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RESEARCH ARTICLE

EFFECT OF *PIMPINELLA ANISUM, SYZYGIUM AROMATICUM* AND *PUNICA GRANATUM* EXTRACTS ON GUT MICROBIOTA AND SOME BACTERIAL PATHOGENS

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ABSTRACT

Pathogenic bacteria is a major health problem that causing a large number of diseases and their treatments with antibiotics lead to the appearance of bacterial resistance to antibiotics. Moreovere, the use antibiotics eliminated the beneficial bacteria from the gastrointestinal tract, thus the effect of some plant extract. Three plants, *Pimpinella anisum, Syzygium aromaticum* and *Punica granatum,* used traditionally as medicine or food additive, were extracted and their inhibitory effects on four pathogenic bacteria and two genera of gut microbiota were determined. It was shown that all tested plant extracts showed an inhibitory effect on pathogenic bacteria, while gut microbiota was affected only by *S. aromaticum* and *P. granatum* extracts. The MIC of the methanolic extracts of the used plant extracts ranged from 3.1 to 93.7 mg/ml and no toxicity was found on *Artemia salina* of *Pimpinella anisum.* Additionally, *P. anisum* was increased the cell count of *Bifidobacterium* by studying the growth curve of gut microbiota.

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INTRODUCTION

The gastrointestinal tract (GIT) contained the largest proportion of the human microbiota specifically the colon (McCartney and Gibson, 2006) and gut microbial ecology employed several important functions for the human host. Gut microbiota performed as defense line against gut pathogens, help in nutrient processing, stimulation and modulation of intestinal immune response and regulation of host fat storage (Bäckhed *et al*., 2005; Palmer *et al*., 2007). The content of the normal colonic microbiota is itself somewhat proportionately in fecal samples (McCartney and Gibson 2006). The contents of the gut microbiota can be modified by changes in diet (Macfarlane and Macfarlane, 2003) and it is know that the GIT is one of the most metabolically and immunologically dynamic organs of the human body (McCartney and Gibson, 2006). Humans have often used plants to treat general infectious diseases, and some of these traditional medicines are still part of the usual treatment of different diseases (Dogruoz *et al*. 2008). Plants comprise a source of novel chemical compounds, which are of probable use in medicine (Gupta *et al*., 2012) and msplant extracts decrease ileal pH value, while increasing the quantity of lactic acid bacteria in the ileum and caecal contents of broiler chickens. It, also decrease the caecal Coliform and *Clostridium perfringens* counts (Vidanarachchi *et al*., 2006; Dalkilic *et al*., 2005).

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Specific types of gut bacteria are amplified in numbers in response to short-term dietary changes, as well as changes in resistant starch content (Martínez *et al*., 2013). Moreover, the search for plants derived antibiotics and dietary supplements have increased in recent years (Aly and Gumgumjee, 2011). Currently, there is a high demand for finding natural substances with antimicrobial activity as an alternative to the existing antibiotics suitable to numerous dangerous problems such as growing drug resistance of bacteria or undesirable side effects on natural gut bacteria. The aim of the present study was determination of the antimicrobial activity and toxicity of *Pimpinella anisum, Syzygium aromaticum*and *Punica granatum* extracts collected from Jeddah on gut microbiota and some bacterial pathogens.

MATERIALS AND METHODS

Pathogenic bacterial strains

Standard local pure culture of *Escherichia coli*, *Pseudomonas* sp. and *Klebsiella pneumonia* were provided by King Abdulaziz Hospital, Jeddah, Saudi Arabia. Also *Salmonella arizona* were obtained from Alborg laboratory culture collection, Jeddah, Saudi Arabia. All cultures were checked up again for purity using API 20 E test.

