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NOVEL PROBIOTIC *BIFIDOBACTERIUM* OVERCOMES SYNERGISTIC EFFECT OF THREE NATURALBIOTIC OMNI DRUG AND ANTIBIOTIC AGAINST SOME UTI PATHOGENS

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ABSTRACT
Extracts of three plants (Turmeric of *Curcuma longa* , Olibanum of *Boswellia luban* and licorice of *Glycyrrhiza glabra*) against 4 bacteria isolated from urinary tract were studied using well and disc diffusion method. To evaluate the synergistic effect of these extracts, the minimum inhibition and bactericidal concentration (MIC and MBC) were done, then compared with antibiotics and *Bifidobacterium* filtrates (Cell Free Supernatant (CFS)). The results showed that all extracts possessed antibacterial activity but not against all test bacteria, *E. coli* and *Pseudomonas* were significantly susceptible to licorice, olibanum while the *E. coli* alone was significantly susceptible to turmeric. Comparative results were carried out and appeared predominant *Bifidobacterium* CFS on synergistic extract and antibiotic. Sensitivity and resistance to extract and antibiotic varied from bacteria to another but *Bifidobacterium* CFS proved active effects against all bacteria without resistance with inhibition zone ranged(8-10) mm when once concentrated and (9-22)mm in twice concentrated, *Acinetobacter* exhibited the higher sensitive(22)mm to these CFS comparative to other bacteria. *Bifidobacterium* effectiveness against test isolates can be considered a probiotic able to inhibit isolate *in vitro*, in the same time provides hope that it can serve as an alternative therapeutic agent *in vivo.*

KEYWORDS: synergistic effect, plant extracts, omni drug UTI

INTRODUCTION

Despite the advances in various field of medicine, urinary tract infections are still considered as serious public health problems and inflict a major burden to health care services around the world and especially in developing countries [1]. Development of resistance against antibiotics and antiseptics is a growing cause of concern which has limited the preventive measures. Therefore, there is a containing need to search for new antimicrobial agents, over the last decade, plant antimicrobial activity has been studied in different regions of the world [2]. *Glycyrrhiza glabra ,Curcuma longa, Boswellia luban* commonly called as licorice, turmeric and olibanum respectively are three of the important traditional medicinal plants grows in the various part of the world , and have several useful pharmacological properties such as antimicrobial, anticancer ,antiviral activities and hepatoprotective effects [3,4]. The synergistic effect of those plants components formed omni drug which put on the market in recent years, especially in Egypt that praised its effectiveness against viruses [5]. Although they contain active substances against bacteria but there are not any previous studies about the effectiveness of UTI, in the present study, we evaluated the antibacterial activity of omni drug against UTI. On the other hand, and according to an expert consultation conducted by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are "Live microorganisms and when they administrated in adequate amounts they will confer a health benefit on the host[6]. The regular intake of probiotic microorganisms has been demonstrated to prevent several infectious, allergic disorders, diarrhea and inflammatory disease such as inflammatory bowel disease [7]. Morereover, probiotics or their metabolites have been suggested to play an important role in the formation or establishment of a well-balanced, indigenous, intestinal microbiota in human, among probiotics, *Bifidobacterium* is one of the favorite genera in lactic acid bacteria (LAB) and focused on the prevention of gastrointestinal infection and is often used in fermented dairy products or food supplement [8]. According to our knowledge, no previous studies were suggested to use *Bifidobacterium* probiotic as treatment or external drug against the UTI pathogens therefore; the aim of the present study is to investigate the effectiveness of this treatment as compared with omni drug and antibiotics effectiveness.

MATERIALS & METHODS

Procurement of plant material: The plants (*Curcuma longa ,Glycyrhiza glabra ,Boswellia luban)* were collected from local market (AL-Shourja), the samples were identified by qualified taxonomists.

Preparation of plants powder: Plants were cleaned and air dried for 2 days the dried samples were again dried in a hot air oven at $50C^0$ for 24 hrs. then ground into powder and pass a sieve with nominal mesh size of 2mm in a diameter. The produced powder of each plant is called Turmeric, Licorice and Olibanum of *Curcuma longa, Glycyrrhiza glabra* and *Boswellia luban* respectively.

Plant powder extraction: The air –dried materials were finally pulverized and extracted by percolation with Ethanol for 1,2 ,3 days (of turmeric, licorice, olibanum respectively) at room temperature .The comined extracts were filtered and concentrated to obtain a crude extract .4

concentrations from stock solution were taken (100, 200, 300, 400, 500) mg /ml for antibacterial activity.

Bacterial strains: Reference microbial strains including *E. coli, Klebseilla, Pseuodomonas, Acinetobacter* were obtained from high studies laboratory in collage of science, Al-Mustansiriyah university that isolated from urinary tract of human, these isolates were identified by the chemical test and the stock culture were kept in refrigerator 4^0C on nutrient agar.

Bacterial suspension preparation: Respective suitable slant medium was used to activate the bacterial isolates to be tested by means of sterile operation and inoculate it into the corresponding liquid medium it was taken as stock solution after culturing in the constant temperature incubator at the most suitable temperature for 16_18 hrs. Bacterial suspension containing bacteria of about 10^6 cell/ ml was prepared with sterile physiological saline for further use.

Antibiotic used and susceptibility testing : Antibiogram of the UTI isolates was ascertained on Muller Hinton Agar using disc diffusion method .Four antibiotics most commonly used for the treatment UTI were employed .The diameter of the zone of inhibition produced by each antibiotic disc was measured ,recorded and the isolates were classified as resistant or sensitive based on (CLSI) [9].

