nutritionists prescribed specific foods and diets as remedies for illness and for their disease preventive value.

**Objective:** The present study deals with the evaluation of *in vitro* antibacterial potential of three different extracts of Algerian *Black Corinth* raisins variety against clinical isolates of extended-spectrum beta-lactamase ESBL-producing *Enterobacteriaceae*.

**Methods:** The total phenolic content of raisins extracts was determined with Folin-Ciocalteu. For the antibacterial activity agar diffusion methods and minimum inhibitory concentration (MIC)-determination were used.

**Results:** Results revealed the richness of the different extract by polyphenol. Indeed, content varies from 101.53 ± 0.96 to 644.43 ± 14.64 mg GAE/100g of fruit. According to the results of the antibacterial activity, all the phenolic extracts showed antibacterial effect against all extended-spectrum beta-lactamase ESBL-producing *Enterobacteriaceae*. *Escherichia coli* was the most susceptible bacteria to all extracts. Pronounced antibacterial activity was observed by the methanolic extract with MIC = 19.74 µg/ml.

**Conclusion:** This study highlights the importance of Algerian *Black Corinth* raisins variety as a source of antibacterial agents to be used in traditional medicine to treat or protect humans against infection diseases. Therefore, their extracts have the potential to be developed into dietary supplements and nutraceuticals.

**Keywords:** Sun-Dried Raisins; *Enterobacteriaceae*; β-lactamase; polyphenols; antibacterial activity.

## ACTIVITÉ ANTIBACTERIENNE DES EXTRAITS DE RAISINS SECS DE VARIÉTÉ ALGÉRIENNE VIS-À-VIS DES SOUCHES D'ENTÉROBACTÉRIES PRODUCTRICES DE BETA-LACTAMASES À SPECTRE ÉTENDU (BLSE)

### Résumé

**Description du sujet:** Jusqu'au début du 20<sup>ème</sup> siècle, les nutritionnistes prescrivait des aliments et des régimes alimentaires spécifiques comme remèdes contre certaines maladies pour leur effet préventif.

**Objectives :** La présente étude porte sur l'évaluation, *in vitro*, du potentiel antibactérien de trois différents extraits de la variété algérienne Corinth-noire de raisin sec vis-à-vis de six souches d'entérobactéries productrices de bêta-lactamases à spectre élargi BLSE.

**Méthodes:** La teneur en composés phénoliques totaux des extraits de raisins secs a été déterminée avec la méthode de Folin-Ciocalteu. Pour l'activité antibactérienne, la méthode de diffusion sur gélose et la détermination de la concentration minimale inhibitrice (CMI) par microdilution ont été utilisées.

**Résultats :** Les résultats ont révélé la richesse des différents extraits en polyphénol, dont la teneur varie de 101,53 ± 0,96 à 644,43 ± 14,64 mg EAG / 100 g de fruit. Les résultats de l'activité antibactérienne, ont montré que tous les extraits présentaient un effet antibactérien vis-à-vis au moins d'une souche parmi les souches testées. *Escherichia coli* était la souche la plus sensible à tous les extraits. Une activité antibactérienne remarquable a été observée avec l'extrait méthanolique avec une CMI = 19,74 µg/ml.

**Conclusion:** Cette étude a montré l'importance de la variété algérienne Corinth-noire de raisin sec comme source d'agents antibactériens, ce qui valorise son utilisation en médecine traditionnelle pour traiter ou protéger les êtres humains contre les maladies infectieuses. Par conséquent, leurs extraits peuvent être transformés en compléments alimentaires et en nutraceutiques.

**Mots clés :** Raisins secs ; *Enterobacteriaceae*; β-lactamases; polyphénols; activité antibactérienne.

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## INTRODUCTION

Most fruits are generally consumed for their nutritional value. In fact, fruits considered as the reservoir of many plant secondary metabolites, possess various health beneficial effects including antibacterial effect [1]. Raisins are dried grapes of *Vitis vinifera* L. (*Vitaceae*) fruits [2]. The human health benefits associated with this dried fruit are widely known and linked to the richness of phenolic compounds such as gallic acid, catechin, anthocyanins and resveratrol, and a wide variety of procyanidins [3]. These compounds, and especially resveratrol, have been demonstrated to have a wide range of biological effects, including anticarcinogenic, and antiatherogenic, antiinflammatory, antimicrobial, and antioxidant activities [4]. Resveratrol can modulate epigenetic patterns by altering the levels of S-adenosylmethionine and S-adenosylhomocysteine or by directing the enzymes that catalyze DNA methylation and histone modifications [5]. It also activates the deacetylase sirtuin and regulates oncogenic and tumor suppressor micro-RNAs [5].

