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STUDIES ON THE STIMULATION OF DATES ON RAT IMMUNE RESPONSE AND THEIR EFFECTS ON SOME BACTERIAL STRAINS

(With 8 Tables)

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دراسة التمر كمحفز مناعي على الاستجابة المناعية فى الفئران وتأثيراته على أنواع مختلفة من البكتريا

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على القرعاوي ، سحر عبد اللطيف

لقد تمت دراسة تأثير كل من التمروالنوى كرافع للمناعة وأيضاً تأثيرهم المباشر على أنواع مختلفة من البكتريا المعزولة من الحيوانات ولقد تمت الدراسة على المستوى المعملي وعلى حيوانات التجارب. بالنسبة للدراسة على حيوانات التجارب فقد تم إجراء التجارب على عدد ٦٠ فأرا تم تقسيمهم إلى أربع مجموعات متساوية المجموعة الأولى وهى المجموعة الضابطة والثانية تناولت العليقة الأساسية إلى جانب ١٠% من التمر المجروش والثالثة تناولت العليقة الأساسية بجانب ١٠% من النوي. أما المجموعة الرابعة فلقد تغذت على العليقة الأساسية إلى جانب ١٠% من خليط التمر والنوى وقد أجريت اختبارات قياس الكفاءة البلعومية لخلايا النيتروفيل وأيضاً تم الاختبار الكمي لجلوبيولينات المناعية IgM, IgG (Immunoglobulins) وقد أظهرت نتائج الاختبارات المناعية عن زيادة فى القدرة البلعومية لخلايا النيتروفيل فى المجموعات التى تناولت التمر والنوى وأيضاً الخليط بالإضافة إلى زيادة ملحوظة فى هذه المجموعات السابقة بالنسبة لجلوبيولينات المناعية IgG، IgM وذلك بالمقارنة بالمجموعة الضابطة. أيضاً تم دراسة التأثير المباشر لكل من التمر والنوى على نمو أنواع مختلفة من البكتريا الممرضة والمعزولة من أصل حيواني. ولقد روعي أن تكون البكتريا المختارة متنوعة وكانت على النحو التالي: مكورات عنقودية ذهبية، بكتريا السل الكاذب، باسيلاس سائليس (موجبات الجرام) وسالمونيلا تايفيميوريام وسالمونيلا دبلين وكليبسيلا (سالبات الجرام). وقد أظهر المستخلص المائي للنوى أثراً مثبطاً على المكورات العنقودية و بكتريا السل الكاذب حتى تخفيف ١/٣٢٠ وعلى باسيلاس سائليس حتى تخفيف ١/٤٠ بينما أثر المحلول المركز على السالمونيلا بدرجة طفيفة ولم يؤثر على

الكليبسيلا. أما مستخلص التمر فقد أظهر أثراً مثبطاً ملحوظاً على بكتريا السل الكاذب (١/٢٠) وكان أثره طفيفاً على باسيلاس ساتليس والمكورات العنقودية بينما لم يكن له أثر على السالمونيلا والكليبسيلا ذات قيمة. من ذلك يستدل على أن نوى التمر له تأثير مثبط مباشر على نمو البكتريا وخاصة موجبة الجرام بينما لم يؤثر التمر تأثيراً مباشراً ملحوظاً إلا على بكتريا السل الكاذب وهى أيضاً موجبة الجرام. ويبدو أن هناك تأثيراً لنوع الجدار الخلوي البكتيري وكون البكتريا متبذرة أم غير متبذرة على أثر التمر والنوى على البكتريا.

