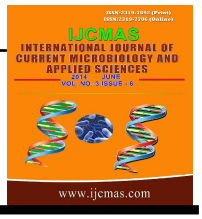
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 6 (2014) pp. 810-818**
http://www.ijcmas.com



Original Research Article

In Vitro and In Vivo Antibacterial Activity of Aqueous and Alcoholic Extracts of *Punica granatum* Peels against some Burn Infections Bacteria

Khadija Kh. M. Barzani[1]*, Souadkh.Ibrahim[2] and Sawsan M. A.Sorchee[1]

[1]Department of Biology, College of Education, University of Sallahaddin, Iraq
[2]Department of Biology, College of Education - Ibn Al-Haitham, University of Baghdad, Iraq
Corresponding author

A B S T R A C T

In current study 100 isolates of different bacterial species were isolated from 200 patients with burn infection within four months from Jun to September,2013 inNuman hospital in Baghdad city/Iraq. All isolates identified depending on morphological, cultural and biochemical tests and the results showed that the *P. aeroginosa*was the most common injuries burns with percentage 35% ,followed by *K. peumoniae, S. aureus,E. coli* (30%, 20%,15%) respectively and the susceptibility of the above isolates to nine antibiotics were tested, the results showed that the Ciproflaxacin and streptomycin were the highest effective antibiotics with a percentage 52.75%, 51.75% respectively, while all of the isolated bacteria were resistant to trimethoprim 95.25% and variable susceptibility to other antibiotics.As well as the antibacterial activity of aqueous and alcoholic extracts of*Punicagranatum* peels were studied against all isolated bacteria and the results found that the MIC of aqueous and alcoholic extractswere ranged between 80-140mg/ml and 40- 80 mg/ml, While the inhibition zonewere ranged between 11-18 mm and 9- 22 mm respectively . Furthermore, the results of the in vivo assay on thirty mice model showed that the treatment of mice with the 100mg/ml of aqueous extract and Chloramphenicolhave more effectiveness than other concentrations and the number of bacteria *P. aeroginosa*was reduced significantly ($P \leq 0.001$) during the treatment days.

Keywords

Punica granatum peels, Antibacterial activity, MIC, Inhibition zone, In vivo (mice model).

Introduction

Infection is an important cause of morbidity and mortality in hospitalized burn patients (1). In spite of considerable advances in medicine and specific treatment of burn, infection continues to pose the greatest danger to burn patients and approximately 73% of all deaths within the first 5 days post burn are directly or indirectly caused by septic processes (2). The rate of nosocomial infections is higher in burn patients due to various factors like nature of burn injury itself, immunocompromised status of the patient (3), age of the patient, extent of

injury, and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, colonization of the burn wound site, systemic dissemination of the colonizing organisms (4). In addition, cross-infection results between different burn patients due to overcrowding in burn wards (5). The burn wound represents a susceptible site for opportunistic colonization by organisms of endogenous and exogenous origin; thermal injury destroys the skin barrier that normally prevents invasion by microorganisms. This makes the burn wound the most frequent origin of sepsis in these patients (6). Currently the common pathogens isolated from burn patients are *Psudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella spp*. and various coliform bacilli (7,8). Multidrug-resistant bacteria have frequently been reported as the cause of nosocomial outbreaks of infection in burn units or as wound colonizers in burn patients (9).

Medicinal plant**s** are the source traditional medicine, herbal medicine and various dietary supplements. World-wide pharmaceutical scientist pushing strong demand to find out active pharmaceutical ingredients from medicinally important plant (10). *Punica granatum* L., commonly known as pomegranate, is a fruit-bearing deciduous shrub or small tree, native to Asia and belongs to the family Punicaceae (11). Different parts of the plant such as bark, leaves, immature fruits and fruit rind have medicinal significance (12). *Punica granatum* is widely employed in various countries as a source of therapeutic agent against a variety of pathogenic microbes (13).

