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ANTIOXIDANT, ANTIHEMOLYTIC AND ANTIBACTERIAL EFFECTS OF DRIED AND FRESH *PRUNUS DOMESTICA* L.

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Abstract: Introduction. The nutritional value of *Prunus domestica* is becoming increasingly important. Also, this fruit is known for its therapeutic potential. The fruit presents also a source of vitamins, phenolic compounds and dietary fiber. The antioxidant activity of the plum and prune was mainly studied. Also, antibacterial and anti-hemolytic effects of this fruit have to be investigated. **Materials and methods.** In vitro antioxidant power from plum and prune methanolic extracts was determined using DPPH radical scavenging activity method, reducing power and iron chelating effect test. Yet, the ability of *Prunus domestica* fruit to protect erythrocytes against lysis was evaluated. In addition, two methods were performed to evaluate antibacterial effect (MTT assay and discs diffusion). Two modes of action of antibiotics present in our extracts were investigated. **Results and discussion.** The prune methanolic extract exhibited higher antioxidant capacity than that of plum methanolic extract. 46.08 ± 2.47 mg GAE/g of fresh weight of prune may be probably responsible for its powerful antioxidant activities, suggesting the involvement of these bioactive molecules in the health benefits related to this fruit. Even the three methods used for the assessment of antioxidant activity show an important antiradicalaire power in prune extract comparing with plum extract. Also, prune DMSO extract presents an important protective activity against erythrocytes hemolysis (31.35 ± 0.28 %) at a concentration of 400 $\mu\text{g/ml}$, comparing with DMSO plum extract, suggesting the potential of our extracts as a new source of natural neuro-protective agent. Antibacterial tests showed a remarkable power, especially, against *Staphylococcus aureus* and *Escherchia coli* for the two extracts studied. **Conclusions.** Prunes can be considered as a potential source of different antioxidant compounds, which are not exploited at the moment, but could find practical applications in many industrial branches.

Keywords: *Prunus domestica*, Antioxidant, Antibacterial, Hemolysis Inhibition.



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INTRODUCTION

The damage caused by free radicals in living cells induces several diseases (atherosclerosis, Parkinson's disease, Alzheimer's disease, cancer...) [1]. As a model system for oxidative damage attacking biological membranes, erythrocytes oxidation has been studied [2]. This damage may be inhibited in vivo and in vitro by some antioxidants, especially, Vitamin C [3]. In fact, some food components from vegetables and fruits have been also observed [4, 5].

In other hand, the risk of bacterial infections threatens human health. The treatment of these diseases is mainly based on antibiotics. The prolonged and inappropriate use of these antibiotics can lead to the development of resistance [6]. Finding an alternative treatment has become an urgent issue. Tests used for identification of the antimicrobial compounds are generally limited to the degree of microorganisms growth inhibition by diffusion or dilution assays used to screen essentially antimicrobial compounds. However, the simple study of the inhibition of microbial growth is not enough. Further studies are needed to demonstrate the mode of action of the extracts tested, such as the effects on bacterial cell membranes, synthesis of the fungal cell wall, DNA replication and repair, connecting ribosome protein synthesis and metabolic enzymes. It is then important to study the mode of action of antibiotics after the positive screening of microbial growth inhibition by plants biomolecules.

This work focused on the assessment of the biological activities of *Prunus domestica*, especially, antioxidant power. In fact, there is a diversity of varieties, maturity, climatic conditions and storage conditions of prune. In addition, a great variation in extraction processes can radically change results: type of solvent (water, ethanol, methanol, acetone, ethyl-acetate), temperature (20-60 ° C) and extraction time (5 minutes, 24 hours).

However, the objectives of this work are also to analyze antioxidant potential of local plum comparing with prune, to study in vitro erythrocytes hemolysis inhibition of the plant extract which will probably give an idea about its cyto-protective activity, and to test the antimicrobial potential of our extracts against five bacteria strains. This antibacterial potential was followed by some tests in order to understand the modes of action of these plant antibiotics, in perspectives, their identification.

MATERIAL AND METHODS

Plant material

This study is realized using *Prunus domestica* fruit also known as the common or European plum belonging to Rosaceae family. This round purple fruit is up to 8 cm long with a hard stony pit and a glaucous bloom on the surface. Plum is known by its interesting benefits, especially, as laxative and anti-tumoral agent.

