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Study of Antibacterial Activity of some Date Seed Extracts

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Abstract: Abstract: Eight species of pathogenic and contaminated bacteria were Isolated and identified with the biochemical test and make sure of purity with VITIC2 Technical. The bacteria were *Acinetobacter baumannii*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoni*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus* and *Streptococcus pyogenes*. Investigation effect of aqueous, ethanolic, methanolic of Halawi, Khadrawiand Zahdi date seed extracts on the growth of isolated bacteria, the results showed that ethanolic extract was most effective extract compared to other extracts in influencing on the growth of bacteria using Agar Well Diffusion. The most active extract against *P. aeruginosa* strain was ethanol extract from Zahdi seed with a 22.3+ 0.32mm inhibition zone followed by 20.2 + 0.22 mm for *Escherichia coli*.

Key words: Halawi, Khadrawi, Zahdi date seed, Antibacterial activity.

Introduction

Date seeds that has been considered as a good example of functional food that rich in natural antioxidant involved: Selenium, Phenolic Acids, Carotenoids, when used as crude extracts of active compounds. However; purification and identification many of these materials still pharmacists, chemists and biologists concern, wherever trends to effect of date seed extracts on many of bacterial species besides of antioxidant activity and their a large role of food avidities and extend of shelf life (Fathi,2005).These active compounds in date seed extracts as protein, polyphenols, polysaccharides, lignans, and flavonoids possesses high antibacterial activity as pointed (Habib*et al*., 2014), that herbal medicine has its roots in every culture around the world; One of the most important medicinal herbs is dates palm (*Phoenix dactylifera* L.) which is well documented worldwide possesses several highly beneficial properties specially date seed were suggested that must be used as an antibiotic to treat bacterial infections (Saleh, 2016). The aim of this study was to investigate the effects of date seed extracts on contaminated and pathogen bacteria to be used of date seed extracts as antibiotic and additives food.

Materials & Methods

Plant material

Halawi, Khadrawi and Zahdi of *Phoenix dactylifera* date seed were obtained from local

market, Basrah. Date and seed have been separated from each other, and then washed and dried. After that, grinding to smooth powder has been done and stored them in a sterilized container for using.

Preparation of date seeds extract

water extracts

Followed of method of Ratheesh & Helen (2007). A weighed 20g of date seed powder and their add 500ml of distilled water, placed in shaking incubator for 24 hours at 28˚C then centrifuged at 2500 rpm for ten minutes, then filtration by Whitman No.1, concentrated of filtrate byrotary vacuum evaporator tothick liquid, then placed in incubator at 37˚Cfor 48 hours get at dry powder of extract. Put in glass containers and refrigerated at 4˚C.

Ethanolic extract

Preparation of ethanolic extracts by weighted 100g of date seed powder which dissolved by 500ml ethanol (98%) and mixed them well and left for 24 hours at laboratory temperature (25-30)˚C. The mixture has filtrated by Whatman No.1 and concentrated by rotary vacuum evaporator at 40˚C and left at laboratory temperature to get dry powder. Put in glass containers and refrigerated at 4˚C (Elmastas *et al*., 2015).

Methanolic extract

Followed steps in above paragraph (Ethanolic extract)to get at ethanolic date seed extracts.

Antibacterial activity

Bactrialisolates

A pathogenic and contaminated food bacterium was isolated in the Microbiology Laboratory at Al-Sadr Teaching Hospital, Basrah.These isolates are *Bacillus subtilis*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Acinetobacter baumannii*.

Each one bacterial isolates were initially identified depending on the forms of their developing colonies on the various plant media. This included the height of colonies (curved or flat), their shape, nature of their edges, their transparency, their colours, and their pigmentation, uses of MacConkey agar and Blood agar for recognize positive or negative bacteria and there were used other media for recognize *E. coli* from other negative gram bacteria such as Eosin methylene blue agar and Mannitolsalt agar was selective and differential for organisms such as *Staphylococcus* species which can live in areas of high salt concentration and Mueller-Hinton agar for antibacterial activity test.Some biochemical diagnostic tests were based on, such as catalysis, oxidase testing, coagulation enzyme testing, and sugar fermentation testing (Holt *et al*., 1994).Then the VITIK technique was used to diagnose the Gram-negative and Gram-positive bacteria after been confirmed by initial biochemical tests, consisting of a cassette holder and 64-hole Reagent cards represents the base material or medium for testing, plastic tubes as well as Densichek device and the input and output units, and it is considered as one of the best devices to identify the types of bacteria in a short period and very accurately (Ligozzi*et al*., 2002).