However, to date no studies regarding the antimicrobial activity of *P. granatum* peels have been conducted in Iraq. Therefore, the goal of this study is to evaluate the an

Materials and Methods

Collection of samples

Tested bacteria *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were obtained from 200 patients with burn infection and with different ages ranged between 20-50 years within four months from Jun to September, 2013 in Numan hospital in Baghdad city/Iraq. All isolates identified depending on morphological, cultural and biochemical tests as described by (14)

Antibiotic Sensitivity Test

The sensitivity of the isolated bacteria to different antimicrobial agents (Ampicillin (AP) 10µg/disc, Refapin (Rif) 5µg/disc, Chloramphenicol (Cm)10µg/disc, Tetracyclin (TE) 30µg/disc, Streptomycin (Sm) 10µg/disc, Ciprofloxacin (Cip)5µg/disc, Erythromycin (Er) 10µg/disc, Trimethoprim (Tm) 10µg/disc, Nalidixic acid (NA) 30µg/disc were determined by using method of Kirby-Bauer as described by (15).

Source of *Punica granatum* L.

Peels of *P.granatum* were identified and purchased from alzahraa Herbarium to cure by DrAlliAlmosawee, science collage, Biology Department, University of Baghdad.

Plant Extraction

The peels of P. *granatum*were air dried and powdered, and then 25g were extracted with 500mL of water and ethanol for 72h on soxhlet apparatus separately. After cooling the contents of

flasks were filtered and concentrated at 40^{o}C to obtain crud extract and stored at 4oC in dark vials until it used(16).

Phytochemical screening methods

The presence of alkaloids, flavonoids, tannins, glycosides and steroidal terpenes were determined as described by (17, 18).

Bacterial susceptibility testing
Agar diffusion test

Muller Hinton Agar (MHA) (HiMedia, India) was used to determine the diameter of inhibition zone (ID) by the well diffusion methods. The plates were inoculated with the standardized suspension (comparing with McFarland tube 0.5) of the test isolates. The plates were allowed to dry in the incubator for 30minutes at 37^{o}C and with the aid of a sterile standard core borer, 5 wells with 5mm in diameter were bored at equidistant. The bottoms of the wells were sealed with sterile molten nutrient agar to prevent seepage of the extract under the agar. Each of the aqueous and ethanolic crude extract was dissolved in Dimethyl sulfoxide (10% DMSO), final concentration 0, 2.5,5, 12.5, 25, 50, 100, 200mg/ml and 25µl of each extract was introduced into the appropriate well in the inoculated plate, in addition to sterile water and DMSO were used as negative controls.

The prepared plates were incubated at 37^{o}C for 24h. The resulting zones of inhibition were measured using a ruler calibrated in millimeters. The average of the three readings was taken to be zone of inhibition of the bacterial isolates in question at that particular concentration (19,20).

Determination of minimum inhibitory concentration (MIC)

The above mentioned extracts that showed antimicrobial activity were later tested to determine the MIC for each bacterial isolates. Four bacterial isolates *P. aeruginosaK. pnemoniae, S. aureus*and *E.coli*were grown in nutrient broth tubes separately for 6 h. Afterwards , 100 µL of 10^{6} cells/mL of growth culture were inoculated in nutrient broth tubes containing different concentrations of the extracts (0,10, 20, 30,40,50,60,70,80,90,100,110,120,130,140 ,150 mg/ml) for 24 h at 37 °Cseparately for each plant extract and each bacteriain addition to control samples, thenthe bacterial growth was evaluated on the basis of the turbidity of the suspension and all tubes were read by spectrophotometer (LKB- PYEUNICAM-SP 6.55, England) at 620nm. However, the MBC was determined by subculturing the test dilutions onto fresh solid medium and incubated further for 18– 24hours (21).