Nowadays, the evidence that the increasing number of microorganisms resistant to the available antibiotics is an emergent problem and subject for researchers and clinicians from all over the world. It is estimated that at a minimum of 25000 patients in Europe, and 23000 in the USA die each year from infections caused by antibiotic-resistant bacteria [6]. Extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. In general, bacteria possess the genetic ability to transmit and acquire resistance to therapeutic agents. Resistance to penicillins and other  $\beta$ -lactams is due to one of four general mechanisms: inactivation of antibiotic by  $\beta$ -lactamase, modification of target Penicillin – Binding Protein (PBPs), impaired penetration of drug to target PBPs and efflux.  $\beta$ -lactamase production is the most common mechanism of resistance [6, 7].

To overlap the disadvantages of the available antimicrobial drugs, other drugs with new mechanisms of action should be developed. Nutritional approach may prove a likely solution to this problem.

*In vitro* and epidemiologic studies suggest that consumption of food rich in phenolic compounds might significantly inhibit or delaying the growth rate of a wide range of bacteria and microfungi [8, 9]. So, that they might be used as alternative food for practicing a preventive or curative effect. In particular, phenolic compounds may represent a novel class of safe and effective drugs. Following desiccation, grapes (*Vitis vinifera*) become more resistant to microbial-based degradation, suggesting that raisins may be a source of antimicrobial compounds.

To investigate this hypothesis, four different extracts of an Algerian raisin variety namely “Black Corinthe” were studied in this work to determine their polyphenolic content and antibacterial activity by using the Folin–Ciocalteu assay agar diffusion methods and minimum inhibitory concentration (MIC)-determination.

## MATERIALS AND METHODS

### 1. Collection of samples

Algerian raisin variety namely “*Corinthe noire* or *Black Corinth*” was collected from local market in Kolea located in the south-west of Algiers (Algeria) and was confirmed by ITAF (Technical Institute for Fruit Trees) experts. The fruit was first washed with tap water followed by washing with distilled water.

### 2. Extract preparations

#### 2.1. Methanolic and Acetone Extracts

The samples were prepared according to the method described by Debib *et al.* [8] with slight modifications. The dried fruits (50 g) were grinded and homogenized in a blender and extracted with 250 mL of 80% methanol or 60% acetone containing 1% 2,6-di-*tert*-butyl-4-methylphenol, using an ultrasonic bath. The extraction was repeated three times. The same extracts were pooled and filtered through Whatman No.1 filter paper and evaporated by using a rotary evaporator to give the crude dried extract. The dried extracts were stored at  $-20^{\circ}\text{C}$  until used.

#### 2.2. Aqueous and Petroleum Ether Extracts

Dried fruits (50 g) were added to 500 mL distilled water or petroleum ether, homogenized and shaking for 24 h, at room temperature. The extracts were filtered through Whatman No.1 filter paper and evaporated using a rotary evaporator or freeze dryer, respectively to give the crude dried extract. The dried extracts were stored at  $-20^{\circ}\text{C}$  until used.

### 3. Total Phenolic Content

The Folin–Ciocalteu method was used to determine the total phenolic content of the extracts according to Singleton *et al.* [10]. The sample (2.5 mL) was diluted to 25 mL with distilled water. An aliquot of the solution (2 mL) was mixed with Folin–Ciocalteu reagent (10-fold diluted with distilled water, 10 mL). After 5 min, a 7.5% (w/v) sodium carbonate solution (8 mL) was added. After 2 h, the absorbance was measured at 765 nm against a blank prepared as described above with distilled water (2 mL), Folin–Ciocalteu reagent and sodium carbonate solution. Values of total phenolic contents were estimated by comparing the absorbance of each sample with a standard response curve generated using gallic acid (0, 12.5, 25, 50, 100 and 200 µg/mL). The results were expressed as mg gallic acid equivalents (GAE)/100 g of fruit. All the measurements were taken in triplicate and the mean values were calculated.