Media

Sterilize all the media in the autoclave device at 121 °C and press 15 lb.kg $^{-2}$ for 15 minutes. All media used in insulation were prepared according to the manufacturer's instructions (Table 1).

Biochemical test

1- Catalase Test: Is a test for demonstrating the presence of catalase enzyme by decomposition of hydrogen peroxide to oxygen and water, the reagent used for qualitative catalase testing is 3% hydrogen peroxide, though up to 6% is acceptable, the test can be done by mixing a

Table (1): Culture media used in study.

S	Media	Manufacture
1	Blood agar	(DIFCO) U.S.A
2	Eosin methylene blue agar	(DIFCO) U.S.A
3	MacConkey's agar medium	OXOID
4	Mannitol salt agar medium	(DIFCO) U.S.A
5	Mueller-Hinton agar medium	(DIFCO) U.S.A
6	Nutrient agar medium	(BDH) England
7	Nutrient broth	(BDH) England
8	Pepton water	(DIFCO) U.S.A
9	Simmon's Citrate agar	OXOID
10	Sodium Citrate Agar	(DIFCO) U.S.A
11	Triple Sugar Iron agar	OXOID
12	Urea agar	OXOID

Sample No.	Species	Company	Origin	Remarks
1	Shad (*Tenualosa ilisha*)	Tahani	India	Packed in Iraq within the validity period
2	Catfish (*Pangasianodon hypophtholmus*)	Al- Baraa	Iraq	within the validity period
3	Rainbow trout (*Oncorhynchus mykiss***)**	Kaskin Oglu	Turkey	Within the validity period
4	Mackerel (*Trachurus trachurus*)	Americana	Thailand	Packed in UAE, within the validity period)

colony with a few drops of H_2O_2 in a slide and looking for the formation of bubbles within 10 sec.

2- Oxidase Test: The test identifies the presence of cytochrome c oxidase or indophenols oxidase, the test is based on the principle of redox (Reduction-oxidation), the oxidase test often uses a reagent as an electron donor, when the reagent is oxidised it changes from colourless to a dark blue or purple compound, indophenol blue.

3-Indole Production Test: Indole is an aromatic heterocyclic organic compound with asix-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring, which is derived from tryptophan using the enzyme tryptophanase, Indole can be detected easily with Kovacs reagent.

4- Methyl-Red Test: The MR test looks for the ability of bacteria to produce large amounts of acid resulting in significant decrease in the pH of the medium below pH 4.4, this acidic nature

is indicated by methyl red indicator which is yellow above pH 5.1 and red at pH 4.4. The test is done on glucose phosphate peptone water.

5- Voges -Proskauer test: VP test is actually extension of MR test and looks for the ability to produce butylenes products. Acetoin is an intermediate in the reaction which is looked for using 40% KOH and alpha-naphthol. If Acetoin is present, it is oxidised in the presence of air and KOH to diacetyl which reacts with guanidine components of peptone, in the presence of alpha-naphthol to produce a red colour.

6- Citrate Utilization test: The test looks for the ability of a bacteria to utilise citrate as a sole source of carbon . The organism is inoculated into simmonscitrate agar, simmons citrate agar contains sodium citrate as the sole source of Carbone, ammonium dihydrogen phosphate as the sole source of nitrogen, and the pH indicator bromothymole blue. The bacteria convert the ammonium dihydrogen phosphate to ammonia, at pH 7.5 or above bromothymol blue turns royal blue which is otherwise green.

7- Sugar Ferment Test: The test medium is Triple Sugar Iron agar (TSI), the test medium contains 3 sugars-Glucose (0.1%), lactose and sucrose (1% each). Phenol red serves as the indicator, the medium contains a butt and a slant, Ferrous sulphate serves as an indicator for H_2S production. The medium is inoculated with a stab method on the butt and stroke method on slant. The organisms ferments glucose but does not ferment lactose or sucrose, the slant becomes red and butt remains yellow. It is reported as K/A(Alkaline slant/Acid butt), the organisms in addition to glucose ferments lactose and sucrose. The slant and butt remain yellow, it is reported A/A(Acid slant /Acid butt), if the organisms in non-fermenter, it is reported K/NC (Alkaline slant/ No change), in addition to the above gas and H_2S is reported.