Gut microbiota collection

The fresh human faecal sample were collected in sterile containers, placed on anaerobic jar with new anaerogen sachet

as quickly as possible. The sample have been diluted to 10% (w/v) using sterile phospphate buffer and homogenized in the vortex and was put directly into the anaerobic jar with new anaerogen sachet to avoid contamination and loosing the anaerobic microflora (Noor *et al*., 2010). The method described by Noor *et al*. (2010) with some modification was used. One ml of faecal slurry was serially diluted from 10^{-1} to 10^{-6}. All procedures were carried out in an anaerobic conditions. Two different media were used for the bacterial isolation (MRS agar for *Lactobacilli* sp. and Beeren's agar for *Bifidobacterium* sp.) and incubation consitions for each bacterium were summarized in Table 1. The two bacterial isolates were stained using Gram stain, examined under light microscope and characterized (Hussein *et al*., 2001).

Collection of plant materials

The dried fruit of *Pimpinella anisum* and dried flowers of *Syzygium aromaticum* in addition to *Punica granatum* peels were bought from spices store and supermarket, Jeddah Saudi Arabia during summer 2014 (Table 2, Figure 1). All the plant materials were identified at Biology Department, Faculty of Scince, KAU, Jeddah.

Extraction procedure

The extraction was determined using the methods described by Khan and Hanee (2011) with some modification. Plant materials were washed individually with distilled water, dried and each plant was grinded into fine powder using electrical glinder. Each plant material was extracted using methanol extraction (125 g of plant material in 250 ml of 99% methanol for 48 hours at laboratory temperature). The supernant were then filtered and concentrated on a rotary evaporator at 42°C for methanol elimination. Under refrigerated conditions, the residue was dissolved in DMSO and kept in sterile bottle until use.

Susceptibility testing using Agar well diffusion method

Agar well diffusion method as described by Holder and Boyce (1994) was used for the test. Preculture of each test organism was prepared using the nutrient broth medium for bacteria. A sterile pipette was used to add 100 µl of the preculture containing 4×10^6 CFU/ ml of bacteria to each Petri dish containing 15 ml of already prepared Muller Hinton agar medium. Wells of 7 mm in diameter were made in the seeded agar using sterile cork borer. About 80 µl of the extract were added to each well. DMSO was used as negative control and the suitable antibiotic was used as positive control. Finally, incubation was carried out according to appropriate conditions of each bacterium.

Disc diffusion method (antibiotic sensitivity testing)

Antimicrobial susceptibility was studied using Muller Hinton agar by employing the method described by Bauer *et al* (1966). The test microorganisms were also tested for their sensitivity against the antibiotics. The cultures were enriched in sterile nutrient broth for 18-24 h at 37ºC. Using sterile cotton swabs, the cultures were aseptically swabbed on the surface of sterile Muller Hinton agar plates. Using an ethanol dipped and flamed forceps, the antibiotic discs were aseptically placed over the seeded agar plates sufficiently separated from each other to avoid overlapping of inhibition zones. The plates were incubated at 37ºC for 24 hours and the diameter of the inhibition zones was measured in mm.

Broth microdilution method

Broth microdilution method as described by Bonnavero *et al.* (1998) with some modification. This test was done to determine MIC of the tested plant extracts for the selected bacterial pathogens. Muller Hinton broth was used to grow the bacteria overnight and the growth was diluted to approximately10^4cell/ml. The tested bacterial samples were used to evaluate the inhibitory activity of an antibiotic and/ or plant extracts. Seeded broth with some drops of phenol red indicator was added into 12 wells in a microtiter plate (125 µl/ well), then 125µl of the selected plant extract was added to well 1 and the mixture was mixed. For serial dilution, about 125 µl of the well 1 was transferred to well 2 and so on and keep diluting the mixture, in this manner, through well 11. The last well (12) serves as a growth control. Incubate the microtiter plate at 37°C overnight in the shaking incubator. Determine the MIC results, by changing in colors of the broth or by using an ELISA reader. Presence of pink color indicates the MIC.