SUMMARY

To better understand, the effect of dates and pits as immuno stimulant agent and their effects on different species of pathogenic bacteria of animal origin, were investigated In Vivo and In Vitro. In Vivo experimental sixty Albino rats were divided into four equal groups, group one as control, group two fed a basal diet supplemented with 10% minced dates, group three fed a basal diet supplemented with 10% pits, group four fed a basal diet supplemented with 10% mixed minced dates and pits. Phagocytic percent and phagoaytic index were assessed. Also, quantitative detection of immunoglobulins (IgG-IgM) were detected. Immunological investigations revealed significant increase in phagocytic activity of neutrophils in groups treated with dates, pits and mixed compared with control. Also, the same treated groups exhibited (Hyperimmuno-globuinaemia) significant increase in values of immunoglobulins (IgG and IgM) compared with control animals. Also, the direct effects of dates and pits, on different species of pathogenic bacteria of animal origin, were investigated. Test bacteria were selected to represent different species. The tested bacteria included *Staphylococcus aureus, Corynebacterium pseudotuberculosis* and *Bacillus subtilis* (Gram-positive) and *Salmonella typhimurium, Salmonella dublin and Klebsiella species* (Gram-negative). The water extract of pits showed clear inhibition for both *Staphylococcus aureus and Corynebacterium pseudotuberculosis* until 1/320 dilution. *Bacillus subtilis* was also inhibited but up to higher concentration (1/40). The effect of concentrated extract was minute on salmobnellae while no effect was detected with klebsiella. On the other hand, water extract of dates showed a detectable inhibitory effect on *Corynebacterium pseudotuberculosis* until the concentration 1/20. The effect of date extract was minute on *Staphylococcus aureus* and *Bacillus subtilis* while no effect was observed against the Gram-negative species. From these results, it can be concluded that date seeds (pits) have a direct inhibitory effect on Gram-positive bacteria while dates had affected

Corynebacterium pseudotuberculosis only which is also Gram-positive bacterium. It looks like that it affects the bacterial cell wall composition.

Key words: *Immunology, phagocytic activity, dates, rats.*

INTRODUCTION

Dates, the fruit of the date palm *(phoenix dactylifera)*, represents an important highly nutritive source for human and animals. This has been attributed to the constituents of the dates. Of these constituents, sugars that are considered the most important source of energy Additionally, dates contain considerable ratios of vitamins (A and B complex), minerals, proteins and fibres (Saway and Miski, 1983; Eromosele *et al.*, 1991; Alshahib and marshall, 2003).

It has been reported that consumption of dates by human beings has a lot of healthy benefits, it improves vision and hearing nerves, growth rate of children and blood vessel capability. Also dates have been prescribed for athletics, pregnant women, heart problems and gastric ulcer, (Al Qarawi *et al., 2005)*. Minerals in dates help in development of teeth and bones while fibers protect against enteritis and cancer (Mills *et al.,* 1989; Ishurd *et al.,* 2004).

Furthermore, some studies indicated that dates stimulate the immune system of human being (Puri *et al.,* 2000). Not only date flesh but also the date pits have been found to be of high nutritive value that pits could compete cereals in formations of non traditional animal rations for different purposes (Alwash *et al.,* 1982).

Concerning microbial pathogens, dates were described, in the public medicine for treatment of respiratory diseases of bacterial causes such as bronchitis and pneumonia. Also, dates showed antibiotic- like effects on some types of bacteria (Abdel salam, 1994).

In the present study, immunological effects of dates and pits were studied In Vivo using rat as model of experimental animals. Also, the direct effect of dates and pits on some pathogenic bacteria were studied. This was to evaluate probability of their applications as immunastimulant agents and in controls and prevention of certain bacterial diseases in human and animals.

MATERIALS and METHODS

Materials:

1- Experimental animals:

A total of 60 male Albino rats weighing from 150 to 170 gm were used. The rats were housed in plastic cages under good hygienic condtions and fed on balanced ration and water ad- libitum.

Experimental Design: -

Two experiments were carried out in the present study, the first In Vivo and the second in vitro.

Experiment (1): this experiment was conducted to evaluate the effect of dates and pits on the immune response on rats.

Sixty rats were divided into four groups (15 rats each).

Group 1: served as a control fed on a commercial basal diet.

Group 2: rats fed on a basal diet supplemented with 10% minced dates.