Chemicals

All chemicals used were of at least analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Bacteria used for study

All microbial strains were procured in LIP-MB. Stock cultures of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis* and *Enterobacter* were subcultured and maintained in nutrient broth at 4°C.

Preparation of Methanolic extract

Plums and prunes were ground to fine powder. 5 g of the powder was homogenized in 50 ml Methanol followed by shaking for 24 h in dark, then the extract was centrifuged at 3500 rpm for 30 min and the upper layer was dried in speed vacuum and stored at 4°C until further use assays.

Preparation of extracts with DMSO

About 5 g of peeled prune and plum were grinded using methanol. The finely ground paste was soaked in 100 ml of methanol and was wrapped with parafilm to prevent the evaporation of volatile compounds. A centrifugation was necessary to recuperate supernatant and dry it in speed vacuum. DMSO was then added and this solution was stored at 4°C.

Methods

Antioxidant Activities

Total phenolics and flavonoids contents determination

The total phenolic content of Garlic extracts was determined using the Folin-Ciocalteu colorimetric method, based on the procedure of Singleton and Rossi [7] using Gallic Acid as a standard phenolic compound. The results were expressed as mg of Gallic Acid equivalents per gram of fresh weight. The determination of flavonoids was performed according to the colorimetric assay of Dewanto et al., [8]. The results were expressed as mg of catechin equivalent per g of fresh weight.

DPPH free radical scavenging assay

The antioxidant activity of the extracts, based on the scavenging activity of the stable DPPH free radical, was determined by the method described by Braca et al., [9]. DPPH free radical scavenging ability (%) was calculated using the formula:

$$\text{Scavenging Activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

With A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Trolox was used as the positive control. Three replicates were made for each test sample.

Reducing power

The reducing power of prunes and plums extracts was determined according to the method of Oyaizu [10] with some modifications. Three replicates were made for each test sample. Increased absorbance for the reaction mixture indicated increased reducing power. BHT was also assayed at the same concentration for comparison purpose.

Iron chelating effect

The ferrous ion-chelating potential was evaluated following the method described by Decker and Welch [11]. Three replicates were made for each test sample. EDTA was used as a positive control. The control contained all the reaction reagents except the extract or positive control. The percent of inhibition of ferrozine-Fe²⁺ complex formation was calculated according to the equation below:

$$\text{Chelating activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

With A₀ is the absorbance of the control and A₁ is the absorbance of the extract.

In vitro inhibition of human erythrocyte hemolysis assay

Blood samples (80 ml) were collected from a good health patient in the laboratory of medical analysis at Rabta Hospital (Tunisia). The procedure described by Barreira et al. [12] was adopted to evaluate the inhibition of erythrocyte hemolysis by the different extracts of plum and prune (*Prunus domestica*).

Antibacterial Activity

Antibacterial effect of *Prunus domestica* extracts: MTT Assay

A modified protocol for the MTT reduction assay was developed by Wang et al. [13]. The antibacterial activity percent was calculated by the equation [14]:

$$\% \text{ antibacterial activity} = [(A_{\text{Blank}} - A_{\text{Sample}}) / A_{\text{Blank}}] \times 100$$

Antibacterial activity by diffusion method

The antibacterial activity of DMSO extracts of prune and plum was evaluated by the diffusion method. Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing solid and sterile LB agar medium (25 ml, pH 7). The DMSO extracts were deposited in wells perforated in LBA, previously inoculated with a sterile microbial suspension, in order to increase the volume (comparing with the method of diffusion in disks). All Petri dishes were incubated at 37 °C for 24 h, followed by the measurement of the zone diameter of the inhibition expressed in mm. Ampicillin was used as antibiotic reference product.

Modes of action

Effects on bacterial cell membranes

The inner membrane permeability assay is measuring the release of cytoplasmic galactosidase from *E. coli* and *Staphylococcus aureus* into the culture medium using *O*-nitrophenyl- β -D-galactoside (ONPG) as the substrate. 500 μ l of bacterial culture (in LB) were incubated with a volume of the DMSO extract of prune so as to have a concentration equal to the CMB (minimum bactericidal concentration). A centrifugation of 10 min at 13000 rpm was required to separate supernatant and the cell pellet. 100 μ l of ortho-nitrophenol galactoside (20 mM) were added to 950 μ l of A Buffer (phosphate buffer pH 6.8 50 mM, MgCl₂ 1 mM). 50 μ l of supernatant were added to the mix. An incubation of 10 min at 30°C was required to accelerate enzymatic reaction. 250 μ l of (Na₂CO₃ 1M, EDTA 10 mM) were added to stop the reaction. The amount of *o*-nitrophenol formed can be measured by determining the absorbance at 420 nm. One blank for each strain was also tested containing all test reagents, but with supernatant of *Escherichia coli* (the other blank with *Staphylococcus aureus*) without DMSO extract of prune. A calibration curve was plotted using ONP (ortho- nitrophenol) used as substrate for this reaction.