Toxicity assay of plant extracts

Brine shrimp lethality bioassay was carried out to investigate the toxicity of the plant extracts using Artemia *salina* (shrimp larvae) as test organism. Different dilution of plant extracts in DMSO at varying concentrations was made (100- 400 mg/ ml were made. After 6 h the average number of larvae that survived in each dish was determined under sterio microscope. The mean percentage of mortality was plotted against the logarithm of concentrations, the concentration killing fifty percent of the larvae (LC_{50}) was determined from the graph (Adoum, 2009).

Effect of some plant extracts on gut microbiota growth curve

The plant extracts which were none inhibitory for *Lactobacillus* sp. *Bifidobacterium* sp. were selected. The effect of these plants on bacterial growth curve was determined and compared to control. About 250 µl of the extract was added to 20 ml of sterile MRS broth in fermentation tube and inoculated with 1ml of each bacterial suspension ($4x10^4$ cfu/ ml) and control without plant extract was also prepared. Absorbance was made at 630 nm using spectrophotometer. Cultures were incubated under the appropriate condition of the microbe. Samples were withdrawn every 2 h, between 0-24 h for *Lactobacillus* sp. and 0-54 h for *Bifidobacterium* sp. Then three serial dilutions were performed using sterile diluent. Enumerations of viable cells were then performed using colony counter (Filocamo *et al*., 2012).

Statistical analysis

All values are the mean of three reading and were expressed as mean ±SD. Statistical analysis was performed with two samples, t-test, using R for statistical programing and $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

The emergence of antibiotic resistance has encouraged researcher to use medicinal plants, not only to determine claims of effectiveness and safety, but also to discover substitute candidates for drug development.

Table 1. The used bacteria and their growth conditions

Bacteria	Selective agar medium	Incubation temperature	Incubation period	Incubation condition
Lactobacillus	MRS	37ºC	1 day	Aerobic
Bifidobacteria	Beeren's	37ºC	3days	Anaerobic

Table 2. Plants and their scientific, common names, families and parts used

Scientific name	Common name	Plant family	Extracted part
Pimpinella anisum	Anise	Apiaceae	Fruits
Punica granatum	Pomegranate	Lythraceae	Peels
Syzygium aromaticum	Cloves	Myrtaceae	Flowers

Table 3. The antimicrobial activity (diameter of the inhibition zone, mm) of the tested plant extracts against some pathogenic Gram negative bacteria using the Agar Well Diffusion method

Methanolic extracts	Pathogenic isolates				Bacterial index*
	Klebsiella pneumoniae	*Pseudomonas aeruginosa*	*Salmonella arizonae*	*Escherichia coli*	
Pimpinella anisum	17.6±0.5	13.6±0.5	13.3±1.5	12.3±0.5	14.2**
Syzygium aromaticum	13±1.0	12.6±0.5	13.3±1.5	14.6±1.1	13.37**
Punica granatum	14.3±0.5	18.3±1.1	17±1.7	18±1.0	16.9**
Cefaclor	39.3±1.5	30±1.0	42±0.0	34.6±1.5	36.4**

*Bacterial index: Total activities against bacteria divided by the number of the tested bacteria; *, significant results at $P \leq 0.05$ as compared to the control (DMSO).

Table 4. The antimicrobial activity (diameter of the inhibition zone, mm) of the tested plant extracts against gut microbiota using Agar Well Diffusion method

Methanolic Extract	Gut Microbiota		Bacterial index*
	Lactobacillus sp.	*Bifidobacterium* sp.	
Pimpinellanisum	ND	ND	0.0**
Syzigium aromaticum	10.6±1.5	10.6±1.5	10.6**
Punica granatum	10±2.0	10±1.0	10.0**
Cefaclor	45	64	54.5

*Bacterial index: Total activities against bacteria divided by the number of the tested bacteria;
**, not significant results at $p < 0.05$ as compared to the control (DMSO)