Group 3: rats fed on basel diet supplemented with 10% minced pits.

Group 4: rats fed a basal diet supplemented with 10% from minced mixed dates and pits.

Two blood samples were obtained from retroorbital venous plexus at 15, 30 and 45 days through the experiment. The first sample was collected into a plastic centrifuge tube containing heparin 20 I.U/ml blood for measuring the phagocytic activity of neutroplils for cell-mediated immune response assessment using Dextran, 5000,000 M.W, from Sigma according to the method described by Wilkinson (1981). The second sample of blood was taken into dry tube, serum was collected by centrifugation at 3000 r.p.m for 15 mintes for qualitative detection of serum immunoglobulins, IgG, IgM: Specific Rat Radial immunodifussion plates (The Binding site, BIND A RID, Birmingham, U.K.) were used to quantitate immunoglobulins (IgG and IgM) in sera mg/ml according to Mancini *et al.* (1965).

(A) Dates and pits:

Dried dats and pits were obtained from Egypt, Assuit type and minced into small pieces for addition to ration.

(B) Dates and pits:

Two hundred grams of semidried date flesh of the sokkary brand were minced into small pieces, exposed to ultraviolet for 4 hours, and soaked in sterile distilled water (1/5) for an overnight. Ground pits were treated similarly and both extracts were serially diluted two fold in sterile distilled water (1/5, 1/10, until 1/2560).

Bacterial cultures:

Staphylococcus aureus, Corynebacterium pseudotuberculosis, Bacillus subtilis Salmonella typhimurium, Salmonella dublin, and Klebsiella spp. were, separately, tested against each dilution of both date and pit extracts. With the exception of *Bacillus subtilis*, bacterial species represented isolates from clinically diseased animals (Table 1) and identified through standard bacteriologic methods (Quinn and Carter, 1994).

Bacterial culture inoculates:

Each bacterial isolate was cultivated onto brain heart infusion agar plates and incubated at 37°C for 24 hours. Few colonies were picked from each bacterial growth and transferred to a tube containing 3 ml of Mueller-Hinton broth. The inoculated tubes were incubated at 37°C for few hours until a turbidity matched that of McFarland tube number 0.5 to be used in the micrbiological assays (Quinn and Carter, 1994).

Bacteriological assays:

Diffusion method:

Four plates of Mueller-Hinton agar were used for each bacterial species. The plates were evenly seeded with the bacterial inoculates using swabs emersed in the corresponding Mueller-Hinton broth cultures. The inoculated plates were allowed to dry at room temperature for 10 minutes. Five wells (8 mm in diameter) were punched in each plate using a sterile cork borer. From each dilution of either date or pit extract, 100 µl were delivered into a corresponding well of the inoculated Mueller-Hinton agar plates. The test plates were incubated at 35°C for 18 hours after which they were examined for bacterial growth inhibition around wells charged with date or pit diluted extract. Inhibition zone diameters were measured using a transparent ruler. (Stokes and Ridgway, 1987).

Microdilution susceptibility assay:

Both dates and pits were tested to detect the minimal concentration that can inhibit bacterial multiplication. This was carried out by using sterile 96-well microculture plates in which 50 µl of either date or pit diluted extract were delivered into wells of corresponding columns (1-10). Equal volumes of each bacterial broth culture were delivered into wells of corresponding rows (A-F) to reach a bacterial concentration of 10^5 bacterial cell/ 1 ml. Columns 10 and 11 as well as rows G and H were left as controls for bacteria and extracts, respectively. After mixing by gentle tapping, the inoculated plates were incubated at 35°C for 18 hours. After incubation, wells were checked for

bacterial growth inhibition indicated by clearance of the mixture. The minimal inhibitory concentration was expressed as the highest dilution that could inhibit the bacterial growth (NCCLS Subcommittee, 1980).

RESULTS

Table 1: Phagocytic percentage and phagocytic index of rat neutrophils fed with 10% dates, pits and mixed at 15,30 and 45 days.