Inhibitory effect of *P. granatum* peels *on* growth of *P. aeruginosa* (*In vivo)*

Thirty male Balb/c mice (26 – 28 g) aged between 12 and 16 weeks were used for all in vivo experiments. They were kept in a light, food and temperature-controlled room and all mice were acclimatized for at least 1 week prior to beginning the experiments.The dorsal back skins of the mice were shaved and ethanol (70%) was used as antiseptic for the shaved region and then burned by using inflamed knife and then contaminated with bacteria *P.aeruginosa* (1.5×10^{8} bacteria /ml), which was the more common species in burn infection in present study. After two days of injury the inflammation, redness and suppuration region were observed and

the experimental mice were randomly divided into the following groups (n = 30)

-Group 1: untreated mice (control).
- Group 2: burn mice treated with ointment composed of antibiotic(Cm) .
- Group 3: burn treated with ointment (concentration 50mg/ml) consisting of 0.5g of aqueous extract added to 9.5g of Vaseline.
- Group 4: burn treated with ointment (concentration 100mg/ml) consisting of 1g of aqueous extract added to 9 g of Vaseline.
Group 5: burn treated with ointment (concentration 200mg/ml) consisting of 2g of aqueous extract added to 8 g of Vaseline.
Group 6: burn treated synergistically with ointment Cm and 100mg/ml of aqueous crude extract of *P.granatum*.

The treatment continued twice each day during 15consecutive days and the numbers of bacteria was calculated.

Statistical analysis

Results were expressed in Mean±SD and were analyzed by one way analysis of variance (ANOVA) and post hoc analysis was done using Tukeys using SPSS package version 16. P value< 0.05 was considered to be significant.

Results and Discussion

In present study, 100 isolates of different bacterial species were isolated from 200 patients with burn infection and *P.aeroginosa* was the most common injuries burns with percentage 35% (Figure 1), followed by *K.peumoniae* which achieved second place (30%), as well as recorded *S. aureus* proportion 20%, while the least isolated bacteria was *E.coli* (15%), these results were similar to that found by (22).

On the other hand, isolated bacteria showed a variation in their sensitivity and resistance to used antibiotics and the results revealed that Ciprofloxacin and streptomycin were the highest effective antibiotics against all of the isolated bacteria with a percentage (52.75%, 51.75%) respectively for both antibiotics, while all of the isolated bacteria were resistant to trimethoprim (95.25%) and theampicillin and nalidixic acid were located in the second gradewith percentage (79%, 78.75%) respectively, moreover the results of the present study showed that isolated bacteria gave variable sensitivity to other antibiotics as appeared in table (1).These finding were similar to resistant pattern of bacterial isolates in previous studies presenting public health problem (23,24). The high resistance of the bacterial isolates in this study to different antibiotics may be related to the presence and disseminations of the plasmids within heterogeneous populations of these bacteria(25), or may be due to that majority of the populace sampled purchases antibiotics in the open markets without any medical prescription, use of wrong concentration and for wrong diseases. The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and microbes (26).

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. The main objective of the present study was to evaluate the ability of *P. granatum* peel extracts to inhibit the growth of