### 4. Microorganisms Test

The microorganisms used are clinical isolates from patients hospitalized in various departments of the Hospital Center of Kolea (south-west of Algiers, Algeria) and Yessad Khaled hospital, Mascara (situated in the north east of Algeria). All isolates were presumptively identified by primary phenotypic identification methods based on colony morphology, catalase and Gram staining. The API 20E system (bioMérieux, France) was also used to confirm identification of *Enterobacteriaceae*.

### 5. Tests for $\beta$ -Lactamase production

Clinical isolates were screened for  $\beta$ -Lactamase production by performing the following tests:

#### 5.1. Double-disc synergy test

A third generation cephalosporin, viz., ceftazidime or cefotaxime (30 µg/disc), and a disc of co-amoxiclav (20 µg amoxicillin/10 µg clavulanic acid) were placed 20 mm apart on Mueller Hinton agar (MHA) plate on which 0.5 McFarland of test organism was swabbed. In case of an ESBL producer strain, the zone diameter of cephalosporin disc was found to extend by  $\geq 5$  mm towards the co-amoxiclav disc [11].

#### 5.2. Phenotypic confirmatory test

Disc tests were performed for phenotypic confirmation of the presence of ESBLs by using cefotaxime (30 µg) or ceftazidime discs

(30 µg) with or without clavulanate (10 µg) as recommended by the Clinical and Laboratory Standards Institute (CLSI). In case of ESBL-producing bacteria, the zone diameter of cephalosporin/clavulanate disc was at least 5 mm greater than the zone for cephalosporin disc [11].

### 5.3. Iodometric Method

Penicillin solution was dispensed in 0.5 mL volume in small test tubes. Test bacteria were removed with a loop from an overnight culture on solid medium and suspended in the penicillin solution to give a density of at least  $10^4$  CFU/ml. After one hour at room temperature two drops of starch indicator was added to the suspension, followed by one drop of iodine reagent. Positive reaction was indicated by the disappearance of blue color immediately. Persistence of blue color for longer than 10 minutes constituted a negative test [12].

### 6. Antibacterial Activity Assay

Antibacterial activity was determined by the agar disc diffusion assay (NCCLS, 2009). The extracts were dissolved in dimethyl sulfoxide (DMSO) or distilled water. Petri plates were prepared with 20 mL of sterile Mueller Hinton agar (Sigma, Paris, France) surface inoculate by suspension of cell (200 µL) adjusted by McFarland 0.5 method ( $10^6$  cfu/mL). Sterile filter paper discs of 6 mm diameter were impregnated with 20 µL of the extract solution. The plates were incubated at 37°C for 24 h. Gentamicin (15 µg), amoxicillin (25 µg) and erythromycin (15 UI) were used as positive controls. Negative controls were performed using paper discs loaded with 20 µL of the solvents used (DMSO and water). The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs. After that, the inhibition zones were measured in millimeters by Vernier calipers. All tests were repeated three times to minimize test error. An inhibition zone of 14 mm or greater (including diameter of the disc) was considered as high antibacterial activity [11].

### 7. Broth Microdilution Assay for Minimum Inhibitory Concentration (MIC)

Determination of the minimal inhibitory concentration (MIC) was determined by microdilution assays in 96-well microtiter plates [13].

Amount of substance used in MIC determination was calculated after evaporating the solvent of 1 mL of extract and then solubilizing the dry extract in 20% v/v DMSO. The solution was subsequently diluted for 10-fold with Mueller Hinton broth. One hundred microliter from broth bacterium solutions and dilutions were transferred into microtitration plates and incubated for 24 h at 37°C. The positive control contained 100 µL of bacterium solution plus 100 mL Mueller Hinton broth. Negative control contained only 100 µL dilute plus 100 µL of extract without bacteria. Positive and negative results were evaluated according to turbidity occurred after 24 h by comparing to the control well. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, which is a clearly erased. All extracts were tested in triplicates.

## 8. Statistical Analysis

Values are given as arithmetic means  $\pm$  standard error of the mean. Data were statistically analyzed by using one-way analysis of variance (STAVIEW version 5.0, Abacus Concepts, Berkeley, CA) and Student's *t*-test.