Effects on DNA

This test was performed using FastDNA kit extraction. The four pellets (blanks and samples) handling described in the β galactosidase assay were resuspended in 200 μ l of Nuclease Free Water. After extraction, DNAs obtained are revealed in agarose gel (0.8%) electrophoresis.

Data Analysis

All determinations were conducted in triplicates and results for each measured parameter were expressed as mean \pm SD. Quantitative differences was assessed by ANOVA procedure (at $P < 0.05$) followed by Duncan's multiple range test. Calculations were performed using the SAS v. 9.1.3 program.

RESULTS AND DISCUSSIONS

Assessment of antioxidant power

Polyphenols and flavonoids contents

Total phenolic content was expressed as Gallic acid equivalents (**Table 1**). The total phenolic contents were 18.46 ± 0.83 mg GAE/g fresh weight and 46.08 ± 2.47 mg GAE/g) fresh weight, respectively in plum and prune. This important difference may be explained by the concentration of bioactive compounds in prune (chlorogenic acid, neochlorogenic acid, caffeic acid, coumaric acid, rutin [15], and proanthocyanidin [16]).

Flavonoids content in prunes was significantly higher compared to flavonoids content in plums extract (**Table 1**). The total flavonoid content of prunes and plums were 37.33 ± 1.42 , $1.93 \pm$

0.32 mg equivalent Catechin/g fresh weight respectively. Consequently, flavonoid content in prune could be powerful antioxidant potential and may play a key role against environment stress [17].

Table 1: Total phenolic and flavonoids contents. A: Polyphenols contents of prune and plum methanolic extracts. Each value is expressed as a mean of three values ± standard deviation (n=3). B: Flavonoids contents of prune and plum methanolic extracts. Each value is expressed as a mean of three values ± standard deviation (n=3).

	Polyphenols (mg Gallic Acid Equivalent/g Fresh Matter)	Flavonoids (mg Catechin Equivalent/ g Fresh Matter)
Prune	46.08± 2.47 ^a	37.33 ± 1.42 ^a
Plum	18.46 ± 0.83 ^b	1.93 ± 0.32 ^b

In each column, values followed by different letter are significantly different (p <0.05).

Many researches showed that polyphenols contribute in antioxidant activity of prunes with only 23% of the total antioxidant activity [18], depending on their composition and their chemical structure [19] and the number of OH groups [20].

DPPH radical scavenging activity

The change of absorbance produced by reduced DPPH was used to evaluate the ability of the test compounds to act as free radical scavenger. **Figure 1** shows a decrease in the concentration of DPPH radical due to scavenging ability of our extracts. A difference between the inhibition profiles of both Trolox and methanolic extracts of the studied plant seems to be very clear. The methanolic extract of plum has a relatively low antiradical activity compared to that of Trolox, a standard antioxidant, which has the most pronounced inhibition at low concentration (IC₅₀ = 3.85 ± 0.02 µg / ml) . However, the methanolic extract of prune has a strong antioxidant activity which is similar to that of Trolox (IC₅₀ = 4.55 ± 0.05 µg / ml).