8- Urease test :Urease is an enzyme belong to the superfamily of amidohydrolases and phosphotriesterases. It catalyses the hydrolysis of urea into ammonia and carbon dioxide. The formation of ammonia causes alkalinisation of the medium and the pH change is indicated by a change to pink at pH 8.1 (Betty *et al*., 2007).

3-Test assay for antibacterial activity of date seed extracts

The antibacterial activity was determined by using agar well diffusion method. A volume of 0.1 ml of eight different strains was inoculated to the test tube , and incubated overnight. Muller Hinton agar was prepared in a conical flask and sterilized in autoclave. The sterile medium was poured into petri plates, Bacterial cultures were uniformly spread over the media using cotton swab, then wells of about 6mm in diameter were punched in Muller Hinton agar by using cork borer. Then, the wells was loaded with 50 µl of seed extract at concentration (5mg*l*ml).The inoculated plates were incubated at 37˚C for 24 hrs. The diameters of inhibition zones were measured for each plate(Perez *et al*., 1990).

Statically analytics

The data analyzed by ANOVA test and Analysis of Variance at probability level $P<0.05$ within SPSS Statistical Package of Social Sciences and used RLSD (Al-Rawi & Khalaf-Allah, 2000).

Result & Discussion

Biochemical test

1-*Acinetobacter baumannii*

Negative for Gram stain, Oxidase, Indole test, Methyl Red, Voges-Proskauer Test, Urease and H_2S gas, Indole, and has non-spores, while is positive for catalase, Glucose fermented, non-Lactose or sucrose fermented. It was an opportunistic pathogen in humans, affecting people with compromised immune systems becoming increasingly important as a hospital-

derived (nosocomial) infection (Holt *et al.*, 1994).

2- *Bacillus subtilis*

It is positive for gram stain, catalase, citrate, oxidise, Voges Proskauer Test, It has the ability to ferment the lactose, sucrose, andglucose, while it is negative for indole, urease, methyl red and H_2S gas.

3- *Escherichia coli*

It is negative for gram stain, citrate test, Voges Proskauer Test, oxidase, urease,H_2S test, while it is positive for Indole test, catalase, methyl red test and It has the ability to ferment the lactose, sucrose, and glucose.

4-*Klebsiella pneumonia*

It is positive for catalase, citrate, Voges Proskauer Test, urease test, while is negative for gram stain, oxidase, indole, methyl red, H_2S gas. Also it is fermented the glucose, lactose and sucrose.

5- *Proteus mirabilis*

Proteus mirabilis is negative for gram stain, glucose ferment, oxidase, Voges Proskauer Test, Indole test, non-lactose, sucrose ferment, while it is positive for citrate, catalase, urease, Methyl Red and H_2S test.

6-*Pseudomonas aeruginosa*

It is positive for citrate, catalase, oxidase test, while negative for H_2S gas, urease, indole, voges-proscauer, methyl red test, and non - lactose, glucose and sucrose ferment.

7-*Staphylococcus aureus*

It is positive for gram stain, catalase, citrate, urease, Voges-Proskauer, Methyl Red Test and lactose- sucrose- glucose ferment, while negative for Indole, oxidase and H_2S gas test.

8-*Streptococcus pyogenes*

These bacteria is Gram-positive, negative for gatalase, oxidase, urease, Voges-Proskauer, Indole, H_2S gas test while is positive for Methyl Red and Citrate test also fermented for lactose, sucrose, and glucose.

2- Analysis of VITIK2 Technique

The VITIK2 system was used for rapid identification of isolates and result calculated. Also, comported with results a store in instrument which was involved with many of strains grow in different conditions and isolated from variety places. System showed results of tester as +, -, (+) and (-). The results in parentheses indicate weak testing, and defined of identification level for organisms by test mapping and compared with mental adjective of system and was given probability percentage and accuracy level reach to 96-99%.

3-Antibacterial of Halawi date seed extracts

The results obtained have showed that most of seed extract possesses a potential antibacterial activity against the tested bacteria; However, ethanolic and methanolic of Halawi date seed extracts had a significant ($P<0.05$) antibacterial agents than the aqueous extracts (Table 3). These extracts (ethanolic and methanolic) inhibited all organism, the methanolic extracts was the maximum rate of effectiveness inhibitory towards *P. mirabilis* diameter 20mm, then *K. pneumonia* diameter 15mm, and other organism which was 11mm and 12 mm, while ethanolic extracts was the maximum rate of effectiveness inhibitory against *P. aeruginosa* diameter 17mm, and the rang of inhibition zone of other strains between 10-12mm. However, the minimum effect was for aqueous extract which showed low zone of inhibition against *P. aeruginosa, K. pneumoniae, A.*

Table(2): Biochemical test of isolated bacteria.

