Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University.

Microbiology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers related to the research across the whole spectrum of the subject. These including bacteriology, virology, mycology and parasitology. In addition, the journal promotes research on the impact of living organisms on their environment with emphasis on subjects such a resource, depletion, pollution, biodiversity, ecosystem…..etc

www.eajbs.eg.net

Citation: *Egypt. Acad. J. Biolog. Sci. (G. Microbiolog) Vol.8 (1)pp.7 -12(2015)*

Egypt. Acad. J. Biolog. Sci., 8(1): 7 – 12 (2016)

Egyptian Academic Journal of Biological Sciences
G. Microbiology

ISSN: 2090-0872

www.eajbs.eg.net

Evaluation of Antimicrobial Activity of *Punica granatum* Pericarp Extracts against *Helicobacter pylori* Resistant to Clarithromycin and Metronidazole

Zeena G. Faisal
Department of Biology, College of Education, Iraqi University, Baghdad, Iraq
Corresponding author g-mail: Zeenaalbayati76@ gmail.com

ARTICLE INFO

Article History
Received: 16/3/2016
Accepted:16/4/2016

Keywords:
Helicobacter pylor
Punica granatum L.
Bioactive components
Antibacterial activity

ABSTRACT

Helicobacter pylori (*H. pylori*) is the responsible bacteria for many gastric disorders such as gastritis, gastric ulcer, and gastric cancer. Half of the world's population is infected with *H. pylori*. The recent surge in multidrug resistant bacteria necessitate the need for additional preventative and therapeutic options to conventional drugs. Interestingly, the use of medicinal plants such as *Punica granatum*, commonly known as pomegranates, is being increasingly used throughout the world because of their efficacy and low toxicity.

The present study indicates the presence of various bioactive components in aqueous and ethanolic crude extracts such as alkaloids, flavonoids, glycosides, tannins, and saponins. Ethanolic extract contains all detected chemical compounds compared with aqueous extract, making the ethanolic extraction more potent than aqueous extraction. The aqueous and ethanolic extracts of *P. granatum* pericarp were tested for their antibacterial activity against ten identified clinical isolates of *H. pylori* (have resistance pattern to clarithromycin (25 µg) and metronidazole (5µg), or one of them) by disc diffusion technique. There is inverse relationship between the concentration of the aqueous or ethanolic extract of the pomegranate peel and the growth of bacteria, whereas, high concentration decrease growth of bacteria. Ethanolic extract significantly reduces the growth of *H. pylori* in a higher degree (with best MIC 2mg/ml), compared with aqueous extract (with best MIC 3mg/ml). In conclusion, present study showed that pomegranate peel extracts were capable to inhibit the growth of *H. pylori in vitro*, possibly via its high antioxidant activity that it contain.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram negative spirally to coccid shapes, microaerophilic bacterium which is commonly found in the stomach of half of the world's population (Czinn, 2005). The bacteria's shape and the way they move allow them to penetrate the stomach's protective mucous lining, where they produce substances that weaken the lining and make the stomach more susceptible to damage from gastric acids (Wyatt and Dixon, 1988).

It is a major etiologic agent in chronic gastritis, peptic ulcer, and gastric cancer (Voravuthikunchai *et al.*, 2006) . Even though triple therapies consisting of two antibiotics and proton pump inhibitor demonstrate high eradication rate, antibiotic resistance rates are now increasing (Ulmer *et al.*, 2003). Furthermore, undesirable side effects such as diarrhea, nausea, vomiting, epigastric pain, and abdominal discomfort are often unavoidable (Wermeille *et al.*, 2002). Therefore, it is necessary to introduce alternative remedial regimens that are effective and free from side effects. One of these resources is medicinal plants that some of their therapeutic properties have been recognized in folk medicine. Most people have positive attitude toward natural products due to their natural origin and lesser toxicity (Naik *et al.*, 2003) .