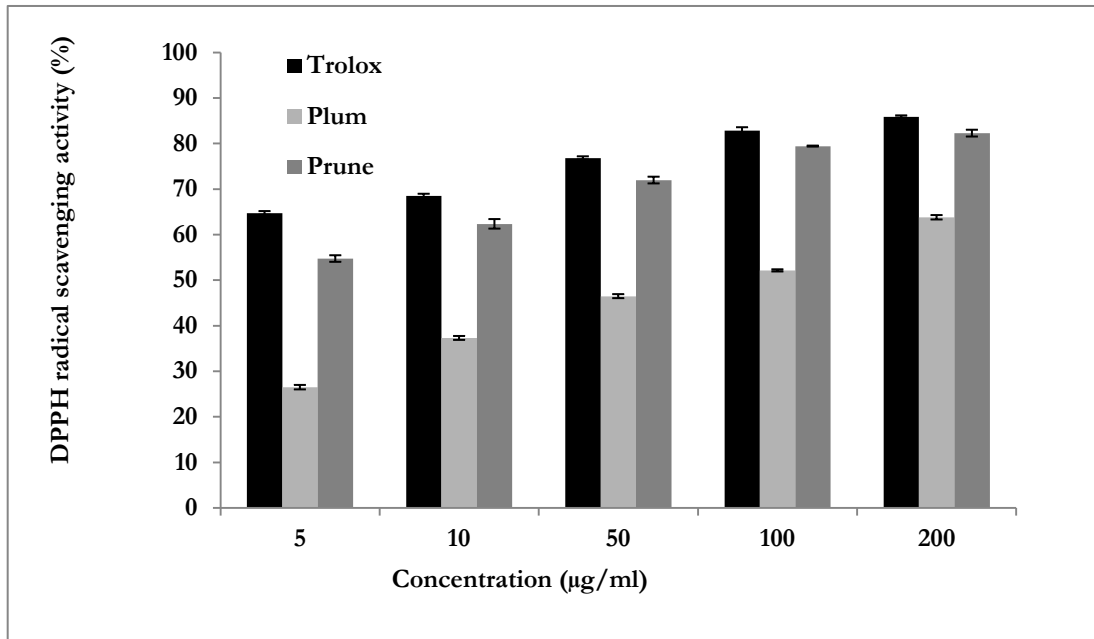


Fig.1: Scavenging activity (%) on DPPH radicals of prune and plum methanolic extracts. Each value is expressed as mean ± standard deviation (n=3).

Reducing power

The Fe^{3+} - Fe^{2+} transformation in the presence of our extracts of prune and plum was investigated. **Figure 2** demonstrates the dose-response curves for the reducing power of our extracts. Their reductive potential revealed a powerful relationship with concentration. Curves obtained show an increasing variation of the reducing power according to the concentration of extracts until 0.1 mg/ml. It is important here to note that this variation is weak comparing to EDTA which is used as a positive control because of its high capacity to reduce oxidized molecules.

It must be emphasized that the methanolic extract of prune exerted stronger reducing abilities than their methanolic extracts of plum. Prune extract has an important reducing power which may surpass the activity of many natural or synthetic standards antioxidants like EDTA.

Comparing with polyphenolic composition of our extract, we can suggest that there may be highly significant positive relationship between total phenols and antioxidant activity in many plant species [21].

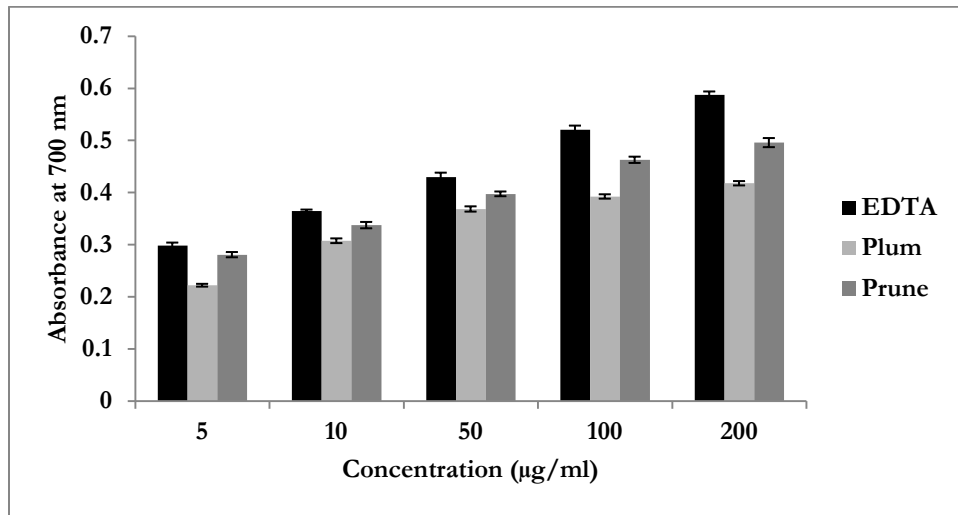


Fig.2: Reducing power of prune and plum methanolic extracts. The absorbance values were directly plotted as the mean of replicate absorbance values \pm standard deviation (n= 3).

Iron chelating effect

Curves obtained show an interesting chelating activity in prune and plum, changing in a concentration dependent manner (**Fig. 3**). Metal chelating activity is significant as it reduces the concentration of the catalyzing transition metal in lipid peroxidation through the Fenton reaction [22]. In the present assay, all the extracts exhibited high metal chelating activities. Many studies have reported that metal chelating potency plays a minor role in the overall antioxidant activities of some polyphenols [23].

