ANTIMICROBIAL ACTIVITY OF POLYPHENOL EXTRACTS FROM ALGERIAN *FICUS CARICA* DRIED FRUIT.

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Abstract

In vitro antimicrobial and chemical properties of methanol, acetone, Petroleum ether and aqueous extract of dried figs against 9 strains resistant human pathogens were evaluated. An Algerian variety fig was extracted with water, methanol, Petroleum ether and 80% acetone. Preliminary phytochemical analysis of extracts revealed the presence of antimicrobial compounds such as tannins, flavonoids and coumarins, The total amount of polyphenols (milligrams of gallic acid equivalents (GAE) per 100 g dried fruit) in fig fruit extracts was determined by using the Folin-Ciocalteu assay, Tannins were determined by precipitation with casein or hide powder and flavonoids by the aluminium trichloride method. The antimicrobial activity of the extracts was evaluated and based respectively on the inhibition zone using the disc-diffusion assay, minimal inhibition concentration (MIC), minimum bactericidal concentration (MBC) assay. Results showed that the phenolic compounds and flavonoids were abundant in acetone and Petroleum ether extract when compared to other extracts. The four extracts possessed antibacterial activity against tested Grampositive and Gram-negative bacteria particularly Petroleum ether their broad spectrums may be due to the presence of coumarins and flavonoids. Candida albicans was more susceptible to all figs extracts.

Keywords: Ficus carica (dried figs), Antimicrobial activity, MIC/MBC, "Taamriout" Algerian variety.

1. Introduction

The increasing frequency of antibiotic-resistant bacterial infections poses an alarming dilemma to healthcare today. Antibiotic resistant "superbugs" threaten public health worldwide and are associated with rising numbers of patients suffering from serious infection and even death (Coast et al., 1996). There is a clear need for a faster route to the discovery and development of new antimicrobial compounds. One possible solution to this problem can be nutritional approach, Natural products such as herbs, fruits and vegetables which contain antioxidant and antimicrobial properties such as phenolics, include flavonoids, phenolic acids and tannins (Klimczak et al.,2007) become popular in recent years due to public awareness and increasing interest among consumers and scientific community (Thaipong et al., 2006). *Ficus carica* L.

(fig) belongs to the mulberry Moraceae family which is one of the oldest fruits in the world (Vinson J.A, 1999). They are widely consumed in Algeria fresh, either peeled or not. Fresh fruits naturally have a short, post-harvest life of 7-10 days Figs are also very popular as dried fruit, since drying prolongs their storability.

This type of diet is considered one of the healthiest and is associated with longevity (Trichopoulou, and al., 2006). Previous reports concerning the nutrient composition of dried figs have indicated that it has the best nutrient score among the dried fruit, being an important source of minerals and vitamins (USDA, 2002). The presence of phytosterols (433 mg/100 g dry basis) has also been reported in fig fruit (Jeong and Lachance, 2001). The fresh and dried figs also present relatively high amounts of crude fiber (5.5%, w/w) and polyphenols (Vinson, 1999; Vinson et al., 2005). Some recent works have reported that fig antioxidants can protect lipoproteins in plasma from oxidation and produce a significant increase in plasma antioxidant capacity for 4 h after consumption (Vinson et al., 2005). Also, Solomon et al. (2006) showed that the higher the polyphenols contents, especially anthocyanins, in fig fruit, the higher was their antioxidant activity. In this study, *Ficus carica* dried figs was chosen for its abundance of phenols, coumarins, tannins and flavonoids, which are effective on bacteria through compounds produced such as resveratrol, psoralen and bergapten.

These compounds have demonstrated antibacterial activity and could be used commercially as a means of

phytopathogenic bacteria control (Ulate-Rodriguez et al., 1997; Salameh et al., 2004; Zao et al., 2005) Antimacterial activities, total phenolics, total flavonoids, total tanins of methanol, acetone, Petroleum ether extracts of dried figs of a white Algerian Variety «Taamriout» wereinvestigated.

2. Material and methods

2.1. Samples of dried figs

Samples of Algerian Variety (Taamriout) dried figs were obtained from ITAF (Technical Institute for Fruit Trees) of Mohammadia (Wilaya of Mascara, Algeria).

Figure 1: Representation of the *"Taamriout"* Algerian variety dried and fresh figs

2.2. Physicochemical analysis of dried figs.

Dried fruits were analyzed for water and dry matter contents (%), pH value, the total content of dry matter was determined by drying at 105°C and weighing by means of an analytical scale Technica. The pH values were obtained with a digital pH-meter after calibration with pH 4.0 and 7.0 standard buffers.