Time	15 days		30 days		45 days	
Group / Parameters	Phagocytic%	Phago index	Phago %	Phago index	Phago %	Phago index
Control	47± 0.91	1.27 ± 1.2	49 ± 1.2	1.4 ± 0.02	49 ± 2.4	1.4 ± 0.05
Dates	51± 1.8	1.3± 0.02	60 ± 3.5 *	1.8 ± 0.02 **	65 ± 1.7 **	1.9 ± 0.08 **
Pits	52± 1.2	1.01 ± 0.03	53 ± 1.6 *	1.7 ± 0.01 *	60 ± 2.1 *	1.8 ± 0.06 *
Mixed	52 ± 2.1	1.3 ± 0.10	57 ± 3.4 *	1.8 ± 0.05 **	63 ± 2.1 **	1.9± .07**

* p < 0.05. ** p < 0.01.

Table 2: Serum immmuno globulins IgG (mg/ml) in sera of Albino rats fed with 10% dates, Pits and mixed at 15 days, 30 days & 45 days.

IgG (mg/ml)

Groups / Time	15 days	30 days	45 days
Control	10.9 ± 0.08	10.9 ± 0.12	10.9 ± 0.12
Dates	11.7± 0.4	12.38 ± 0.6*	12.4 ± 0.6*
Pits	14.5± 0.3**	14.9 ± 0.2**	14.9 ± 0.2**
Mixed	13.6 ± 0.4**	14.2 ± 0.09**	14.42 ± 0.09**

* p < 0.05. ** p < 0.01.

Table 3: Serum immunoglobulins IgM (mg/ml) in sera of Albino rats fed with 10% dates, pits and mixed at 15, 30 and 45 days (IgM (mg/ml).

Groups / Time	15 days	30 days	45 days
Control	2.1 ± 0.3	2.2 ± 0.06	2.2 ± 0.06
Dates	2.8± 0.05 **	2.93 ± 0.05**	3.01 ± 0.08**
Pits	2.95± 0.03**	2.96 ± 0.07**	3.04 ± 0.07**
Mixed	2.97 ± 0.06 **	2.97 ± 0.05 **	3.05 ± 0.06**

* p < 0.05. ** p < 0.01.

Table 4: Sources of bacterial isolates tested in the study.

Bacterial species	Source
Staphylococcus aureus	Abscess in a camel kidney
Corynebacterium pseudotuberculosis	Lymph node abscess in a sheep
Bacillus subtilis	ATCC 6633
Salmonella typhimurium	Cattle
Salmonella dublin	Cattle
Klebsiella spp.	Chicken

Table 5: Effects of different dilutions of pit-water-extract on bacterial growth In Vitro.

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Staphylococcus aureus	+	+	+	+	+	+	+	-	-	-
Corynebacterium pseudotuberculosis	+	+	+	+	+	+	+	-	-	-
Bacillus subtilis	+	+	+	+	+	-	-	-	-	-
Salmonella typhimurium	-	-	-	-	-	-	-	-	-	-
Salmonella dublin	-	-	-	-	-	-	-	-	-	-
Klebsiella spp.	-	-	-	-	-	-	-	-	-	-

+ : Inhibition of the bacterial growth.
- : No inhibition of the bacterial growth.

Table 6: Effects of different dilutions of date-water-extract on bacterial growth in vitro.

	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-
Corynebacterium pseudotuberculosis	+	+	+	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-	-	-
Salmonella dublin	-	-	-	-	-	-	-	-	-	-
Salmonella typhimurium	-	-	-	-	-	-	-	-	-	-
Klebsiella spp.	-	-	-	-	-	-	-	-	-	-

+ : Inhibition of the bacterial growth.
- : No inhibition of the bacterial growth.

Table 7: Zone diameters of inhibition produced by pit-water-extract on different bacterial isolates.

