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INHIBITORY ACTIVITY OF POMEGRANATE PEEL EXTRACT AGAINST BACTERIA *CRONOBACTER SAKAZAKII* ISOLATED FROM DIFFERENT FOOD

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ABSTRACT

The present study was conducted to demonstrate the effectiveness of the pomegranate peel aqueous extract against *Cronobacter sakazakii* (*C. sakazakii*) isolated from different food sample, *C. sakazakii* was isolated and identified with biochemical tests. On the other hand, active compounds were extracted from pomegranate peel and a concentration of (0.8,1.6,3.2,6.4,12.8,25.6,51.2,100) mg/ml was prepared. The effectiveness of the pomegranate extract was then evaluated against the growth of the isolates under study using Minimum inhibitory concentration method, the results showed a sensitivity of *C. sakazakii* to all concentrations. The results showed a difference in the inhibitory effect according to the concentration used. The bacteria were more sensitive to the concentration of 100 mg/ml and less sensitive to the concentration of 25 mg/ml by measuring the diameters of the bacterial inhibition zones of the different concentrations of pomegranate peel extract, the largest diameter inhibition was 37 mm at 100 mg/ml, while the 25 mg/ml concentration showed the lowest diameter was 15 mm. The results of the study suggest that pomegranate peel extracts have an inhibitory effect on *C. sakazakii* and increase this activity by increasing the concentration used.

Keywords: Food sample Pomegranate peel extracts, Minimum inhibitory concentration.

الفعالية التثبيطية لمستخلص قشر الرمان ضد بكتريا *Cronobacter sakazakii* المعزولة من أغذية مختلفة

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الخلاصة

أجريت الدراسة الحالية لبيان فعالية المستخلص المائي لقشر الرمان ضد *C. sakazakii* المعزولة من عينات غذائية مختلفة، وتم عزل *C. sakazakii* وتشخيصها بالاختبارات الكيموحيوية. ومن ناحية أخرى، تم استخلاص المركبات الفعالة من قشر الرمان وتم تحضير تركيز (0.8،1.6،3.2،6.4،12.8،25.6،51.2 و100 ميكروجرام/مل). تم بعد ذلك تقييم فعالية مستخلص الرمان مقابل نمو العزلات قيد الدراسة باستخدام طريقة التركيز المثبط الأدنى، أظهرت النتائج حساسية C. sakazakii لجميع التراكيز. أظهرت النتائج اختلافاً في التأثير المثبط تبعاً للتركيز المستخدم. وكانت البكتيريا أكثر حساسية لتركيز 100 ملغم/مل وأقل حساسية لتركيز 25 ملغم/مل وذلك بقياس أقطار مناطق التثبيط البكتيري للتراكيز المختلفة لمستخلص قشر الرمان، وكان أكبر قطر تثبيط 37 ملم عند 100 ملغم/مل، بينما التركيز 25 ملغم/مل أظهر أقل قطر 15 ملم. وتشير نتائج الدراسة إلى أن مستخلصات قشر الرمان لها تأثير مثبط لبكتيريا Cronobacter sakazakii وتزيد من هذا النشاط عن طريق زيادة التركيز المستخدم.

الكلمات المفتاحية: عينات غذائية، مستخلص قشور الرمان، التركيز المثبط الأدنى.