2.3. Extraction of polyphenols

Methanolic, Acetone and Petroleum ether Extraction : The samples were prepared according to the method described by Escarpa and Gonzalez (1998) with little modification: 100 g of dried fruit were homogenized and extracted with solvent containing 1% 2,6-ditert-butyl-4-methylphenol (BHT), using an ultrasonic bath. Samples were extracted with 250 ml of solvent for 1 h, 100 ml for 30 min, and finally 50 ml for 30 min. The three extraction fractions were combined into a final volume of 500 ml and filtered through a 0.25 µm membrane filter and evaporated prior to their utilization. BHT was added to the samples to prevent oxidation during the extraction.

2.3.1. Aqueous extraction

100 g of dried fruit was added to distilled water, homogenized and shaking for 24 h, at room temperature. The extracts were filtered through Whatman No.1 filter paper then centrifuged at 5000g for 10 min and the supernatant was concentrated under reduced pressure at 40°C for 3 hr using a rotary evaporator prior to their utilization.

2.3.2. Phytochemical screening

The methanolic, acetone, Petroleum ether and aqueous extract of dried figs extracts were separately subjected to preliminary phytochemical tests using standard techniques (Evans, 1996, Harborne, J. B. 1998). The usual reagents of characterization that we used, allowed us to put in evidence of the groups of following chemical compounds:

polyphenols $FeCl_3$,%), flavonoids NH_4OH, Chinoda's test), tannins ($FeCl_3$, 1%.) and ammonia-UV test for coumarins.

2.4. Quantification of polyphenols

The total amount of polyphenols (milligrams of gallic acid equivalents (GAE) per 100 g dried fruit) in fig fruit extracts was determined by using the Folin-Ciocalteu assay (Marinova et al., 2005, Balestra G.M, 2009). For each replicate, 1 ml of extract was added to 9 ml of sterile distilledwater (SDW), and 1 ml of gallic acid standard solution (20, 40, 60, 80 and 100 mg/ml) was similarly added to 9 ml of SDW; as control 1 ml of SDWwas used. The Folin-Ciocalteu's phenol reagent (1 ml) was added to the extract solution and to

each standard solution. After 5 min, 10 ml of 7% Na_2CO_3 solution was added to each solution. Then, 4 ml of SDW was added to each one to reach the final volume of 25 ml. After incubation for 90 min at room temperature, the absorbance was determined at 750 nm with a UV spectrophotometer. All samples were analysed in triplicate.

2.5. Quantification of flavonoids

The determination of flavonoids was performed according to the colorimetric assay of (Lamaison and Carnet, 1990 Huang and al., 2004). A calibration curve was prepared with catechol and the results were expressed as mg catechol equivalents (CEQ)/ 100 g dried fruit). An aliquot of 1mL of each sample and standard solution was added individually to equal volumes of solution of 2% AlCl3, 6H2O mixed evenly and allowed to stand at room temperature for 10 minutes. The absorbance was then read at 367 nm.

2.6. Quantification of tannin

Total tannin concentrations were determined by the casein precipitation method (Seigler D.S. and al., 1986, Monteiro, J.M., 2006), which consists of adding 1 g of powdered casein to 6ml samples of extracts diluted with 12 ml of distilled water. Theresulting solution was agitated for three hours at room temperature (25 °C), after which it was filtered through 9 cm Whatman filter paper and the filtrate adjusted to25 ml final volume. Aliquots (8 ml - 12 ml) of this solution where then tested for residual phenolic compounds using the Folin-Ciocalteau method. The quantity of tannins corresponds to the difference in the absorption of these casein precipitated samples and those obtained in the total phenol analysis. The quantity of total phenols and tannins are expressed per 100 g dried fruit.

2.7. Antimicrobial assay

2.7.1. Microorganisms used

The microorganisms had three origins, Algerian Pasteur Institute, the laboratory of microbiology, hospital Yessad Khaled of Mascara and the laboratory of microbiology, Mascara University, Algeria. From eight tested bacteria three were Gram-positive bacteria, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* (ATCC29212) and *Bacillus subtilis* sp. *spizizenii* ATCC 6633 and six Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC27853), Pseudomonas aeruginosa ATCC 10145, *Escherichia coli* (ATCC25922), *Enterobacter cloacae* (ATCC13047), *Salmonella enterica* sp. *Heindelberg* (ATCC 8326), *Klebsiella pneumonia* (Clinical isolated), and yeast *Candida albicans* (Clinical isolated). Test solutions were screened by culturing a single colony in 2 ml nutrient sterile broth for bacteria and Saboraud broth for Candida albicans for 24 h, after which the optical density (OD) at 600 nm for each liquid culture was determined, to obtained microbial

suspensions (10^5 CFU/ ml) and the bacteria were at the start of the log phase when the test commenced such as Mc Farland method.