Types of Bacteria / Biochemical test	*Acinetobacter baumannii*	*Bacillus subtilis*	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Streptococcus pyogenes*	*Staphylococcus aureus*
Gram stain	-	+	-	-	-	-	+	+
Catalase production test	+	+	+	+	+	+	-	+
Oxidase production test	-	+	-	-	-	+	-	-
Detection of Indole production	-	-	+	-	-	-	-	-
Methyl red test	-	-	+	-	+	-	+	+
Voges – proskauer test	-	+	-	+	-	-	-	+
Citrate test	+	+	-	+	+	+	+	+
Urine test	-	-	-	+	+	-	-	+
Glucose fermention	+	+	+	+	+	-	+	+
Sucrose fermention	-	+	+	+	-	-	+	+
Lactosefermention	-	+	+	+	-	-	+	+
H2S	-	-	-	-	+	-	-	-

baumannii and *P. mirabilis*, while inhibition zone of other organisms with7mm, 7mm, 6mm,8mm against to *E. coli*, *S. aureus*, *B. subtilis* and *S. pyogenes* respectively. The antibacterial activity of these extracts due toas alkaloids, sterols, carbohydrates, flavonoids vitamins, tannins and phenolic acids (Eong, 2006). The results agreed with Perveen *et al*. (2012) which found the antibacterial activity of methanolic extract was the maximum rate of effectiveness inhibitory towards pathogenic bacteria than aqueous and acetone extracts. Also, agreed with Saleh (2016) which notice the ethanolic and chloroform extracts of Khastawi date seed was the best than aqueous extract of antibacterial activity, This due to the ability of organic solvents to extraction high level of phenolic compounds (Cox *et al*., 2001). The inhibition of bacterial growth by these extracts could be due to the presence of some active compounds which may act alone or in combination to inhibit bacterial growth (Mohammedi & Atik, 2011). Also table (2) noticed Halawi date seed extracts was effective in influencing the growth of pathogenic or contaminated bacteria such as *B. subtilis* and *S. aureus*. Different results and selective inhibition of extracts against bacteria may due

to extraction condition and type of solvents uses (Habib *et al*., 2014) in addition ethanol was consider common solvent with low poisoning to extract active compound and uses of introduction of many materials such as makeup, flavour and colour in food industry.

4-Antibacterial of Khadrawi date seed extracts

The table (4) showed results in the inhibition effect of aqueous, ethanolic, methanolicextracts of Khadrawi date seed against pathogenic bacteria, also showed the inhibition effect be varied by bacteria species, ethanolic and methanolic of Khadrawi date seed extracts had a significant value ($P<0.05$) antibacterial agents than the aqueous extracts, these two extracts showed inhibition effect against all bacteria, as was the maximum rate of effectiveness

Table (3): Inhibitory zone of Halawi date seed against pathogenic bacteria.

Bacterial Species	Inhibition zone diameter (mm)			L.S.D
	Ethanolic extract	Methanolic extract	Aqueous extract	
Acintobacter baumanii	12	10	weak Inhibition	N.S
Bacillus subtilis	12	11	6	4.8
Escheichia coli	13	12	7	4.5
Klebsiella pneumoniae	9	15	weak Inhibition	5.2
Proteus mirabilis	10	20	weak Inhibition	6.4
Pseudomonas aeruginsa	17	11	weak Inhibition	3.7
Staphylococcus aureus	12	12	7	3.2
Streptococcus pyogenes	11	11	8	2.4
Inhibition Average	12	11.4	7	4.6

inhibitory towards *A. baumannii* by ethanolic extract diameter 14mm. Then *K. pneumoniae, S. pyogenes* diameter 12mm, and other bacteria 11mm, 10 mm, while methanolic bacteria was the maximum rate of effectiveness inhibitory towards *P. aeruginosa*

diameter of inhibition zone was 14mm. The inhibition zone diameter of other bacteria ranged between 10 to 11 mm, and the minimum

effect was for aqueous extract which showed low level inhibition for each *K. pneumoniae, A. baumannii, P. aeruginosa,* while diameter of other species 6mm, 7mm, 8mm, 8mm, 6mm for *E. coli, S. aureus, B. subtilis, S. pyogenes* and *P. mirabilis* respectively. The antibacterial activity of these extracts due to presence phenolic compounds which aromatic compounds contained hydroxyl group and inhibition ability of these compounds increased by rise of these groups (Al-Moussaoui, 2006).