	1/5	1/10	1/20	1/40	1/80	1/160	1/320
Staphylococcus aureus	17*	16	14	12	11	10	9
Corynebacterium pseudotuberculosis	18	16	15	13	12	11	10
Bacillus subtilis	13	12	11	9	9	8**	8
Salmonella typhimurium	8	8	8	8	8	8	8
Salmonella dublin	8	8	8	8	8	8	8
Klebsiella spp.	8	8	8	8	8	8	8

* : Diameters are expressed in millimeters (mm).
** : no inhibition as the well diameter was 8 mm.

Table 8: Zone diameters of inhibition produced by date-water-extract on different bacterial isolates.

	1/5	1/10	1/20	1/40	1/80	1/160	1/320
Staphylococcus aureus	8	8	8**	8	8	8	8
Corynebacterium pseudotuberculosis	14*	12	11	11	8	8	8
Bacillus subtilis	8	8	8	8	8	8	8
Salmonella typhimurium	8	8	8	8	8	8	8
Salmonella dublin	8	8	8	8	8	8	8
Klebsiella spp.	8	8	8	8	8	8	8

*: Diameters are expressed in millimeters (mm).
** : no inhibition as the well diameter was 8 mm.

DISCUSSION

Dates have been evaluated for their nutritive and medical values by many authors (Booij and Piombo, 1992; Abdel Salam 1994; Hussein *et al.*, 1998; Al-Dabeeb, 2005). Concerning pits, a lot of successful studies have been mentioned about their use as essential element in animal rations (Alwash *et al.*, 1982). However a little attention has been paid to their effects on immune responses as well as direct antibacterial effect therefore, this study was planned to test date flesh and pits as immunostimulant agent In Vivo, in addition to their direct effect against different species of bacteria.

Phagocytic activity of neutrophils as shown in Table (1) revealed a significant increase in phogocytic percentage and phagocytic index in all treated groups with dates, pits and mixed (dates & pits) compared

with control animal at 30 and 45 days. Also, value of immunogloblins IgG and IgM are presented in Table (2) and Table (3). The tables show that significant increase in IgG and IgM in all treated groups with dates, pits and their mixture compared with control animals during experiment, Highly significant enhancement of immunoglobulins was more prominent in all treated groups (dates, pits and their mixture) at 30 and 45 days.

These results indicate significant immunostimu-latory activity of dates and pits, which agree with the results of Puri *et al.* (2000) who recorded an enhancement of macrophage activation and increased in haemagglutination antibody titres as well as plaque –forming cell (PFC) counts in mice orally administered date extract. The immunostimulatary activity of dates may be attributed to presence of ß-D-glucan in date flesh. Ishurd *et al.* (2002) isolated ß-D-glucan from the fruit of dates. ß-D-glucan is an important candidate molecule of biological response modifier implicated in cancer, it can be obtained from other sources such as yeast, fungi, bacteria and plants. Similar results have been reported by Tokunaka *et al.* (2000) who mentioned that ß-D-glucan has a higher biological effect in activation of alternative pathway of complement, induce interleukin-6-synthesis of macrophage In Vitro, and act as adjuvant effect on antibody production.

Furthermore our results agree with Al.shahib and Marsall (2003) who found that dates contain carbolydrate, fat, proteins, vitamins, fiber and at least 15 minerals, including selenium which play an important role in immune function and prevent cancer. Concerning pits there is no available data explaination about their immunological effects. However, a lot of successful studies have been mentioned about their use as essential element in the animal rations. Al washi *et al.* (1982) and Hussein *et al.* (1998) observed significant increase in body weight and improved feed utilization in chickens.

We elucidate the effect of dates and pits that stimulate the humoral and cellular immume response, immunoreactive and immunostimulant action, through Bcells, T-helper /inducer cell (CD_4 helper and CD_8), T- cells receptor for major histocompatibiliy complex (MHC) molecule that mutate and regulate expression of immunoglobulins gene (Tizard, 1995).