Antimicrobial activity assay

Antimicrobial activity was determined against ten microbial pathogens by the agar disc diffusion assay (NCCLS (National Committee for Clinical Laboratory Standards), 2005). The organic extracts were dissolved in Dimethyl Sulfoxide (DMSO) and the aqueous extract in distilled water then antimicrobial effect of extracts were tested. Petri dishes (measuring 90 mm each side) containing 20 mL of Mueller Hinton agar. At the same time, 6 mm diameter sterile Whatman Antibiotic disc were placed on the surface of the inoculated agar plates, and then appropriate quantity (20µl) of the extracts was applied onto the discs.

The plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the zone of growth inhibition surrounding the discs.

Standard discs of the antibiotic Gentamycin (15 µg) and Amoxicilline (25µg), kanamycine (15µg) and Erythromycine (15UI) served as the positive antibacterial controls. Negative controls were done using paper discs loaded with 20 ml of DMSO and water. After that, the diameter of inhibition zone was 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 (Koshy Philip et al., 2009).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) for bacterial growth was determined by a serial dilution technique using 96-well microtitre plates. 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 dimethyl sulfoxide (DMSO). The solution was subsequently diluted for 10 fold with Mueller Hinton broth. 100 µl from broth bacterium or yeast solutions and dilutions were transferred into microtitration plates and incubated for 24h at 37 °C. The positive control contained 100 µl of bacterium solution plus 100 µl 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 24h 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 clear well. All extracts were tested in triplicates (Elof J. N., 1998).

Minimum bactericidal concentration (MBC)

The MBC of the extracts was determined by Spencer A.L.R. and Spencer J.F.T., 2004 method. Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared PCA (Plate Count Agar), and later incubated at 37°C for 48 h. The MBC was taken as the concentration of the extract that did not show any growth on a new set of agar plates. Finally CMB/CMI report is calculated to know if the extract has bactericidal (CMB/CMI<4) or bacteriostatic (CMB/CMI>4) affect according to Marmonier A. A., 1990 method.

3. Statistical analysis

Results were expressed as the mean ± DS, the statistical analyses were performed by a one-way ANOVA and the Student's t-test to show variations in the various experimental. Differences are considered significant when $p<0.05$.

4.Results

4.1. Physicochemical analysis of dried figs.

Results of physicochemical analysis of dried figs, are according to the CEE-ONU DF 14 dried Figs norm concerning the Marketing and Commercial Quality Control, the studied variety can be classified in the "category; Extra" (number of fruits in the kilogramme should not exceed 65). They are almost uniform from the point of view of the size and free from defects (Fig1), with a conditioning in conformity with the norms.

Table 1: Physicochemical of "*Taamriout"* dried figs variety

	unitary weight	**units / kg**	**Dry matter**	**pH**
Dried figs "*taamriout* " variety	16.1 (±0.1)	62 (± 0.5)	81 (± 1,3)	5.05 (± 0.5)

Phytochemical analysis

The phytochemical analysis of *Ficuscarica*extracts showed the presence of different groups of secondary metabolites viz., coumarins, flavonoids, terpenoids, and tanins, which are of medicinal importance. The tanins groups and flavonoids were rich in ME, AcE and AqE extract and coumarins in PEE extract.

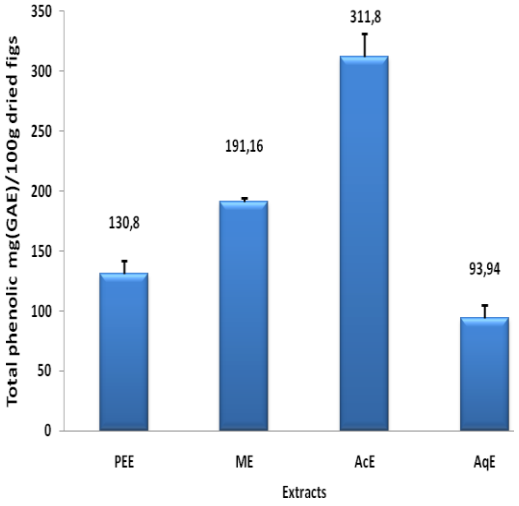
Table 2: Preliminary phytochemical screening of *Ficuscarica* Extracts.