## RESULTS

### 1. Total phenolic contents

Based on the gallic acid standard calibration curve (Fig. 1), total phenolic levels have been determined using regression curve  $y=0.0094x+0.2317$ , with *y*: absorbance and *x*: concentration of gallic acid,  $R^2=0.9977$ . The results were expressed as mg gallic acid equivalents (GAE)/100 g of fruit.

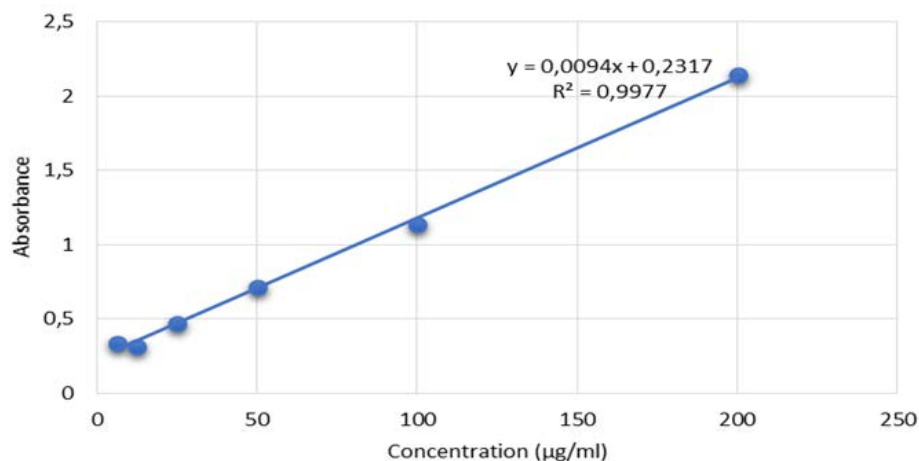


Figure 1 : Gallic acid standard calibration curve.

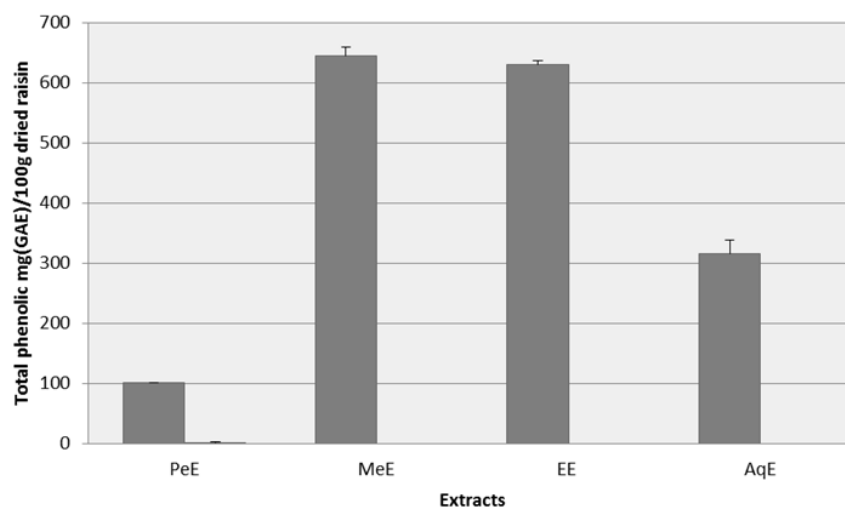


Figure 2 : Total phenolic content as gallic acid equivalent (GAE mg/100g) in dried *Vitis vinifera* fruit extracts (PeE, Petroleum ether extract; MeE, methanolic extract; EE, Ethanolic extract; AqE, aqueous extract).

The data reported in Figure 2 show total phenolic compounds of the different dried raisins extracts. The results show that the levels of total phenolic compounds differed significantly ( $\alpha < 0.05$ ) between ethanolic, aqueous and petroleum ether extracts. However, no significant difference was found among ethanolic and methanolic extract. Generally, the extracts of methanol and ethanol contained the highest polyphenols concentrations ( $630.37 \pm 6$  mg GAE/100 g and  $644.43 \pm 14.67$  mg GAE/100 g of fruit respectively); followed by aqueous extracts ( $315.45 \pm 22.45$  mg GAE/100 g of fruit). Moreover, the lowest phenolic compounds content was registered in the petroleum ether

extracts ( $101.53 \pm 0.96$  mg GAE/100 g of fruit).