A number of medicinal plants have been reported to have antibacterial activity against *H. pylori* (Moghaddam, 2011). However, there have been few detailed studies on their antibacterial mechanisms. Hydrophobic interactions appear to be commonly involved in prokaryotic and eukaryotic cell interactions. The adhesion of pathogenic bacteria on host cell is required in many Gram- negative intestinal pathogenic bacteria-induced infections and can be influenced by the surface hydrophobicity of the microbial cell (Voravuthikunchai *et al.*, 2006). Recently, a series of studies have demonstrated that aqueous extracts of certain plants, such as *Punica granatum* L., *Quercus infectoria*, and *Uncaria gambir* can affect the cell surface hydrophobicity of gram-negative bacteria including *E. coli*, *Acinetobacter baumannii* (Turi *et al.*, 1997), and *H. pylori* (Annuk *et al.*, 1999).

Pomegranate (*Punica granatum* L.) has long been esteemed as food and medicine. It has a long history of antibacterial use dating back to biblical time. It is used in Siddha, Ayurveda, and Unani medicine especially for the treatment of Gastro-Intestinal (GI) diseases (Pradeep *et al.*, 2008). Egyptians used pomegranates to treat a number of different infections (Farmahan, 2004). Over the years, there have been many small studies undertaken in different areas of the world on the bactericidal effects of pomegranate peels on a number of highly pathogenic and drug resistant strains (Prashanth and Amit, 2001). *P. granatum* has been shown to have antibacterial effect (Duman *et al.*, 2009) and can minimize the problem of antibiotic resistance *H. pylori* by increasing the cell surface hydrophobicity of *H. pylori* strains (Voravuthikunchai *et al.*, 2006).

The aim of this study was to investigate whether pomegranate peel extracts have *in vitro* curative effect toward *H. pylori*, and prove the importance of *P. granatum* traditionally used in folk medicine.

Materials and Methods

Bacterial Strain: Ten identified clinical isolates of *H. pylori* were obtained from Baghdad University, College of Science, Laboratory of Biology Department.

Preparation of Pomegranate Peel: Pomegranate peel was collected, washed with distilled water, dried at 60°C overnight, cut into small pieces, and crushed in a mechanical motor. The powder was saved in sterile vials.

Preparation of Crude Extracts

Preparation of Aqueous Extract: 100g of powder were soaked in 500 ml of distilled water. The mixture was put into a vibratory incubator at room temperature for 7 days (**Voravuthikunchai *et al.*, 2006**). The mixture was subsequently filtered through Whatman No.2 filter paper for filtration and sterilization. Thus, we obtained solutions of aqueous in a concentration of (200mg/ml). The extracts were stored at 4°C for while in use.

Preparation of Ethanolic Extract: 100g of powder were soaked in 500 ml of 95% ethanol. The mixture was put into a vibratory incubator at room temperature for 7 days (**Voravuthikunchai *et al.*, 2006**). The mixture was subsequently filtered through Whatman No.2 filter paper for filtration and

sterilization. Thus, we obtained solutions of ethanolic extract in a concentration of (200mg/ml). The extracts were stored at 4°C for while in use.

Phytochemical Analysis of Different Crude Extracts: Crude aqueous and ethanolic extracts were tested for the presence of active principles such as glycosides, alkaloids, flavonoids, tannins, and saponins. Following standard procedures were used (Raman, 2006; Harborne, 2005):

- **Glycosides Detection:** Keller Killiani Test: Test solution was treated with few drops of glacial acetic acid and Ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides.
- **Alkaloids Detection:** Hager's Test: Test solution was treated with few drops of Hager's reagent (saturated picric acid solution). Formation of yellow precipitate would show a positive result for the presence of alkaloids.
- **Flavonoids Detection:** Ferric chloride test: Test solution when treated with few drops of Ferric chloride solution would result in the formation of blackish red color indicating the presence of flavonoids.
- **Tannins Detection:** Gelatin Test: Test solution when treated with gelatin solution would give white precipitate indicating the presence of tannins.
- **Saponins Detection:** Foam Test: Test solution was mixed with water and shaken and observed for the formation of froth, which is stable for 15 minutes for a positive result.

Preparation of Different Concentrations of Aqueous and Ethanolic Extract: Different concentrations [1, 2, 3, 4] mg/ml were prepared by taking a certain amount of aqueous or ethanolic extract (25 µl, 50 µl, 75 µl, and 100 µl, respectively) in 5 ml distilled water. A sterile filter paper discs (6mm) was soaked with 50 µl of the each concentration of pomegranate peel extracts that were prepared. The discs were left for drying.