Extracts / Compound	**PEE**	**ME**	**AcE**	**AqE**
Flavonoïdes	+/-	+++	+++	+++
Condensing tannins	-	+++	+++	++++
Hydrolysables tannins	-	-	-	-
Coumarins	+++	-	-	-

PEE Petroleum ether extract, :**ME** methanolic extract, **AcE** Acetonic extract, **AqE** aqueous extract

Quantification of polyphenols

The amounts of total phenolic compounds in dried figs extracts are shown in Fig 2. The Acetonic extract (AcE) contained a significantly higher amount of total phenolics (311,8 ±19,22) than methanolic and Petroleum ether extract(191,16 ± 3,19 and 130.80 ± 11,12 mg GAE/100g, respectively). On the other hand, total phenolics in aqueous extract was present in lesser extent (93, 94 ± 11.1 mg GAE/100g dried figs)



PEE: Petroleum ether extract, **ME**: methanolic extract, **AcE:** Acetonic extract, **AqE:** aqueous extract

Figure 2: Total phenolic content as gallic acid equivalent (GAE mg/100g) in Ficuscaricadried fruitextracts, each value in the table is represented as mean ± DS (n=3).

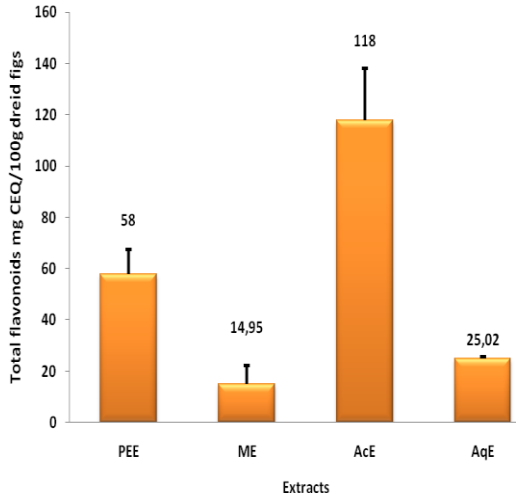
acetonic extract (118mg CEQ ± 20,01) and followed by PEE (58mg ± 9,56), thereafter comes EAq (25.02mg CEQ ± 0.56) followed by EMe (14.95± 7.07 mg).

Quantification of tannins

The total tannins results show the high content of these molecules in the polar extracts, methanolic extract represent the richest extract (158.9mg ±5,33), followed by the aqueous extract (75.96mg ±9,71) and acetonic extract (74mg ± 14,46) (Fig 4).

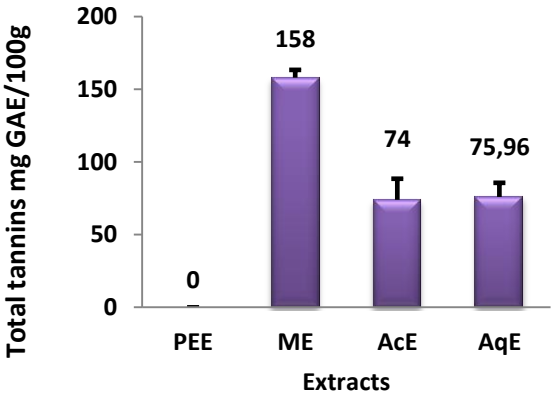
Antimicrobial activity

Antimicrobial activity as summarized in Table 3, we found that the Ficus carica extracts showed antimicrobial activity against most of the tested microbes. The Petroleum ether extract of



PEE: Petroleum ether extract, **ME**: methanolic extract, **AcE:** Acetonic extract, **AqE:** aqueous extract

Figure 2: Total flavonoids content as catechol equivalent (CEQ mg/100g) in *Ficus carica* dried fruit extracts, each value in the table is represented as mean ± DS (n=3).



PEE: Petroleum ether extract, **ME**: methanolic extract, **AcE:** Acetonic extract, **AqE:** aqueous extract

Figure 4 : Total tannins content as gallic acid equivalent (GAE mg/100g) in *Ficuscarica*dried fruit extracts, Each value in the table is represented as mean ± DS (n=3).

F. carica fruit exhibited strong activities against B. subtilis subsp. *Spizizenii* zone diameters; (20.33± 2,3mm) and C. albicans (18±0.30mm), and moderate antibacterial activity against the other bacteria, with exception of K. pneumoniae we registered no effect (Fig 5).