Figure.1 Number and percentage of bacteria detected in burns samples

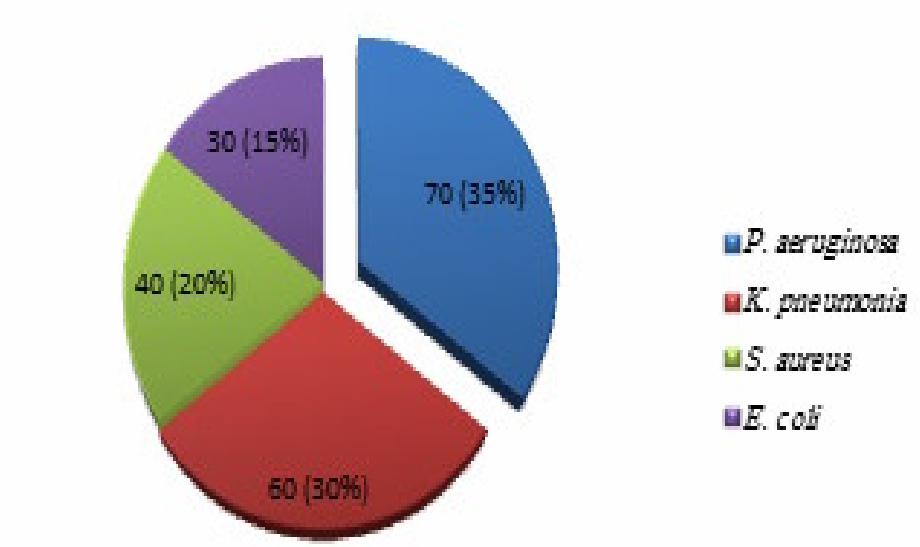


Table.1 Antimicrobial resistance percentage ofisolated bacteria from wounds of burned patients

Isolated Bacteria	No. of isolates	Tm S%	R%	AP S%	R %	NA S%	R%	Cip S%	R%	CM S%	R%	Sm S%	R%	Rif S%	R%	Er S %	R%	TC S %	R%
P. aeruginosa	35	0	100	28	72	28	72	10	90	81	19	81	19	28	72	28	72	10	90
K.pneumonia	30	0	100	18	82	9	81	82	18	18	82	9	91	18	82	27	73	0	100
S. aureus	20	19	81	19	81	10	90	100	0	28	72	82	18	28	72	35	65	82	18
E. coli	15	0	100	19	81	28	72	19	81	28	72	35	65	28	72	10	90	28	72
Total (%)		4.75	95.25	21	79	18.75	78.75	52.75	47.25	38.75	61.25	51.75	48.25	25.5	74.5	25	75	30	70

Figure.2 Minimum inhibitory concentration (MIC) of ethanolicand aqueous extract of *Punica granatum* peel on bacterial burn isolates

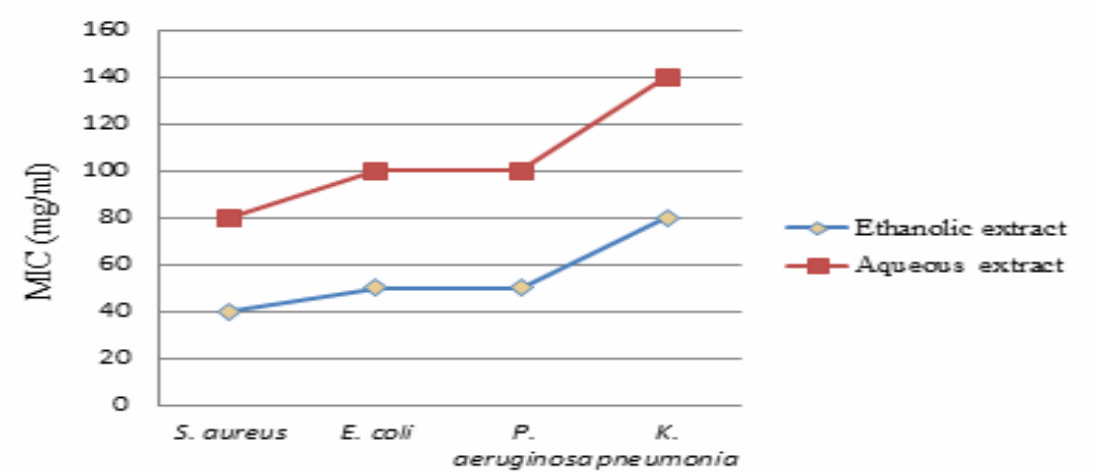


Table.2 Phytochemicals of *Punicagranatum* peel.

Chemical constituents	Ethanolic extract	Aqueous extract
Tannins	+	+
Flavonoids	+	+
Alkaloids	+	+
unsaturated sterols and triterpenes	_	-
carbohydrates and glycosides	+	-

Figure.3 Diameter of inhibition zones (mm) of ethanolic and aqueous extracts of *P. aeruginosa* peel against isolated bacteria.