Table 5. The antimicrobial activity of some antibiotics (diameter of inhibition zone, mm) against some pathogenic Gram negative bacteria using Disk Diffusion method

Antibiotics (µg/disc)	Pathogenic Isolates				Gut flora	
	Klebsiella pneumoniae	*Pseudomonas aeruginosa*	*Salmonella arizonae*	*Escherichia coli*	*Lactobacillus* sp.	*Bifidobacterium* sp.
Amikacin (30µg)	19	23	28	20	30	ND
Amoxillin (20µg)	ND	ND	9	ND	20	38
Kanamycin (5µg)	15	15	20	ND	13	ND
Ciprofloxacin (5 µg)	28	22	38	ND	12	ND
Piperacillin 100µg)	18	20	33	12	24	40
Cefaclor (100 µg)	39	30	42	34	45	64

ND: not detected

Table 6. The MIC (mg/ml) of the tested plants for some pathogenic Gram negative bacteria using Broth Microdilution method and toxicity (LD_{50}) using brine shrimp as test organism

Methanolic extract	Pathogenic Isolates				Toxicity (LD_{50}, mg/ml)
	Klebsiella pneumoniae	*Pseudomonas aeruginosa*	*Salmonella arizonae*	*Escherichia coli*	
Pimpinella anisum	23.4±0.0	46.8±0.0	46.8±0.0	93.7±0.0	>400
Syzygium aromaticum	43.7±15.1	21.8±7.5	70±30.3	8.7±3.8	>400
Punica granatum	3.1±0.0	20.8±7.2	4.1±1.7	6.2±0.0	>400
Cefaclor	3.9±0.0	3.9±0.0	0.79±0.0	1.95±0.0	>400

*Toxic effect, 0.0: no inhibition

The selected medicinal plants were *Pimpinella anisum, Syzygium aromaticum* and *Punica granatum*well known to be used as food additives (Ross *et al*., 2001; Moghtader and Farahmand 2013; Al-Mariri and Safi 2014) or to treat some diseases. In this study, fresh human fecal samples were collected and two bacterial isolates from gut microbiota were obtained on two selective media.

The two isolates were Gram positive and identified as *Lactobacillus* sp. and *Bifidobacterium* sp. (Orrhage and Nord, 2000, Venkatesan *et al*., 2012). The colonies of *Lactobacillus* sp. were large, white, circular, convex, opaque, non pigmented and smooth. Under light microscope, *Bifidobacterium sp.* cells are Gram-positive, rod shaped and in clusters of pairs, or even independently, nonmotile and non spore forming bacterium as seen.

Figure 1. Flowers of *Syzygium aromaticum* (A), Dried peels of *Punica granatum* (B)and Dried fruits of *Pimpinella anisum*(C)