Table 3: Antimicrobial activity of different extracts of *Ficuscarica*dried fruitand standards antibiotic using disc diffusion method (inhibition zones, mm), each value in the table is represented as mean ± DS (n=3).

Extracts/ ATB / Bacteria	**PEE**	**ME**	**AcE**	**AqE**	**AMO**	**GEN**	**KAN**	**ERY**	**DMSO**
E.coli	13.00± 0.4	12.00±0, 01	**0**	0	25± 0,2	28	19	0	0
E. cloacae	12.16± 0.15	11.00±0. 20	10.23 ±0.21	0	0	0	15± 0	0	0
P.aeruginosa ATCC 27853	10.33± 1.52	11.16±1. 02	13.89 ±0.45	18.43 ±1.0	0	24± 0,09	19± 0,3	0	0
P.aeruginosa ATCC 10145	13.70± 0.36	12.23±0. 20	14.56 ±0.14	10.93 ±0.4	0	23± 2,2	20± 0	0	0
K.pneumoniae	0	24.43±0. 41	11.50 ±0.50	0	15	23± 0,5	20± 0,1	0	0
S.enterica subsp.*heindelberg*	10.86± 0.15	0	0	0	18± 1,3	25± 0,08	17± 0	0	0
E. faecalis	13.7±0. 25	09± 0,02	10.76 ±03	12.86 ±0.8	22± 2,1	23± 0,8	18± 1,4	14± 0,8	0
S. aureus	13±0,5	10± 00	09.96 ±0.25	0	0	26± 0	23± 0	27± 0	0
B. subtilis subsp. *spizizenii*	20.33± 2,3	11.5 ±0.4 4	10±00	12±0, 22	24± 0, 6	20± 0	26± 0, 7	12± 0,3	0
C.albicans	18±0.3 0	24.9 ±0.3 6	24.83 ±0.15	23±00	-	-	-	-	0

Concerning, the methanolic extract, it possessed significantly higher antimicrobial activity against K. pneumoniae, zone diameters (24.43 ± 0.41mm) and C. albicans (24.90 ± 0.36 mm). While other bacteria appeared less sensitive. On the other hand this extracts did not show any inhibition against S.enterica subsp. Heindelberg. Except for its negative effect on E.coli and S.enterica, acetonic ory activity C.albicans zone diameters (24.83 ± 0.15 mm) and moderate antibacterial activity on the rest seven bacteria, zone diameters situated between (9.96 ± 0.25 mm) and (14.56 ± 0.14 mm). Aqueous extract showed inhibition against; E. faecalis, P. aeruginosa ATCC 27853, P.aeruginosa ATCC 10145, C.albicans and B. subtilis with maximum inhibition against C.albicans (23±0.0mm) and

mini-mum against P.aeruginosa ATCC 10145 (10.93 ± 0.4 mm) as indicated in table 3.

Minimum inhibitory concentration (MIC)

Table 4 shows the MIC values of the extracts for bacteria resulted with antimicrobial activity, minimum inhibitory concentrations of different extracts against the tested organisms are as follows:

Petroleum ether extracts exhibited MIC ranging from 16 µg/ml (S. aureus and B subtilis) to 257,5 µg/ml (E. cloacae) against the tested organisms. In case of methanolic extracts inhibited the growth of the bacteria used in the study at concentrations ranging from 44,6µg/ml to 357,1µg/ml. it inhibited the growth of both K.pneumoniae, B. subtilis and C.albicans at 44,6g/ml; whereas the S.enterica MIC did not determined (MIC=357,1 µg/ml) Acetonic extract inhibited P. aeruginosa ATCC 27853, P.aeruginosa ATCC 10145 and C.albicans at concentrations of 48,5µg/ml, but did not inhibit the growth of E.coli, E. cloacae K.pneumoniae and S.enterica at any of the concentrations tested. Aqueous extract was effective against six of the tested organisms but the concentrations were were high, it did not inhibit the growth of E. coli , K. pneumoniae and S.enterica.

Minimum bactericidal concentration (MBC)

The four extracts PEE, ME, AcE and AqE carried a extract, rich in coumarins and methanolic extract rich bactericidal or bacteriostatic against the tested organisms (Table 5), in particular the petroleum ether extract, rich in coumarins and methanolic extract rich in flavonoïdes.