## 2. Antibacterial Activity Assay

From antibiotic resistance results (Table 1), we can observe that most of the tested bacterial strains have developed a particular resistance against Amoxicillin (AMX), Amoxicillin + Clavulanic Acid (AMC), Cefazolin (CZ), Cefalexin (CN), Oxacillin (OX), and the Ceftazidime (CAZ). However, we find that different strains are sensitive to the monobactam such as Aztreonam (ATM), and some 3<sup>rd</sup> generation antibiotics (Cefotaxime (CTX), Cefepim (CEF)).

Table 1 : Antibiotic resistance profile of ESBL-producing *Enterobacteriaceae*.

| Strains Designation           | Antibiotic resistance pattern  | MICs of $\beta$ -lactam antibiotics |     |     |
|-------------------------------|--------------------------------|-------------------------------------|-----|-----|
|                               |                                | CTX                                 | CEF | ATM |
| <i>Klebsiella</i> sp.         | AMX, AMC, CZ, CN, OX, CAZ      | 512                                 | 256 | 128 |
| <i>Providencia</i> sp.        | AMX, AMC, CZ, CN, OX, CAZ      | 256                                 | 256 | 256 |
| <i>Salmonella paratyphi</i> A | AMX, AMC, CZ, CN, OX, CAZ, ATM | 512                                 | 256 | R   |
| <i>Shigella</i> sp.           | AMX, AMC, CZ, CN, OX, CAZ      | R                                   | R   | R   |
| <i>Escherichia coli</i>       | AMX, AMC, CZ, CN, OX, CAZ, ATM | R                                   | R   | R   |

The data of antibacterial effects of all extracts against ESBL-producing *Enterobacteriaceae* reported in figure 3, showed that bacterial inhibitor activity was recorded by ethanolic and methanolic extracts against *Klebsiella* sp.

and *E. coli* as the highest values, although these two strains were resistant to the most of antibiotics tested, whereas *Providencia* sp. was resistant to the ethanolic extract.

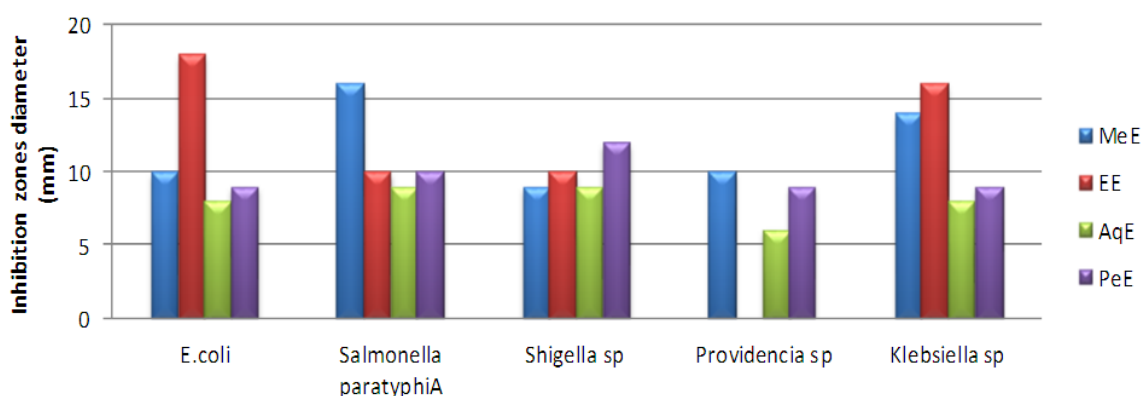


Figure 3 : Antibacterial activity of the crude extracts in agar diffusion assay.

(PeE, Petroleum ether extract; MeE, methanolic extract; EE, Acetonic extract; AqE, aqueous extract).

## 3. Broth Microdilution Assay for Minimum Inhibitory Concentration (MIC)

Table 2 presents the results of Broth Microdilution Assay for Minimum Inhibitory Concentration (MIC) of the 4 extracts. All extracts showed variable degree of activity against the tested bacterial strains. It was observed that the *E. coli* strain manifested more sensitivity to the investigated extracts

than the other bacteria strains. For the most part, among the investigated extracts the methanol extracts exhibited the highest antibacterial effect. It was interesting to note that the most remarkable antibacterial activity with MIC value of  $19.74 \mu\text{g/mL}$  was noticed for the methanol and ethanol extracts against *E. coli*. On the other hand, the lowest antibacterial activity was observed for the

aqueous as well as Petroleum ether extract extracts.