INTRODUCTION

Cronobacter spp (previously known as *Enterobacter sakazakii*) are Gram-negative, catalase positive, oxidase negative, motile by peritrichous flagellae, rod shaped, non sporforming bacteria belonging to the *Enterobacteriaceae* family. This ubiquitous microorganism has been associated with severe neonatal infections; these include meningitis, meningoencephalitis, sepsis, and necrotizing enterocolitis. It is also associated with serious sequelae including brain abscess and impaired sight and hearing (**Farmer *et al*., 1980**). The bacterium was first implicated in a case of neonatal meningitis in 1958 when an outbreak resulted in the death of two infants in England (**Maclean *et al*.,2008**). Although the frequency of infection generally tends to be low, the prognosis is poor with case-mortality rates varying from 33-80% among infected infants (**Lai *et al*.,2001**). Consequently, *Cronobacter* spp. have been ranked as a 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' by the International Commission for Microbiological Specifications for Food (ICMSF), which places this pathogen in the same group as *Listeria monocytogenes, Cryptosporidium parvum* and *Clostridium botulinum* types A and B (**ICMSF, 2002**). , the species of E. sakazakii was defined to include 5 genomogroups, which were differentiated according to the division of the 15 *E. sakazakii* biogroups. Accordingly, in 2007 the Cronobacter genus was first defined, and this definition was subjected to more revisions in 2008 and 2012. Currently, Cronobacter genus consist of 7 species; *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis, C. universalis and C. ondimenti* (**Iversen *et al*., 2007a; Joseph *et al*., 2011**). It is known that *C. sakazakii, C. malonaticus* and C. turicensis are the only species associated with clinical incidence and particularly with neonatal infections so far (**Stephan *et al*., 2010; Hariri *et al*., 2013; Asato *et al*., 2013; Holý *et al*., 2014**) ,*Punica granatum* L. (pomegranate) fruit belongs to the Punicaceae family and is distinguished by its high nutritive benefits assignable to their bioactive components of phenolic acids, flavonoids and tannins (**Coronado *et al*., 2021**) In this regard, pomegranate plant has been assayed for possible healing effects recording antiradical, antimicrobial, anti-inflammatory, hypolipidemic, antiproliferative and hypoglycemic properties (**Di sotto A. *et al*., 2019**) .The potential antiradical and antitumor efficiency of pomegranate peel extracts has been directly assigned to their phytoactive constituents of polyphenolic compound (**MasciA ,*et al*,. 2016**). Punica granatum L. peels were previously described to exhibit antimicrobial efficiency against food borne pathogens involving *Escherichia coli*, *Bacillus subtilis, Penicillium italicum* and *Fusarium sambucinum* (**Hunter *et al*., 2008**) .Moreover, pomegranate exhibited strong antioxidant activity due to the prevalence of several active phytochemicals as polyphenols, flavones, flavonoids, anthocyanins and catechins in seeds, fruits and peels of pomegranate (**Kothary *et al*., 2009**).Furthermore, pomegranate has been reported as a potential source of anti-tumor agents owing to the prevalence of many active phytochemicals as polyphenols and flavonoids (**Al-Lami *et al*., 2015**). The aim of this study to demonstrate to demonstrate the effectiveness of the pomegranate peel aqueous extract against *Cronobacter sakazakii* isolated from different food sample.

MATERIAL AND METHODS

Sample Collection

After recording the labeling information name of company, commercial name of product, contents, origin, date of expired and production, batch number, 50 samples were collected from various food sources form local market in Baghdad during period January 2021, to December 2022. The samples included 10 gm from powdered infant formula, Hash meat, spices, and dairy products and 5 gm from Cereals and vegetable as show in table (1).

Table (1): Food samples used for isolation of *Cronobacter spp*.

Food sample	Type of sample	Amount (gm)
Powdered Infants formula	almudhish, dialac, Nido, Similac novolack,rainbow	10
Spices, herbs and dried food	thyme, cumin,cinnamon, ginger,tea,chamomile semolina,sesame	10
Cereals and flour	flour, rice adass, homs, corn,	5
Meats and meat products	hash spiced meat, chicken meat, sausage, processed meat	10
Dairy Products	cheese, yogurt, cream	10
Vegetables	cucumber Vegetables carrot, green olive,	5
Total Number		50

These samples were collected in packages and bags (Plastic or paper) sterilize. After completing the sampling, the following information was documented before being sent to the laboratory and then transported to the laboratory. Food samples were kept at 4 °C until use.

Culture Media

The following culture media were present in this study for isolate *C. sakazakii* include Violet red bile glucose agar, Brain heart infusion broth ,Enrichment Enterobacteraceae broth ,Tryptic soy agar and chromogenic agar all this media were prepare according to the manufacturer's instructions and use medium EEbroth for activation ,TSA and chromogenic agar used as differential medium ,All culture media was prepared according to the manufacturing company instructions pH was adjusted with 0.1 N NaOH or 0.1N HCl , The culture media used in this study was sterilized, which needs to be sterilized by autoclaving at 121ºC (15 pound/Inch $_2$ pressure) for 15min , except Violet Red Bile Glucose Agar (VRBGA) sterilized by boiling(**kim *etal*., 2008**).