Discussion

Phenolic compounds have been extensively investigated since the past 30 years, these substances are present in a variety of plants utilized as important components Mediterranean diet . The health benefits associated with the consumption of fruits and vegetables have been partly attributed to the phenolic content (Lampila P,.and al., 2008). Some phenolic compounds, with reported pharmaco-logical properties have already been isolated from fig, namely furanocoumarins like psoralen and bergapten (Innocenti, G et al 1982), flavonoids like rutin, quercetin, and luteolin, phenolic acids like chlorogenic, cinnamic acid (Tsanova-Savova S. et al., 2005, del Caro et al., 2008, Harnly J. M.et al., 2006), also phytosterols like campesterol and stigmasterol (Jeong W. S. and Lachance P. A., 2001). In this study, Phytochemical investigation of petrol ether, methanol, acetonic and aqueous extracts of F. carica fruit reveled differences in their phyto- constituents. The tanins groups and

Table 4 :MBC/MIC report values, **PEE;** Petroleum ether extract, **ME** ; methanolic extract, **AcE;**Acetonic extract.

Extracts / Micro-organism	**PEE** MBC/MIC	**ME** MBC/MIC	**AcE** MBC/MIC	**AqE** MBC/MIC
E.coli	257 ,5/64,3	178,5/98,2	**>388/>388**	977,5/977, 5
E. cloacae	257,5/257, 5	178,5/178, 5	**>388/>388**	488,7/488, 7
P.aeruginosa ATCC 27853	64,3/64,3	98,2/98,2	97/48,5	977,5/122
P.aeruginosa ATCC 10145	64,3/64,3	178,5/98,2	>388/48,5	ND
K.pneumoniae	128,7/128, 7	178,5/44,6	>388/>388	977,5/977, 5
S.enterica sp.*heindelberg*	357/128,7	>357/>357 ,1	>388/>388	977,5/977, 5
E. faecalis	257,5/64,3	357,5/98,2	194,1/97	1955/488,7
S. aureus	64,3/16	178,5/178, 5	194,1/194, 1	488,7/488, 7
B. subtilis sp. *spizizenii*	128,7/16	98,2/44,6	194,1/97	1955/244,3
C.albicans	64,3/64,3	98,2/44,6	97/48,5	488,7/244, 3

AqE; aqueous extract, **MIC** ; Minimum inhibitory concentration, **MBC**; Minimum bactericidal concentration, **ND;** not determined

Khan and Al, 2005], and the resveratrol, psoralen and the bergapten largely present in figs showed an antibacterial activity [Ulate-Rodriguez and Al, 1997; Salameh and Al, 2004; Zao and Al, 2005]. The results of antibacterial studies particularly of the petroleum ether extract could be attributed to the coumarin ring as such natural products are known since the 1950 to exert their effects by inhibition ofbacterial nucleic acid synthesis (Rosselli, S. and al., 2007). It has been proved that the addition of a prenyl group to the furanocoumarin skeleton results in an increase in lipophilicity of the molecule, facilitating its passage though the thick bacterial membrane to its target (Stavri, M.; Gibbons, S.,2005).

Table 5: bactericidal or b**acteriostatic** effect of extracts by different solvents,

Extracts / Bacteria	**PEE**	**ME**	**AcE**	**AqE**
E.coli	bacteriost	bactericid	**ND**	ND
E. cloacae	ND	bactericid	**ND**	bactericid
P.aeruginosa ATCC 27853	bactericid	bactericid	bactericid	bacteriost
P.aeruginosa ATCC 10145	bactericid	bactericid	ND	ND
K.pneumoniae	bacterici	bacteriost	ND	ND
S.enterica	ND	ND	ND	ND
E. faecalis	bacteriost	bactericid	bactericid	bacteriost
S. aureus	bacteriost	bactericid	bactericid	bactericid
B. subtilis subsp. *spizizenii*	bacteriost	bactericid	bactericid	bacteriost
C.albicans	bactericid	bactericid	bactericid	bactericid

PEE; Petroleum ether extract, **ME** ; methanolic extract, **AcE;** Acetonic extract, **AqE;** aqueous extract, **ND;** not determined

Finally, these findings may provide the Algerian traditional uses of this fruit in pulmonary and inflectional diseases.

Conclusion

As far as we know, this was the first study concerning the antimicrobial activity and total phenolic content of extract of *F. carica* dried fruit. Our results reveal the great potential of this fruit for curative and preventive treatment, in spite of the fact that they have not been completely investigated. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of this fruit as an antimicrobial agent and isolate the compound responsible for this antimicrobial activity.

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