Table 2: MIC values in µg/mL for the investigated extracts

| Strains                       | Extract | MeE    | EE     | AqE    | PeE    |
|-------------------------------|---------|--------|--------|--------|--------|
| <i>Shigella</i> sp.           |         | 315.87 | 315.87 | 631.74 | 631.74 |
| <i>E. coli</i>                |         | 19.74  | 19.74  | 315.87 | 157.93 |
| <i>Klebsiella</i> sp.         |         | 39.48  | 39.48  | 157.93 | 157.93 |
| <i>Providencia</i> sp.        |         | 78.96  | 157.93 | ND     | ND     |
| <i>Salmonella paratyphi</i> A |         | 39.96  | ND     | 315.87 | 315.87 |

## DISCUSSION

Almost 25 centuries ago, Hippocrates, the Father of Medicine, stated, "Let food be thy medicine and let medicine be thy food". Supporting this statement, at present, there has been an enormous interest worldwide in nutraceuticals, which are known to play a pivotal role in health management. Many different studies have shown the beneficial effects of a range of different fruits, vegetables, and spices [14]. On this basis, our present study was carried out to evaluate the possible antibacterial potential of four different extracts of Algerian dried *Vitis vinifera* L. fruit against clinical isolates of Extended-Spectrum Beta-lactamase ESBL-producing *Enterobacteriaceae*. It is important to mention that the existing knowledge about the tested variety namely "*Corinthe noire* or *Black Corinth*" is in many cases very limited. To the best of our knowledge, this is the first study concerning the antibacterial activity and phenolic contents of this Algerian variety. The dried fruits were extracted with 4 different solvents (methanol, ethanol, distilled water and petroleum ether). Our results show that the methanolic and ethanolic extracts were found to have more phenolic compounds compared to the aqueous and the petroleum ether extracts. Similar findings were also reported by other studies [15, 16]]. Compared to the total phenolic concentrations reported for other raisins, values found in the Algerian *Black Corinth* variety tested were comparable to concentrations found by other authors in other raisin varieties [17, 18]. However, the values obtained in this study were lower than the values quantified by Breksa *et al.* [19] in of 16 raisin grape (*Vitis vinifera* L.) cultivars produced in the USA.

The ethanol and methanol remain good extraction solvents of plant secondary metabolites, furthermore the phenolic compounds solubility is affected by the solvent polarity used which makes it difficult to establish all plant phenolic compounds extracting process. It was interesting to note that polyphenols are hydrophilic phytochemicals, and hydrophilic solvents are more effective agents for the extraction. According to the literature, it is known that the ultrasonic extraction generally increases the efficiency extraction. Thongson *et al.*, reported that ultrasonic extraction took only 5 minutes to obtain the bioactive components that exhibited the antimicrobial activity from medicinal plant [20].

Our results are not in agreement with Jayaprakash *et al.* [21] who reported that two different solvents system i.e. acetone: water: acetic acid (90: 9.5: 0.5) and methanol: water: acetic acid (90: 9.5: 0.5) were used for extraction and their results revealed that both extracts had activity against gram positive bacteria but not against gram negative bacteria .

Our data showed that all extracts showed variable degree of activity against the tested strains. This observed effect could be attributed to the high content of phenolic compounds. It is known that these substances include flavonoids and phenolic acids are synthesized by plants in response to bacterial infection. Currently it is known by the literature that the inhibition of bacteria are included several mechanisms such as adsorption to cell membrane, destabilization of cytoplasmic membrane, permeabilization of plasma membrane, interaction with enzymes, substrate and metal ion deprivation, and inhibition of extracellular microbial enzymes [22].

The presence of the hydroxyl group in phenolic compounds plays an important role in antimicrobial activity [23]. The hydroxyl group reacts with the cell membrane and disrupts the structure of it, and the cell components are lost from the interior.

## CONCLUSION

On the basis of the experimental results and discussion, it can be postulated that the Algerian *Black Corinth* dried raisins variety possesses the potent antibacterial properties and indicate their potential as natural drug products to prevent and treat infectious human diseases. Further investigations should be done for confirm antibacterial effects of other varieties against pathogenic bacteria from different sources, and to the bioactive compound that related to these effects and other beneficial activities.

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