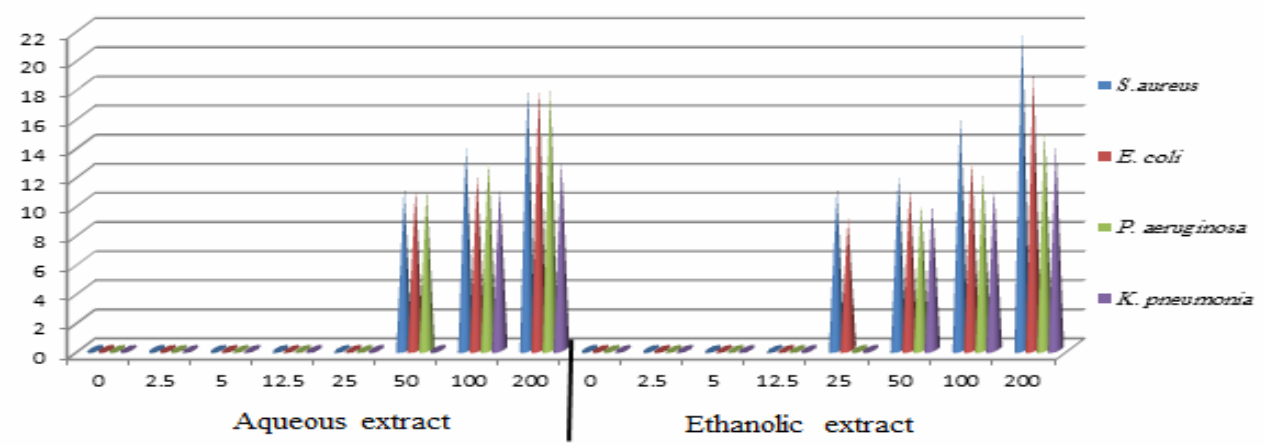


Table.3 Numbers of *P. aeruginosa* between the mice groups.

Duration of treatment per day	Group(1) Control	Group(2) CM	Group(3) Aqueous extract 50mg/ml	Group(4) Aqueous extract 100mg/ml	Group(5) Aqueous extract 200mg/ml	Group(6) CM + Aqueous extract 100mg/ml
3	0	150	180	171	162	120**
6	0	120	85	75*	88	5*
9	0	29	19	12	37	5*
12	0	0	3	1	6	0
15	0	0	0	0	0	0

* Significant at P≤0.001

isolated bacteria and to explore possible future use of these extracts as alternatives to common antibiotics and to determine their ability to enhance activity of antibiotics.

The MIC of both ethanolic and aqueous extracts was presented in (Figure 2). It found that the aqueous extract of the peels was effective against all tested bacteria with the MIC ranged between 80-140mg/ml.Moreover, the ethanol extract was the more effective against isolated bacteria with the MIC ranged between 40-80 mg/ml, this might be results due to some components present in peel extract(Table2) and this results were in agreement with(27).

Different concentrations of both extracts of pomegranate peels exhibited different inhibitory activity against all tested bacteria and the highest inhibition zones were between9-22 mm and 11-18 mm for both of ethanol and aqueous extracts respectively. However, the results showed that there were a significant differences at $P<0.05$ between concentrations (50,100, 200mg/ml) for aqueous extract respectively except for bacteria *K. pneumonia* which the affect were found only between 100- 200mg/ml.While for ethanolic extract it was showed a stronger effect than aqueous and there were a significant differences at $P<0.05$ between concentrations (25, 50,100, 200mg/ml) for *E. coli* and *S. aureus* respectively except *P. aeruginosa* and *K. pneumonia* which the affect were found between 50,100, 200mg/ml (Figure 3).These results may be due to the possibility of containing all active compounds and various concentrations had inhibitory effects on all types of microorganisms in vitro such as flavonoids, tannins, alkaloids, saponins and glycosides (28). Antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the bacterial cell wall, they may get attached to enzyme sites rendering those inactive(29), similar results were reported by(30,31).