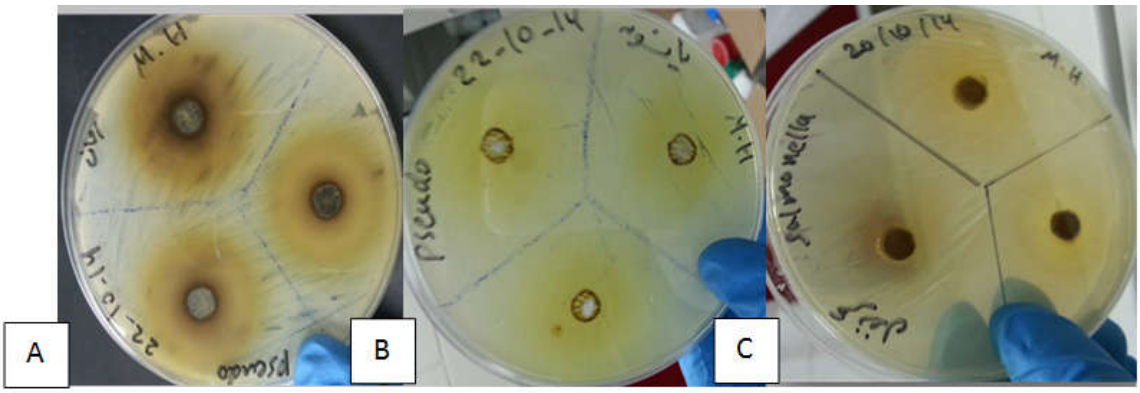

Figure 2. The antimicrobial activity of *P. granatum* extract against *Pseudomonas aeruginosa* (A), *P. anisun* extract against *Pseudomonas aeruginosa* (B) and *S. aromaticum* extract against *Salmonella arizonae* (C)

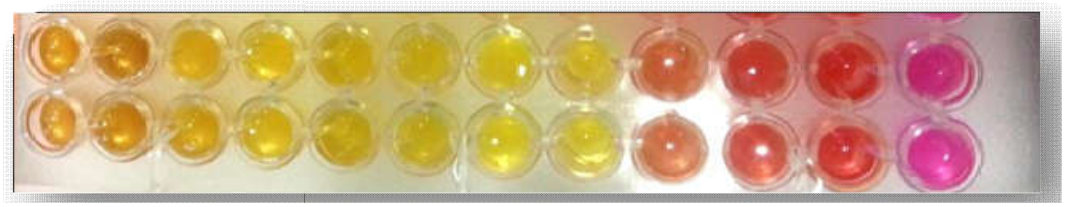

Figure 3. MIC of *P. granatum* against *E. coli* detected using microtiter plate, changing the color from yellow to pink indicated MIC valuess

Identification of some pathogenic bacterial isolates, obtained from microbiology laboratory, was confirmed using API 20 E system and some physiological characters in addition to Gram stain. The identification of the examined pathogenic bacteria was confirmed as *Klebsiella pneumonia, Pseudomonas earuginosa, Salmonella arizonae* and *Escherichia coli*. The antimicrobial activity of the methanolic extracts was tested against some pathogenic bacteria and some gut microbiota using Agar Well Diffusion method. The inoculated plates were incubated for 24 hours at 37°C, mean diameter ± SD of developing inhibition zones were calculated and taken as criterion for antimicrobial activity (Table 3, Figure 2). *Pimpinella anisum* extract gave inhibition zones ranged from 12.3±0.5 to 17.6±0.5 mm with mean antibacterial index of 14.2 mm against the pathogenic isolates. On contrast, no antimicrobial activity was noted for *P. anisum*, extracts against *Lactobacillus* sp. and *Bifidobacterium* sp (Table 4). In the present study, the methanolic extract of *Punica granatum* peels has a significant antibacterial activity against all tested bacteria while the effect on gut microbiota was not significants.

Ates and Erdoğrul (2003) were studied the antibacterial activities of the alcohol, ethyl acetate, acetone and chloroform extracts of *P. anisum* seed and they were tested *in vitro* against 13 bacterial species and strains by the agar diffusion method. The results indicated that the alcohol extracts of *P. anisum* seeds showed antibacterial activity against *Micrococcus luteus* and *Mycobacterium smegmatus*. The alcohol extracts did not inhibit *Bacillus subtilis*, *B. brevis*, *Enterobacter faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. The ethyl acetate extracts did not inhibit *B. subtilis* or *Y. enterocolitica*, and the acetone extracts did not inhibit *E. faecalis*, *L. monocytogenes*, *P. aeruginosa* or *Y. enterocolitica*. The chloroform extracts showed no inhibition effect against *P. aeruginosa* or *Y. enterocolitica*. *Syzygium aromaticum* extract showed *in vitro* antimicrobial activity against all tested bacterial pathogenic isolates and the effect was not significant on gut microbiota. The high antibacterial activity of the investigated plant was recorded against *E. coli*. Preliminary screening of the antimicrobial activity *in vitro* of some antibiotics was studied against 4 pathogenic bacteria using Disc Diffusion method.