Bacterial Susceptibility Testing:. Campylobacter selective agar (CSA) (Oxoid) plates were prepared by pouring 15ml of sterile molten media into sterile petridishes. The plates were allowed to solidify for 15 minutes. 100µl of *H. pylori* suspension in log phase (48hrs) (approximately 10^8 cfu/ml by using Mc-Farland's tube) were spread on plates, allowed the inoculums to dry, and prepared discs of different pomegranate extracts concentrations were placed on inoculated plates. Clarithromycin (25 µg) and metronidazole (5µg) are the antibiotics most commonly used in clinical practice and hence were used as positive control, while discs containing distilled water were used as negative control. All plates were incubated anaerobically at 37°C for 48-72 hr. The antibacterial activity was evaluated by measuring the radius of the inhibition zone (Erdogrul, 2002). Minimum inhibitory concentration MIC values were reported as the lowest concentration of the pomegranate extract that produced complete suppression of colony growth (Moghaddam, 2011).

RESULTS AND DISCUSSION

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substance from other sources including plants and microbes (Khan, 2009). Interestingly, *P. granatum* has been shown to have antibacterial, anti-inflammatory, and anticancer activity by different mechanisms (Rahimi and Arastoo, 2011). According to these properties, *P. granatum* or its active components may have a protective role on gastritis, gastric ulcer, and gastric cancer diseases which is induced by *H. pylori*. It has been shown that *P. granatum* has anti adhesive activity against *H. pylori* and can inhibit attachment of the *H. pylori* to gastric

mucosa by altering the cell surface hydrophobicity of this bacterium, so it will possibly inhibit *H. pylori* infection by this mechanism (Khan, 2009; Rahimi and Arastoo, 2011).

The phytochemical screening of *P. granatum* aqueous and ethanolic crude peel extracts has revealed the presence of alkaloids, flavonoids, glycosides, tannins, and saponins (Table 1). All of which are known as antioxidant, and have antibacterial activity (Khan, 2009). It is obviously in Table1 that the ethanolic extract was generally more potent in extraction of bioactive components than the aqueous extract, probably because the active principles in the plant dissolved more in less polar solvent (ethanol) than in water (Farmahan, 2004).

The preliminary phytochemical testes are helpful in finding chemical constituents in the plant material that may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compounds.

Table 1: Qualitative Analysis of some Bioactive Components in Crude Extracts

Bioactive components	**Glycosides**	**Alkaloids**	**Flavonoids**	**Tannins**	**Saponins**
Aqueous extract	+	-	+	-	+
Ethanolic extract	+	+	+	+	+

+ positive test
- negative test

Ten identified clinical isolates were checked for their antibiotic resistance pattern (Table 2). Three isolates of *H. pylori* (Z(2), Z(3), Z(8)) were sensitive to clarithromycin and metronidazole, while four isolates (Z(1), Z(5), Z(9), Z(10)) showed high resistance for them.

H. pylori Z(6) and Z(7) showed resistance to metronidazole and more sensitive to clarithomycin, while *H. pylori* Z(4) showed resistance to clarithomycin and has few sensitivity to metronidazole. This is a reflection for the misuse of antibiotics.

Table 2: Susceptibility of *H. pylori* Strains to Antibiotics Used in the Study

	inhibition zone of antibiotics (mm) of radius	
Bacterial Strain	Clarithomycin(25 µg)	Metronidazole(5 µg
H. pylori Z(1)	Resistance	Resistance
H. pylori Z(2)	13	10
H. pylori Z(3)	10	12
H. pylori Z(4)	Resistance	8
H. pylori Z(5)	Resistance	Resistance
H. pylori Z(6)	13	Resistance
H. pylori Z(7)	10	Resistance
H. pylori Z(8)	12	13
H. pylori Z(9)	Resistance	Resistance
H. pylori Z(10)	Resistance	Resistance