The in vivo assay revealed that the treatment of mice with the different concentrations ofaqueous extract of *P. granatum* peel and Cm antibiotic during 15 daysand it was found to have marked effects on the number of *P.aeroginosa* especially the group six which represent 100mg/ml of aqueous extract and Cm antibiotic which showed more effectiveness than other groups and the number of bacteria was reduced significantly ($P\leq0.001$) from 120 to 5×106 cell/ml during the treatment days and this results due to P. granatum extract which have antibacterial activities and exhibited synergistic effects when used with antibiotic Cm as well as have wound healing properties. In addition, according to the results presented in table 3 it was noted that the group four (100mg/ml aqueous extract) significantly ($P\leq0.001$)was more effective than other groups and generally the skin of the mice was healed after nine days of treatments. Therefore, the current data clearly demonstrated the importance of plant extracts in the control of burn infection resistant bacteria which are becoming a threat to human health and the aqueous extracts of pomegranate peels are reported to have therapeutic properties.

References

1. McManus AT, Mason AD, McManus WF, Prutt BA (1994). A decade of reduced Gram-negative infections and mortality associated with improved

isolation of burned patients. Arch. Surg. Reig
2. A, Tejerina C, Condina J.(1992).Infections in burn patients. Annals of MBC;5:91–95.129:1306-1309
3. Pruitt BA, McManus AT, Kim SH and Goodwin CW (1998).Burn wound infections: current status. World J Surg. 22: 135-145
4. Pruitt BA, Colonel MC and McManus AD (1984). Opportunistic infections in severely burnt patients. Am J Med. 76: 146-154
5. Gupta M, Gupta OK, Yaduvansh RK and Upadhyahy J (1993). Burn epidemiology: the pink city scene. Burns. 19: 47-51.
6. Mooney DP and Gamelli RL (1989). Sepsis following thermal injury. Comp.Ther. 15:22 29.
7. Lawrence J. (1994).Burn bacteriology during the past 50 years. Burns;18:23–29.
8. Nudegusio L, Algimantas T, Rytis R,.(2004)Analysis of burn patients and the isolated pathogens. Lithuanian Surgery;2(3):190-93
9. Karlowsky JA, Jones ME, Draghi DC. (2004). Prevalence and antimicrobial susceptibilities of bacteria isolated from blood cultures of hospitalized patients in the United States in 2002. Ann ClinMicrobiolAntimicrob;(3):3-7.
10. Karmegam, N., M. Jayakumar and S. Karuppusamy(2012). Synergistic Antibacterial activity of four medicinal plants collected from Dharapuram Taluk of Truppur District, South India. J. Plant Sci., 7: 32-38.
11. Qnais, E.Y.; Elok, A.S.; Abu Ghalyun , Y. Y. &Abdulla,F.A.(2007). Antidiarrheal Activity of the Aqueous Extract of *Punicagranatum* (Pomegranate) Peels.Pharmaceutical Biology, 45(9) : 715–720.
12. Arun N. and Singh D.P. (2012). Punicagranatum: a review on pharmacological and therapeutic properties, IJPSR, 3(5), 1240-1245.
13. Vijayanand, S.andHemapriya, J. (2011).In vitro Antibacterial Efficacy of Peel and Seed Extracts of Punicagranatum L against Selected Bacterial Strains. Int J Med Res., 1(4): 231-234.
14. Arora, B. and Arora, D.R.(2007).Practical Microbiology . First ed. India. Binding House, Noida,UP.
15. Bauer, A.W.; Kirby, E.; Sherris, E.M.; Turk, M. (1966). Antibiotic by standardized single disk method. Am. J. Clin. Path. 45, 493-496(cited byNascimento etal.,2002).
16. Anesini, C.and Perez, C.(1993). Screening of Plant Used in Argentine Folk Medicine for Antimicrobial Activity . 39(2): 119-128.
17. Harbone JB.(1998). Phytochemical methods: A guide to modern techniques of plant analysis. London: Chapman and Hall;. p. 129,189,203.
18. HegdeChaitra, R. ; Madhuri, M. ; Nishitha, S.T. ; Arijit1,D. ; Sourav, B. &Rohit,K.C.(2012). Evaluation of Antimicrobial Properties, Phytochemical Contents and Antioxidant Capacities of Leaf Extracts of *Punicagranatum*L.ISCA J. Biological Sci. 1(2): 32-37.
19. Bilgehan, H. (2004). Klinikmik robiyolojiktanı. _afakMatbaacılık. 4. Baskı. Barı_ Yayınları Fakülteler Kitapevi, _zmir, : 777.
20. Junaid, S. A.;Olabode, A. O; Onwuliri, F. C.;Okwori, A. E. J. and Agina, S. E. (2006). The antimicrobial properties of *Ocimumgratissimum*extracts on some selected bacterial gastrointestinal