Isolation from food sample

The isolation of *Cronobacter spp* from different food samples was done according to the Food and Drug Administration (**2012**) with modification (**kim *et al.*, 2008**). food samplewere cultured on VRBGA media and incubated for a while 24 h at 37°C, after which all samples were streaking on Tryptic soy agar and incubated with temperature25 °C for 72 h, and finally it was cultured on chromogenic agar It was incubated at 37 °C for 24 h.

Identification

Bacterial isolates were identified according to microscopical and morphological features by viewing colonies developing on TSA and chromogenic agar, biochemical tests, and molecular analysis (**Barron *et al.*, 2007; Iversen *et al.*, 2007**)

Pomegranates peel

Pomegranates fruits were collected from different places in the local markets in Baghdad-Iraq, using plastic bags and sent to the laboratory. The pomegranate seeds were removed and separated from the peels, then they were dried using a convection oven, then it was first grounded using a ceramic mortar and then blender using an electric mixer for fine powder. The powder was kept in clean plastic bags and marked with the name of the plant and the date of collection, then kept in a dark sterile place away from moisture at room temperature until it was used later.

Extraction technique:

Aqueous Extract (AE)

The water was extracted from the plant according to the method described by Pin-Der and Gow-Chin,(1997), where 10 g of each sample of the dried plants was taken with 150 ml of distilled water at boiling point and left for 2 h on the magnetic mixture was filtered by funnel through filter paper (What man No.1) and then the concentrated extract was poured into a petri dish and placed in an electric oven a temperature ranging from 45-55ºC until the extract dried ,then the dried powder was scraped and collected in dry sterile containers and kept until use .

Ethanolic Extract (EE)

The extraction was carried out according to the method described by (**Zhou *et al.*, 2005**), as 10 gm of each sample of the mentioned dried plants prepared was taken with 150 ml of 70% ethanol alcohol and left for 2 h on the magnetic stirrer. Filter by funnel through a filter paper (Whatman NO.1), then pour the extract into petri dishes and put in the electric oven at a temperature of 45-55ºC until dry, then the dried powder was scarped and collected in dry test tubes and kept until use.

Preparing the stock solution of the extraction

Weigh 1 g of the obtained extract powder was used, dissolved in 10 ml of solvent distilled water in a sterile glass container and left for 3 d in the laboratory until the extract dissolved well. The concentration of the stock solution of the extract becomes 100 mg /ml.

Preparing the extract concentrations used in the present study

The stock solution of plant extracts (aqueous and alcoholic) was prepared by dissolving 100 mg of dry extract/10 ml of distilled water and the following concentrations were prepared from it: 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2 and 100 mg/ml **(Teramoto *et al.*, 2010).**

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of pomegranate peel aqueous extract against *C. sakazakii* were determined as described previously (**Shi *et al.*, 2018**), with some modifications. Briefly, overnight bacterial culture was diluted 400× in TSB medium (approximately1×10^8 cfu/ml) before 125 ml of the

diluted culture were added to individual wells of a 96-well Microtiter plate. Equal volumes of pomegranate peel aqueous extract solution were gently added to each well to achieve final extract concentrations of 0 (control), 10, 25, 50 and 100 mg/ml. TSB medium containing 0.1% DMSO was used as the negative control. Plates were incubated at 37°C for 24 h, and cell growth was monitored at 600 nm at 1-h intervals using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The MIC of extract was defined as the lowest concentration at which there was no visible growth of *C.sakazakii* (**Jasim *et al.*, 2015**).

Determination of Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration is explained as the least concentration of the pomegranate peel extract showing bactericidal activity. After 24 h incubation, 100 µL from the well of the micro-broth assay plates was cultured onto MHA plates then the plates were further incubated at 37 °C for 24h.The minimum concentration of extracts exhibiting no visible bacterial growth was recorded as MBC (**Saleh *et al.*, 2023).**

RESULTS & DISCUSSION

Through the results of isolation, it was found that 36 bacterial isolates (72%) that were isolated from 50 food samples gave yellow colonies and turbidity (**Mossawi *et al.*, 2015**).All positive samples that were cultured on Violet Red Bile Glucose Agar (VRBGA) the results showed that 10 bacterial isolates (27.8%) grew on VRBGA medium and gave typical colonies of Enterobacter spp. (Cronobacter), which appeared in the form of pink or purple colonies, because this medium contains crystal violet color and bile salts (**Al-joubori *et al.*, 2014**). This is why the color of the colony is pink (FDA, 2002) as shown in Figure (1).