Also, Ali *et al.* (1999) noticed high increase in body weight, plasma testosterone and improved feed utilization in male rats fed date pits at concentration that immunostimulatory activity of pits might be attributed to its constituent that have been already analyzed.

The tested bacteria represented different Gram positive and Gram negative species (Table 4). With the diffusion method, it was found that the effect of date flesh extract was not inhibitory for the tested bacterial isolates except *Corynebacterium pseudotuberculosis* that was inhibited with high concentrations (first 2 wells). This effect can be attributed to the high sugar concentration of the extract as the effect disappeared with the dilution 1/20. This was reported in previous studies in which it was mentined that *Vibrio cholera* was destroyed within 16 days after its mixing with dates (King, *et al.*, 1968). In such experiments, the antibacterial effect of dates was attributed to the high sugar contents which can destroy bacterial cells through the osmotic imbalance and plasmolysis. Eventhough, the inhibition zone diameters around the first two wells of *Corynebacterium pseudotuberculosis* plate were not so wide to be considered. In contrast, the other tested bacterial species did not show any inhibition with either concentrated or diluted date flesh extract (Table 6). This is supported, to a large extent, with the success of many other studies in isolation of different bacterial and fungal species from black dates (Mikky & Yanezo 1997). Thus, to fight pathogenic microorganisms, it can be suggested that maximum benefits can be obtained from date flesh through its indirect effect as immunostimulant. This is in addition to the well known role of consumable dates in the general health condition and fitness of the consumers.

Concerning pits, it was very interesting to detect strong inhibitory effects of pit-extract (until dilution 1/320) on both *Staphylococcus aureus* and *Corynebacterium pseudotuberculosis* (Table 5). Also, there was an inhibitory effect but to a lower extent (until dilution of 1/40) on *Bacillus subtilis*. On the other hand, no inhibitory effects were detected on *Salmonella* and *Klebsiella* species.

It can be, causiously, suggested that water extract of pits selectively affect Gram positive bacteria as the three inhibited species were Gram positive and the three unaffected ones are Gram negative. It is difficult to explain this finding, but it can be speculated that the bacterial cell wall composition has something to do with this interaction. In other words, the active principle(s) of pits inhibit the Gram positive bacteria through its effect on the cell wall formation. How this can happen, it can be through one of the pit constituents that have been already analyzed such as chlorine, sulpher or tannins (Booij & Piombo, 1992). Otherwise, the active principle might be something else beyond the capabilities of the analytical methods. This is not unlogic because some antibiotics such as penicillin affect only Gram positive bacteria by

inhibiting their cell wall formation. This means that the water-soluble antibacterial active principle (s) of the pits might have a penicillin-like effect. Absence of the inhibitory effect of pit-water extract on Gram negative bacteria tested in this study may reflect absence of the susceptible target in their cell walls (Table 7).

The results of the microdilution susceptibility assays were in agreement with those of the diffusion assays as bacterial growth was indicated by turbidity after the pit extract dilutions of 1/320, 1/320 and 1/40 with *Staphylococcus aureus, Corynebacterium pseudotuberculosis* and *Bacillus subtilis*, respectively.

Conclusively, in addition to its use in ration formulation, date pit flour can be used for direct therapeutic purposes against diseases caused by *Corynebacterium pseudotuberculosis* and *Staphylococcus aureus* at this situation. The first species has been known for its role in many animal diseases as caseous lymphadenitis in sheep and goats. The second species is a well known pyogenic organism in both man and animals in addition to the human food poisoning (Hirsh and Zee, 1999; Quinn & Carter 1994). It can be concluded that dates and pits may activate the humoral and cellular immune response.

However, this was the first trial in this concept and as enhancing results were obtained more research is recommended to answer many questions about dates and pits and the limits of their direct application in microbial disease therapy. Also, the real dilemma of pit-bacterial interaction is to be investigated as much as possible. This only can be achieved by cooperative studies between microbiologists, pharmacologists, chemists and clinicians.

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