The results agreed with Mossa *et al*. (1986) were found alcoholic extract of date seed showed antibacterial activity on *K. pneumoniae* growth, also agreed with Saddiq & Bawazir (2010) were noticed alcoholic extract of date seed possesses antibacterial activity against *S. aureus, Proteus* and *Bacillus.* In addition, was more effective in inhibiting growth of most tested bacteria as compared with antibiotics and

Table (4): Inhibitory zone of Khadrawidate seed against pathogenic bacteria.

Bacterial Species	Inhibition zone diameter (mm)			L.S.D
	Ethanolic extract	Methanolic extract	Aqueous extract	
Acintobacter baumanii	14	11	weak Inhibition	2.8
Bacillus subtilis	10	10	8	N.S
Escheichia coli	11	9	6	2.5
Klebsiella pneumoniae	12	11	weak Inhibition	N.S
Proteus mirabilis	10	8	6	2.6
Pseudomonas aeruginsa	10	14	weak Inhibition	3.5
Staphylococcus aureus	11	11	7	3.2
Streptococcus pyogenes	12	11	8	3.4
Inhibition Average	10	10.6	7	2.7

this may refer to difference in resistance of bacteria to anti-tested material due to change in membrane permeability of cells, thereby hindering the entry of enzymes or excretions by the change in the chemical composition of the constituent chemical or by changing the natural of some of their components (Aba Al- Khail *et al*., 2003).

5-Antibacterial of Zahdi Date Seed Extracts

Results showed in table (5) the inhibition effect of aqueous, ethanolic, methanolic extracts of

Zahdi date seed against pathogenic bacteria also showed the inhibition effect be varied by bacteria species, ethanolic and methanolic of Zahdi date seed extracts had a

Table (5): Inhibitory zone of Zahdidate seed against pathogenic bacteria by mm.

Bacterial Species	Inhibition zone diameter (mm)			L.S.D
	Ethanolic extract	Methanolic extract	Aqueous extract	
Acintobacter baumanii	16	10	weak Inhibition	4.2
Bacillus subtilis	12	14	8	2.1
Escheichia coli	20	8	7	5.9
Klebsiella pneumoniae	13	10	weak Inhibition	N.S
Proteus mirabilis	22	9	weak Inhibition	4.6
Pseudomonas aeruginsa	15	16	8	3.4
Staphylococcus aureus	12	10	8	1.9
Streptococcus pyogenes	12	11	8	2.3
Inhibition Average	15.3	10	7.8	3.5

significant (P<0.05) antibacterial agents than the aqueous extracts, as was the maximum rate of effectiveness inhibitor against *P. aeruginosa* and *E. coli* by ethanolic extract diameter22mm and 20mm respectively. These results agreed with Ado *et al*. (2017) about study inhibition effect of active compounds in date seed extract against *Escherichia coli*. Also the extracts showed presence bio active compounds such as Alkaloids, Glycoside and Saponins which responsible of extracts ability of inhibition isolated bacteria *E.coli* 20mm and concentration 1mg.ml^{-1}, while diameter of other bacteria reach 12, 13, 15, 16 mm for *S.aureus, K. pneumoniae, P. mirabilis* and *A. baumannii* respectively, while methanolic extract which was at most activity with 16mm diameter against *P. mirabilis*. Inhibition zone diameter for other bacteria arranged between 8 to 14 mm ,the minimum inhibition activity was aqueous extract showed low inhibition level on *P. aeruginosa, K. pneumonia* and *A. baumannii*. Inhibition zone diameter for other

bacteria 7mm for *E. coli,* 8mm for *S. aureus, B. subtilis, S. pyogenes* and *P. mirabilis*, these results agreed with Al-Moussaoi & Abd-Almotalib (2011) of study about phenolics activity which extracts from different parts of date palm against bacteria caused Blepharitis, showed phenolic compounds extracts high antibacterial activity against isolated bacteria, phenolic compound work on inhibition growth of cells by effect on cell well activity and deposition of cells protoplasm accordingly their death (Gende *et al*., 2008;Trajeno *et al*., 2010).

Conclusions

Phoenix dactylifera seeds extract has antibacterial effect against Isolated bacteria due to abundance of phytochemical in the seeds.

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