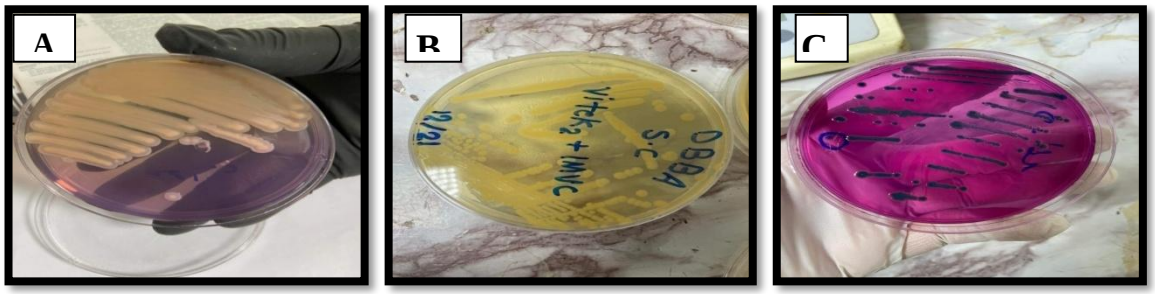

Figure (1): (A)*Cronobacter spp* on VRBGA (B) On Tryptic soy agar (C) On Chromogenic agar.

MIC and MBC of *C. sakazakii* by Pomegranate peel extracted.

The Minimum Inhibitory Concentrations (MICs) for Pomegranate peel extraction against *C. sakazakii* isolates were determined using the micro dilution method in Mueller-Hinton broth, and the results were interpreted after 24 hours of incubation at 37 °C according to the Clinical Laboratories Standards Institute CLSI (2020) (Table 1). The results of MIC confirmed the previous results of disc diffusion method, where the current study showed the high level sensitive of most isolates to the different concentration of pomegranate peel aqueous extract **(Mahindroo *et al.*, 2016).** The result showed strong antibacterial activity against *C. sakazakii*, with an observed MIC of 100 mg/ml for both strains, the effects of

pomegranate peel aqueous extract on the growth of *C. sakazakii* strains are shown in table(2) concentration of 51.2 µg/mL, the lag phase of both *C. sakazakii* cultures was longer than that of the control culture grown in the absence of aqueous extract However, the growth weak of *C. sakazakii* treated with 12.8,6.4, 3.2, 1.6, or 0.8 mg/ml aqueous extract were not different from that of the untreated control.**(Jaber *et al.*, 2015).**

Table (2): MIC and MBC of Pomegranate peel toward ten isolate of *C.sakazakii*.

No	Strain	Origin	MIC (µL/mL)	MBC (µL/mL)
1	*C.sakazakii* ATCC (CFSAN068773)	Infant formula	25.6	51.2
2	A1	Infant formula	25.6	51.2
3	A2	Infant formula	51.2	102.4
4	A3	Hash Meat	51.2	102.4
5	A4	Hash Meat	25.6	51.2
6	A5	Spices	25.6	51.2
7	B1	Blood	51.2	40.2
8	B2	Blood	6.4	12.8
9	CSF1	Cerebrospinal fluid	25.6	51.2
10	CSF2	Cerebrospinal fluid	51.2	102.4
11	CSF3	Cerebrospinal fluid	51.2	102.4

CONCLUSION

Through studying the effect of aqueous extract of pomegranate peels on the growth of *C. sakazakii* bacteria, it was found that growth is affected, and this effect increases with increasing concentration of the extract. We note that it was more sensitive to the concentration of 100 mg and less sensitive to the concentration of 0.8 mg. Therefore, this extract can be used and adopted as one of the methods to control the growth of bacteria. *C. sakazakii*.

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