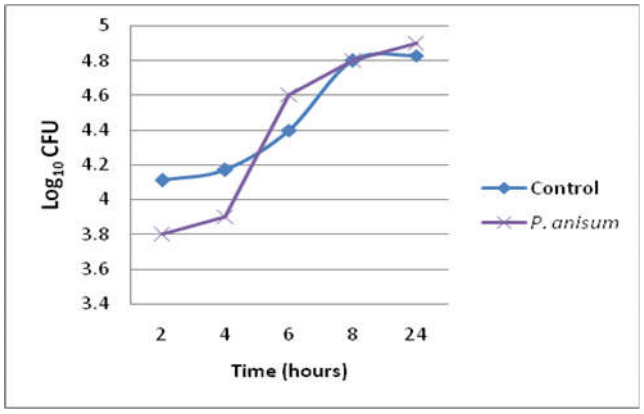

Figure 4. *Lactobacillus* sp. growth (CFU/ml) during 24 h in the presence of *P. anisum* plant extract

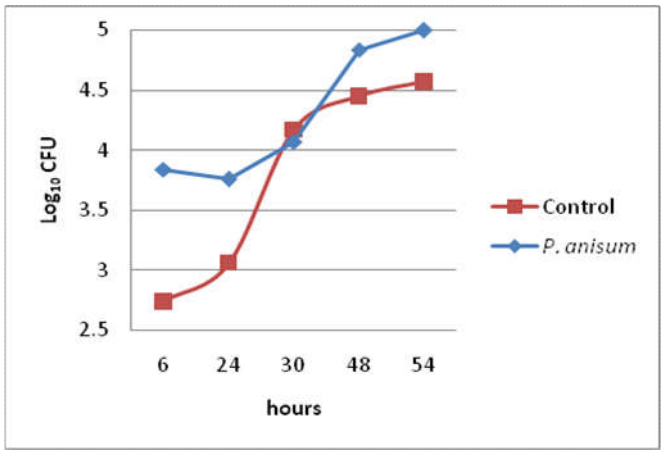

Figure 5. *Bifidobacterium* sp. growth (CFU/ml) during 54 h in the presence of *P. anisum* plant extract

The mean of the zones of inhibition is shown in Table 5. The diameter of the inhibition zones ranged from 9-42 mm. Amikacin gave inhibition zones between 19 to 30 mm against *K. pneumoniae*, *P. aeruginosa*, *S. arizonae*, *E. coli* and *Lactobacillus* sp. Also Amoxillin gave inhibition zones of 9, 20 and 38 mm against *S. arizonae*, *Lactobacillus* sp. and *Bifidobacterium* sp., no antimicrobial activity was observed against *K. pneumonia* and *P. aeruginosa.* However *E. coli* was resistance to Amoxillin, Kanamycin and Ciprofloxaci. Kanamycin gave inhibition zones between 13 to 20 mm against *Lactobacillus*, *K. pneumonia*, *P. aeruginosa* and *S. arizonae*. The Ciprofloxacin inhibition zones ranged from 12 to 38 mm against *Lactobacillus*, *K. pneumonia*, *P. aeruginosa* and *S. arizonae*. Piperacillin gave inhibition zones between12 to 40 mm. Finally Cefaclor gave inhibition zones between 30 to 64 mm. The MIC values of *P. anisum* extract against tested pathogenic isolates were between 23.4 mg/ml for *K. pneumonia* and 93.7 mg/ml for *E. coli* (Table 6, Figure 3). MIC values of *S. aromaticum* extract were ranged between 8.7 mg/ml for *E. coli* and 70 mg/ml for *S. arizonae*. Similarly to our result, Al-Mariri and Safi (2014) found that *S. aromaticum* essential oils showed anti-bacterial activity against *E. coli* O157:H7, *Yersinia enterocolitica* O9, *Proteus* spp., and *K. pneumoniae*. The MIC_{50} values of this essential oil against Gram-negative organisms varied from 1.5 µg/ml (*Klebsiella pneumonia*) to 25 µg/ml (*E. coli* O157:H7). The MIC values of *P. granatum* extract against tested Gram negative bacteria were ranged between 3.1mg/ml for *K. pneumonia* and 20.8 mg/ml for *P. earuginosa* (Table 6).