isolates. *Afr. J. of Biotechnology.*, 5(22): 2315 – 2321

21. NCCLS (National Committee for Clinical Laboratory Standard), (1999). Performance Standards for Antimicrobial Susceptibility Testing, 9 International Supplement. M100-S9, Wayne Pa.
22. Ibrahim,S.K.(2009).Comparative study of the effect of some plants extract and carboxylic acids on contaminating bacteria in burns infection.PhD,.Thesis, Baghdad, Iraq.
23. Tagoe, D. N.; Nyarko, H.; Arthur, S. A. and Birikorange, E. A. (2011). A Study of Antibiotic Susceptibility Pattern of Bacteria Isolates in Sachet Drinking Water Sold in the Cape Coast Metropolis of Ghana .Res. J. Microbio.;(6):453-458.
24. Khan, R. N. and Malik A. (2001). Antibiotic Resistance and Detection of A-Lactamase in Bacterial Strains of *Staphylococci* and *Escherichia coli* Isolated from Foodstuffs. World J. Microbiol. Biotechnol.;17:863-868.
25. Anderson,K.(2005).Is bacterial resistance to antibiotic an appropriate example of evolutionary change?Creat,Res.Soc.Quart.41(4):318 -326.
26. Erdogrul, O.T.(2002). Antibacterial activities of some plant extracts used in folk medicine. Pharmaceutical Biology., 40: 269-273.
27. Nuamsetti,T.; Dechayuenyong, P.& Tantipai bulvut,S.(2012). Antibacterial activity ofpomegranate fruit peels and arils. Science Asia 38 : 319–322.
28. Cowan, M.M.C (1999). Plant products as antimicrobial agents. Am. Soc. Microbiol. 12(4): 546-582
29. S. P. Voravuthikunchai, T. Sririrak, S. Limsuwan, T. Supawita, T. Iida, and T. Honda, (2000)"Inhibitory effects of active compounds from Punicagranatum pericarp on verocytotoxin production by enterohemorrhagic Escherichia coli O157:H7," Journal of Health Science, vol. 51, no. 5, 590–596.
30. Dahham,S.S.; Ali,M.N.; Tabassum H. &Khan,M.(2010). Studies on Antibacterial and Antifungal Activity of Pomegranate (*Punicagranatum*L.). American-Eurasian J. Agric. & Environ. Sci., 9 (3): 273-281.
31. Jocelem,M.S.; Ferreira,B. Rachel,T. b; Fúvia, D.O.B.; Carlos Tadeu,D.S.D. (2012). Increased Antioxidant Content in Juice Enriched with Dried Extract of Pomegranate (*Punica granatum*) Peel.*Plant Foods for HumaNutrition* , 67 (1) : 39 – 43.