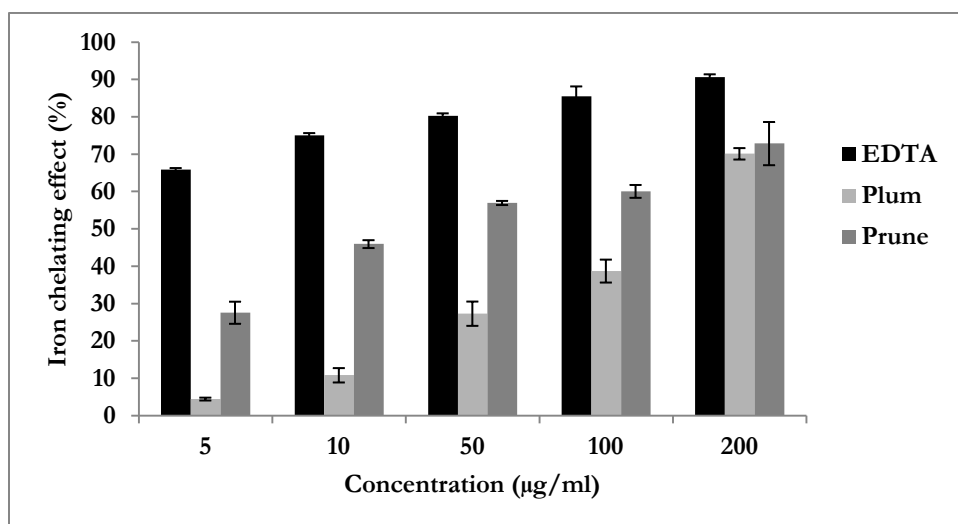


Fig.3: Iron chelating effect in different methanolic extracts (plum and prune). The absorbance values were directly plotted as the mean of replicate absorbance values \pm standard deviation (n= 3).

The chelating effect of prune and plum extracts on ferrous ions increased with increasing concentrations (Fig. 3). At a concentration of 10 $\mu\text{g/ml}$, prune and plum extracts exhibit chelating effect of $45.95 \pm 1.05 \%$ and $10.82 \pm 1.92 \%$, respectively. The IC_{50} values of prune extract was calculated, it is estimated to be $19 \pm 1.27 \mu\text{g/ml}$. The present work suggests that methanolic extracts of prune and plum exhibit good chelating activity ferrous ions.

The antioxidant power of the methanolic extract of prune was evaluated by three methods and correlations between these methods were equally good. These results are not surprising since previous reports on antioxidant capacity of fruits such as strawberry indicate that vitamin C is not the major antioxidant in fruits and polyphenols are mainly responsible for the observed activity [24].

In vitro inhibition of human erythrocyte hemolysis assay

The *in vitro* oxidative hemolysis of human erythrocytes was used as a model to study the free radical-induced damage of biological membranes and the protective effect of extracts from fruits of *Prunus domestica*. Figure 4 shows the value of the erythrocytes hemolysis inhibitory percent. The DMSO extract of prune showed the high protective activity against the erythrocytes hemolysis ($31.35 \pm 0.28 \%$) at a concentration of 400 $\mu\text{g/ml}$ comparing with DMSO plum extract. The amount of hemolysis was reduced in a dose-dependent manner. However, prune and plum extracts could not reach an inhibition of 50 % of erythrocyte hemolysis until a concentration of 400 $\mu\text{g/ml}$.