The antibacterial activity of the aqueous extract and ethanolic extract of pomegranate peel resulted as clear inhibition zone. Ethanolic extract exhibit a higher degree of anti- *H. pylori* activity compared with aqueous extract (Table 3). This is due to the quality and quantity of active components that released and dissolved in ethanol more than in water. The results shown in Table 3 indicate the inverse relationship between the concentration of the aqueous or ethanolic extract of the pomegranate peel and the growth of bacteria, whereas, high concentration decrease growth of bacteria. *H. pylori* (Z(1), Z(5), Z(9), Z(10)) showed resistance to lower concentrations of *P. granatum* peel aqueous and ethanolic extracts, while other strains showed sensitivity to all concentrations used. Therefore, the best MIC of aqueous extract

and ethanolic extracts are 3mg/ml and 2mg/ml, respectively. Distilled water did not have any effect on bacterial growth. It was also noted that leaving the dishes in the incubator for more than 48-72 hr, does not change the radius of inhibition zone.

In addition to antioxidant secondary metabolites, *P. granatum* also contains a number of water-soluble proteins, lectins, and carbohydrates which may bind specifically to sugar residues, polysaccharides, glycoproteins, or glycolipids such as adhesins present on cell surface of *H. pylori*. Previously described that *P. granatum* can increase the cell hydrophobicity of *H. pylori* (O'Mahony *et al.*, 2005). So, modulation of cell surface hydrophobicity of *H. pylori* by *P. granatum* may synergistically facilitate the elimination of the bacterial cells from the human body (Rahimi and Arastoo, 2011).

Table 3: Susceptibility of *H. pylori* Strains to Aqueous and Ethanolic extract of *Punica granatum*

Bacterial Strain	Radius of inhibition zone of aqueous extract(mm)				Radius of inhibition zone of ethanolic extract(mm)			
	1mg/ml	2mg/ml	3mg/ml	4mg/ml	1mg/ml	2mg/ml	3mg/ml	4mg/ml
H. pylori Z(1)	-	-	1	5	-	7	11	17
H. pylori Z(2)	7	10	13	17	9	13	17	22
H. pylori Z(3)	6	9	13	15	6	10	14	19
H. pylori Z(4)	4	7	8	10	6	9	12	15
H. pylori Z(5)	-	-	5	7	-	5	10	15
H. pylori Z(6)	5	7	8	11	7	10	14	18
H. pylori Z(7)	3	5	6	8	8	11	15	18
H. pylori Z(8)	6	10	13	17	9	13	18	23
H. pylori Z(9)	-	-	3	7	-	5	9	11
H. pylori Z(10)	-	-	2	5	-	6	9	13

- No response

The high activity of *P. granatum* peel against *H. pylori* could allow their use in the treatment of *H. pylori* infection. It may enable a treatment that is simple and relatively inexpensive by incorporation into the normal diet of the patient since the plant is already known to be safe and commonly employed in traditional folk medicine with no toxicity have been reported. Alternatively, they could be used in combination with antibiotics, possibly increasing the success of eradication, as has been demonstrated earlier with cranberry juice (Shmuely *et al.*, 2004).

CONCLUSION

The results of this study showed that the *P. granatum* may have a considerable potency at preventing or aiding the treatment of *H. pylori* and has curative potential as an antiulcer, possibly via its high antioxidant activity. The presence of phytoconstituents makes the plant useful for treating different ailments and has a potential of providing useful drug of human use. So you can use them in the production of therapeutic materials (antimicrobial agents), thus reducing the incidence of resistance among bacteria that appear as a result of the misuse and overuse of antibiotics. However, many more studies are needed to confirm the *in vivo* effects of *P. granatum* ingredients. Further studies are in progress to determine more precisely the effects of different fractions of the plant in order to provide an alternative treatment of *H. pylori* infection.

REFERENCES

Annuk, H.; Hirmo, S. ; Turi, E. ; Mikelsaar, M. ; Arak, E. and Wadstrom, T. (1999). Effect on cell surface hydrophobicity and susceptibility of *Helicobacter pylori* to medicinal plant extracts. FEMS Microbiol. Lett. , 172: 41 – 45.

Czinn, S.J. (2005). *Helicobacter pylori* infection: Detection, investigation and management. J. Pediatr. , 146: 21 - 26.