Similary, Naziri, Rajaian, and Firouzi (2012) were prepared the methanolic extracts of sour and sweet pomegranate (*P. granatum*) peels. Antibiogram tests using the disk diffusion technique and serial dilution method were performed against ten pathogenic bacteria isolated from animals, and relative MIC values were also determined for the above compounds. The greatest zone of inhibition induced by the action of pomegranate peel extracts was obtained for *Staphylococcus aureus* and the smallest zone of inhibition was obtained for *Pasteurella multocida*. In addition, the lowest MIC values of pomegranate peel extract were obtained for *Staphylococcus aureus* (7.8 mg/ml).

Brine shrimp (*Artemia salina*) is often used as agent in laboratory assays to determine toxicity values by assessing LC_{50} values (median lethal concentration) (Fichna *et al*. 2009; Sandoval-Chacón *et al*. 1998; Spperoni *et al*. 2007). These larvae are sensitive to toxic substances (Pelka *et al*., 2000). The toxicity of investigating plant extracts was differentiated between toxic and non toxic. There were found toxicity of *S. aromaticum* and *P. granatum* peels Furthermore, *P. anisum* and were non toxic.No toxicity was found using the antibiotic Cefaclor and up to 400 µl/ml. Also, no toxicity was found at 400 µl/ml of *P. anisum* (Table 6). Sökmen (2001) showed that the LC_{50} of the chloroform extract of *Pimpinella anisum*seeds was 595.6 µg/mL.Traditional preparation methods are taking this into account; most remedies are prepared as simple water extracts, thus avoiding potential toxic effects. The effect of *P. anisum* extract was studied in MRS broth. The growth curve of *Lactobacillus* sp. and *Bifidobacterium* sp. was drawn per standard methods in the presence of the methanolic extract and compared with the control. The growth of *Lactobacillus* sp. was reduced in the presence *P. anisum* extract during the first 2 hours of inoculation, then the growth was increased from hours 4 to 24 and become constant up to 30 hour (Figure 4). Logarithmic graphs were drawn by determining the colony counts of *Lactobacillus* sp. *in* the presence of methanolic extract and compared with control. *Bifidobacterium* sp. is anaerobic microorganism and was incubated for 54 hours in MRS broth, and then the growth on Beeren's agar was determined.

The effect *P. anisum* extracts on a *Bifidobacterium* sp. growth was studied.In the control the *Bifidobacterium* growth was beginning to decline after 48 hours, whilst in the presence of the tested extract, *Bifidobacterium* growth continue to increase up to 54 hours (Figure 5). Logarithmic graphs were drown by determining the colony counts of *Bifidobacterium* sp. in the presence of methanolic extract and compared with control. A very fiew study confirms the viability of some plant extracts as a prebiotic on commensals gut microbiota. In a study carried by Filocamo *et al*. (2012), they evaluated *in vitro* effect of a commercial garlic powder, upon the viability of representative gut bacteria. In pure culture studies, *Lactobacillus casei* DSMZ 20011 was essentially found to be resistant to GP whereas a rapid killing effect of between 1 and 3 log CFU/ml reduction in cell numbers was observed with *Bacteroides ovatus*, *Bifidobacterium longum* DSMZ 20090 and *Clostridium nexile* A2-232. Lactic acid bacteria were found to be more resistant to GP compared to the clostridial members of the gut microbiota. While for most bacteria the antimicrobial effect was transient, the lactobacilli showed a degree of resistance to garlic, indicating that its consumption may favour the growth of these beneficial bacterial species in the gut.

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