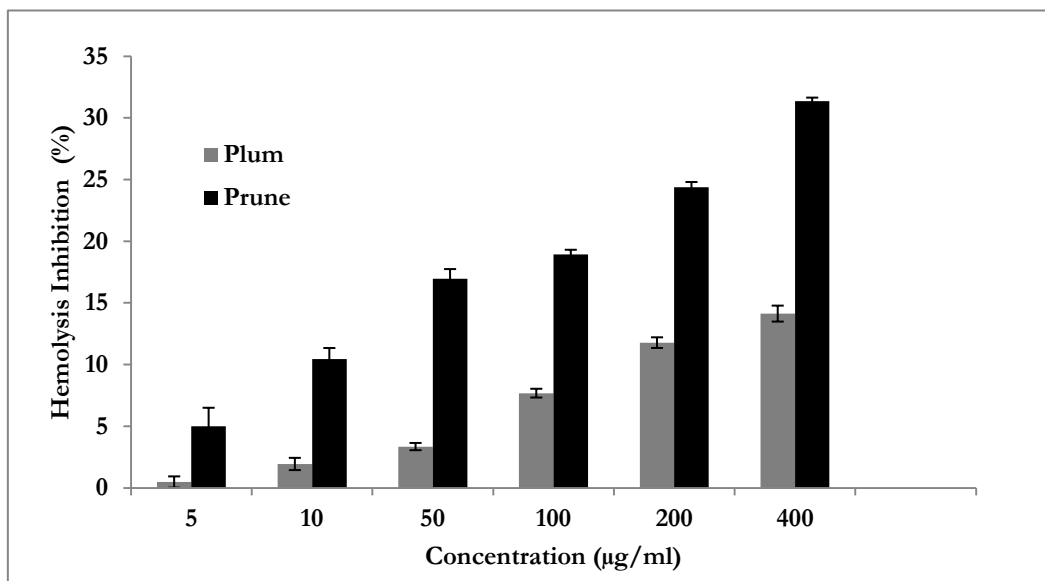


Fig.4: Inhibition of human erythrocyte hemolysis by DMSO prune and plum extracts. The absorbance values were directly plotted as the mean of replicate absorbance values \pm standard deviation ($n=3$).

These results can be explained by the fact that our extracts contain biomolecules able to inhibit the serie of reactions initiated by hydrogen peroxide when it crosses the red blood cell membrane, protecting then red blood cells against lysis [25]. However, the *in vitro* effects of a phytochemical in the inhibition of erythrocytes hemolysis should be appreciated, since the *in vivo* effects may be complicated by many of chemical, physical and physiological factors [26].

Antibacterial potential study

The cleavage of the tetrazolium salt MTT into a blue colored product (formazan) by the mitochondrial enzyme succinate-dehydrogenase is potentially very useful for assaying cell survival and proliferation [27]. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of cells present. **Table 2** shows a variation in the response of our extracts to inhibit bacterial strains. At a concentration of 10µg/ml, prune extract shows an inhibition of *Staphylococcus aureus* growth with 65.88 ± 1.23 %. For plum extract, the best inhibition activity at the concentration of 10 µg/ml was against *Escherchia coli* growth. This activity may be due to the tannins content in plum [28]. In fact, many biological activities and antibacterial promoting effects have been reported for plant tannins, and their investigation is now increasingly relevant [29].

Table 2: Antibacterial activity of DMSO prune and plum extracts (% of inhibition) against bacterial strains. Concentration of all plant extracts were 10 µg/ml. Each value is expressed as a mean of three values ± standard deviation (n=3).

Strains	<i>Staphylococcus aureus</i>	<i>E. coli</i>	<i>Bacillus subtilis</i>	<i>Klebsiella</i>	<i>Enterobacter</i>
Plum	16.07 ± 1.14 ^g	40.56 ± 0.13 ^d	25.25 ± 0.87 ⁱ	0.84 ± 0.37 ^j	34.36 ± 0.56 ^e
Prune	65.88 ± 1.23 ^a	63.66 ± 0.69 ^b	32.25 ± 0.19 ^f	12.61 ± 0.29 ^h	55.39 ± 0.3 ^c

In each column, values followed by different letter are significantly different (p <0.05).

Results from the antibacterial assay using diffusion method are summarized in **Table 3**. Both Gram-positive and Gram-negative bacteria were affected approximately equally by DMSO extracts of *Prunus domestica*, especially by DMSO prune extract (**Fig.5**). They show that DMSO extract from prune exhibit an interesting antibacterial effect by producing a zone diameter inhibition from 10 to 27 mm depending to the susceptibility of the tested organism. These results confirm those obtained by MTT assay. However, The culture medium, the technique of testing, the age of the plant, the state of plant material used (dried or fresh), the quantity of the extract used for the test and the isolation technique are some factors implicated in the great variation of the activity [30].