Duman, A.D; Ozgen, M.; Dayisoylu, K.S.; Erbil, N. and Durgac, C. (2009).

Antimicrobial activity of six pomegranate (*Punica granatum*) varieties and their relation to some of their pomological and phytonutrient characteristics. Molecules, 14: 1808 – 1817.

Erdogrul, O.T. (2002). Antibacterial activities of some plant extracts used in folk medicine. Pharmaceutical Biology, 40: 269 – 273.

Farmahan, H.L. (2004)." Pomegranate", in Recent Trends in Horticulture in the Himalayas, K.K. Jindal and R.C. Sharma, Eds., p.139, Indus, New Dehli , India.

Harborne, J. B. (2005). Phytochemical methods. New Delhi: Springer (India). p: 17.

Khan, S.A. (2009). The role of pomegranate (*Punica granatum* L.) in colon cancer. Pak. J. Pharm. Sci., 22: 346 - 348.

Moghaddam, M.N.(2011).*In vitro* inhibition of *Helicobacter pylori* by some spices and medicinal plants used in Iran. Global J.Pharmacol., 5(3): 176-180.

Naik, G.H. ; Priyadarsini, K.I. ; Satav, J.G. ; Banavalikar, M.M. ; Sohani, D.P. ; Biyani, M.K. and Mohan, H. (2003). Comparative antioxidant activity of individual herbal components used in ayurvedic medicine, Phytochemistry, 63: 97 - 104.

O'Mahony, R.; Al-khtheeri, H.; Weersekera, D.; Fernando, N.; Vaira, D.; Holton, J. and Basset, C. (2005). Bactericidal and anti-adhesive properties of culinary and medicinal plants against *Helicobacter pylori.* World J. Gastroenterol., 11: 7499 - 7507.

Pradeep, B.V.; Manojbabu, m.k. and Palaniswamy, M. (2008). Antibacterial Activity of *Punica granatum* L. against Gastro Intestinal Tract Infection Causing Organisms. Ethnobotanical Leaflets, 12: 1085-1089.

Prashanth, M.K. and Amit, A.(2001).Antibacterial activity of *Punica granatum*. Fitoterapia, 72:171-173.

Rahimi, H.R. and Arastoo, M. (2011). *Punica granatum* is more effective to prevent gastric disorders induced by *Helicobacter pylori* or any other stimulator in humans. Asian J. Plant Sci., ISSN 1682-3974.

Raman, N.(2006). Phytochemical technique. New India publishing agencies : New Delhi. p: 19.

Shmuely, H.; Burger, O.; Neeman, I.; Yahav, J.; Samra, Z.; Niv, Y.; Sharon, N.; Weiss, E.; Athamna, A.; Tabak, M. and Ofek, I. (2004). Susceptibility of *Helicobacter pylori* isolates to the anti-adhesion activity of a high-molecular-weight constituent of cranberry. Diagn. Microbiol. Infect. Dis., 50: 231 - 235.

Turi, M.; Turi, E.; Koljalg, S. and Mikelsaar, M. (1997). Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *E. coli* strains of different origin. APMIS, 105 : 956 – 962.

Ulmer, H. J.; Backerling, A. and Gatz, G. (2003). Recent use of proton pump inhibiter based triple therapies for eradication of *Helicobacter pylori*: abroad data review. *Helicobacte pylori* , 8 : 95 - 104.

Voravuthikunchai, S.; Limsuwan, S. and Mitchell, H.M. (2006). Effect of *Punica granatum* pericarps and *Quercus infectoria* nutgalls on cell surface hydrophobicity and cell survival of *Helicobacter pylori*. J. Health Sci., 52(2): 154 – 159.

Wermeille, J. ; Gunningham, M. ; Dederding, J.P.; Girard, L.; Baumann, R.; Zelger, G.; Buri,P.; Metry, J.M.; Sitavance, R. ; Gallaz, L.; Merki, H. and Godin, N. (2002). Failure of *Helicobacter pylori* eradication: is poor compliance the main cause? Gastroenterol. Clin. Biol., 26: 216 - 219.

Wyatt, J. I. and Dixon, M.F. (1988). Chronic gastritis – a pathogenic approach .J. Pathol. , 154: 113 - 124.