Table 3: Antibacterial activity of DMSO prune and plum extracts measured as zone of inhibition (mm)

	Ampicillin	10 µg		50 µg		100 µg	
		Plum	Prune	Plum	Prune	Plum	Prune
<i>B. subtilis</i>	31	-	12	4	15	11	17
<i>Enterobacter</i>	19	-	10	-	16	-	21
<i>S. aureus</i>	46	-	25	7	25	12	27
<i>Klebsiella</i>	16	-	-	-	-	-	-
<i>E. coli</i>	17	-	-	-	15	-	24

These findings are insufficient for the study of the antibacterial activity. It presents a simple screening which remains general. Researches in the medical field, especially microbiology, for the fight against bacterial infections require a strategy that focuses on "how", in order to follow the modes of action, and especially, at what level of the bacterial life cycle acts our antibiotics. Therefore, we are interested in the rest of the current study to guess the mode of action of antibiotics presents in DMSO extracts of plum and prune. Such study needs to calculate the CMB (Minimum Bactericidal Concentration), essential to ensure a complete inhibition (100%) of studied organisms. This calculation was done by a mathematical method using results found by MTT assay. In fact, curves plotted with the variation of antibacterial activity (%) depending on the concentration of extract (µg/ml) were converted into the variation of antibacterial activity (%) depending on the Log of extract concentration. Straights with an equation $y = ax + b$ were obtained. Replacing y by 100 (total inhibition), the CMB value is deduced to be: $CMB = e^{(100-b)/a}$.

The CMBs of plum and prune extracts for the five bacterial strains studied (10 CMB values) were calculated. However, these values are so high (in kilograms) except for those of prune against *Escherchia coli* (49.56 mg/ml) and *Staphylococcus aureus* (6.96 mg/ml). Thus, we are interested to study the mode of action of our extracts' antibiotics with prune DMSO extract and two bacterial strains: *Escherchia coli* and *Staphylococcus aureus*.

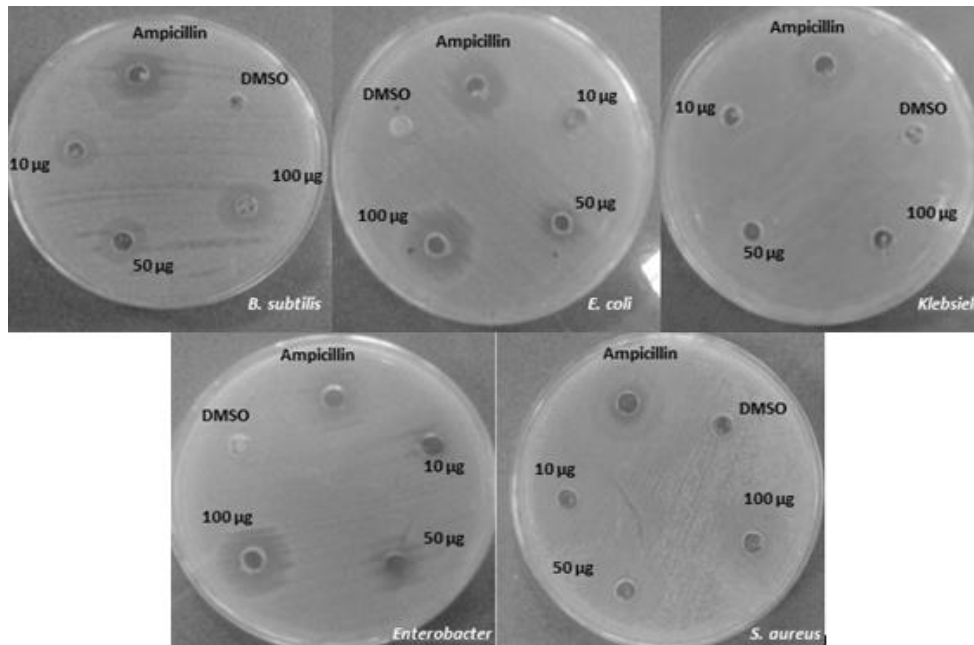


Fig.5: Revelation of the antibacterial activity of DMSO prune extracts on Petri dishes.

Proposed modes of action

Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principle mode of action. The most common modes of action are interference with the cell membrane and cell wall and interaction with nucleic acids [31].

Effects on bacterial cell membrane

Some bacteria are protected by a wall, which is formed when the bacterium divides. This wall has a layer of peptidoglycan. There is synthetic machinery which produces the components of the wall and which is composed of enzymes and transport systems carrying components on the cell surface [32]. There is a set of antibiotics that block various stages of this machinery. Blocking the synthesis of the wall weakens strong outer shell of the bacteria, which become highly susceptible to external stress causing cell lysis. Results of beta galactosidase assay are shown in **Figure 6.A**. It is so clear in these histograms that DMSO extract of prune is able to destroy the bacteria wall and acced to the cytoplasm. According to these histograms, the beta-galactosidase activity increases each time we add our extract. For *Staphylococcus aureus*, the activity increases four times in the presence of the extract (from 0.09 ± 0.016 U/ml without extract to 0.4 ± 0.051 U/ml in the presence of extract). Researches carried out by McManus [33] have shown that antibiotics which inhibit the synthesis of the bacterial membrane are β -lactams by interfering with enzymes required for the synthesis of the peptidoglycan layer. In fact, β -lactams act primarily as inhibitors of the synthesis of the cell wall; by blocking the action of transpeptidases (this mechanism is called "suicide inhibition"). In Gram-positive bacteria,

these enzymes are located in the periplasmic space. In Gram-negative bacteria, β -lactams have to cross the outer membrane. Perturbations induced by the β -lactams in cell wall formation explain the inhibition of growth of the bacteria, but the bactericidal effect results from indirect mechanisms like the activation of autolytic enzymes. It is important to note here that beta lactams are active only against highly dividing bacteria [34].

Effects on DNA

Reproduction of bacteria is achieved by a simple binary division of a cell in active growth stage with an equal sharing of genetic material to produce two identical cells which acquire all structures and properties of parent cell. This mechanism involves the replication of the bacterial chromosome, the elongation and the cleavage of the cell body. The antibacterial effect on DNA replication affects essentially the formation of the double-strand DNA.

Results are shown in **figure 6.B**. The effect of our extract on *Staphylococcus aureus* DNA (lane 2) seems to be so important (Total degradation of DNA) comparing with that on *Escherchia coli* DNA (lane 4). These results suggest that our extract contains compounds able to disturb DNA synthesis. These compounds may be the fluoroquinolones, known by its antibacterial effect, by inhibiting the activity of topoisomerases, enzymes responsible for the supercoiling of DNA (DNA gyrase) and relaxation of supercoiled DNA (topoisomerases IV). Both enzymes have a similar mode, which implies the binding of DNA to the enzyme, the cleavage of the DNA, the passage of the DNA segment through the DNA gate, and finally, the resealing of the DNA break and the release from the enzyme [35]. The main target of fluoroquinolones is DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria.

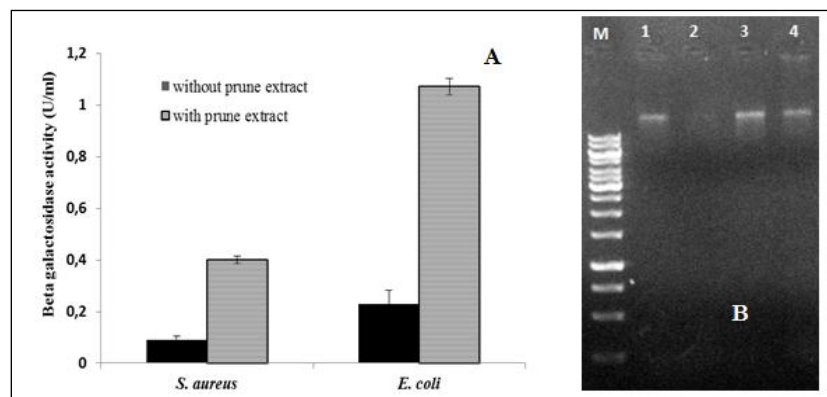


Fig.6: A. Beta galactosidase activity (U/ml). B. DNA damage caused by Prune DMSO extract against *Staphylococcus aureus* and *Escherchia coli* revealed by Agarose gel electrophoresis (0.8%). (M: 1 kb ladder, Lane 1: *Staphylococcus aureus* without Prune extract, Lane 2: *Staphylococcus aureus* with Prune extract, Lane 3: *Escherchia coli* without Prune extract, Lane 4: *Escherchia coli* with Prune extract).

CONCLUSION

In conclusion, this current study presents new data on the evaluation of antioxidant activity of fresh and dried *Prunus domestica L.* extracts. This work focused also on the evaluation of the bioactivity of this plant against cells as bacteria and erythrocytes. In fact, in vitro erythrocytes lysis inhibition test was carried out and results indicate that *Prunus domestica* extracts exhibit a good antihemolytic power. The results of antibacterial activity test indicate clearly that this effect varies with the plant material and the bacteria strain used. This work shows a potent antibacterial and antioxidant properties along with therapeutic potential which can play an important role in drug development and health supplement.

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