***Bifidobacterium* source:** *Bifidobacterium* sp. Was isolated from Activia yoghourt product .Cultured and grew on De Man Rogosa Sharpe (MRS) agar .It was identical according to Wood and Holzapfel,(1995).

Preparation of cell free supernatant (CFS) of *Bifidobacterium* : Strain was incubated in MRS broth for 24 hrs. at $37C^0$ .Bacterial cells were removed by centrifuging the culture at 8000 xg for 5min .the pH value of CFS was adjusted to (6.5) by the addition of 0.1 N(Normality) NaoH .Sterilized, filtered through 0.22*m*M filter and stored at $4C^0$. CFS was concentrated in Freez dryer once and twice and named CFS1 and CFS2 respectivly to be tested for antibacterial activity.

Test for antibacterial assay

***A. Agar* well Diffusion:** The solid agar was punched with 5mm diameter wells. The inoculums were spreed on to the agar plates using sterile swabs, wells were filled with extracts or CFS .The plates were incubated at $37C^0$ for 24 hrs. Inhibition zone was measured bin millimeters .each extract was tested three times.

B. The disk diffusion: This method was used to test antibacterial activity of all extract or CFS against bacterial isolates. Solutions of known concentration (mg/ml) of the test sample were made by dissolving measured amount of the sample in calculated volume of solvent. Dried and sterilization filter paper disc (6mm diameter) were then impregnated with known amounts of the test substances using micropipette and residual solvents were completely evaporated. Discs containing the test materials were placed on to nutrient agar medium uniformly seeded with the test microorganisms .these plates were then Kept at low temperature (4)C^0 for 24 hrs. to allow maximum diffusion of the test material. Then these plates were incubated at 37C for 24 hrs to allow maximum growth of the organism.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC): MIC and MBC were determined by a broth dilution method according to Zhang *et al*. (2010).The MIC was defined as the minimum level of the extract that produced a 90% reduction in growth of the bacteria, MBC was the lowest concentration that killed at least 99.9% of the initial inoculums.

The synergism effect of the three extract: 1ml from the MIC of each extract was taken ,then mixed by vortex to determine the synergism effect in both methods .,except bacteria that did not show any sensitivity by extracts ,1ml was taken from first concentration .

Statistical Analysis: Data from experiments were analyzed using students-t-test and p-value of ≤ 0.05, $\leq 0.01$and $p \leq 0.001$ were considered statistically lower, moderate and high significant differences.

RESULTS & DISCUSSION

The licorice, turmeric, olibanum extract was screened against four test bacteria from UTI by both methods, disc diffution (DD) and well diffusion (WD) .Table (1) show the antibacterial activity of licorice extract concentration by DD and WD, two of these organisms were found to be sensitive to the extract. However, licorice exhibited high significant differences ($p \leq 0.001$) in its effectiveness against *E. coli* with inhibition zone (23)mm compared with *Pseudomonas* that showed moderate significant differences with inhibition zones ranged between (6-19)mm, whereas *Klebseilla* and *Acinetobacter* did not appear any sensitive by licorice, so they proved the less(or lower) inhibition($p \leq 0.05$).,these results obtained by DD method .In the other side and by WD method, licorice exhibited antibacterial activity against Pseudomonas just in (500, 400) mg/ml concentration and with high significant differences in inhibition zone (19) mm compared with other bacteria and other concentrations, Figure (1,2).

TABLE 1: Antibacterial activity of licorice extract by disc diffusion and well dif method

Plant con. mg/ml	*E.coli*		*Klebseilla*		*Acinetobacter*		*Pseudomonas*	
	DD	WD	DD	WD	DD	*WD*	DD	WD
500	23	R	R	R	R	R	19	19
400	22	RRR	R	R	R	R	17	18
300	7	R	R	R	R	R	12	R
200	6	R	R	R	R	R	7	R
100	6	R	R	R	R	R	6	R

DD, WD= Disc dif fusion, Well diffusion respectively

Evidence showed that due to the presence of glabrene (licoisoflavone B, isolicoflavonol, gancaonin I) it showed significant activity against these bacteria. Several studies agreed with us about the antibacterial activity of licorice, generally, and *G. glabra* is well known [11, 12, 13]. In the same time other studies has been reported that glabridin

posses this activity against some strains [14, 15, 16], this may be explain why some strain did not response to the licorice in our study. Also, each of flavonoids, saponins, coumarins, stilbenoids and other miscellaneous compounds have an important role in licorice activity[17] and clearly indicates the presence of potent bioactive principles in these extractives, which might be very useful as anti-inflammation or other bioactive agents [18].

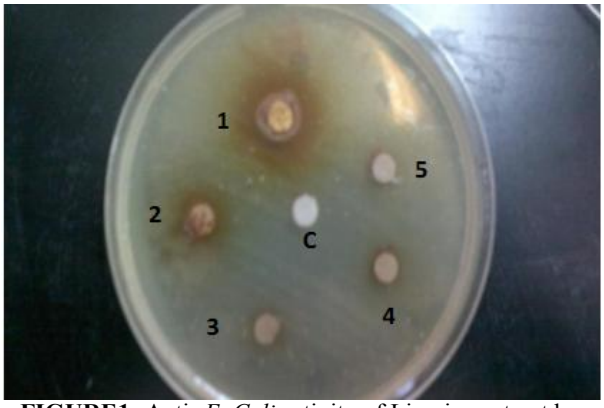

FIGURE1: Anti- *E. Coli* activity of Licorice extract by disc diffusion method**.**
1,2,3,4,5: Concentration of licorice (500,400,300,200,100)mg /ml respectively C:control

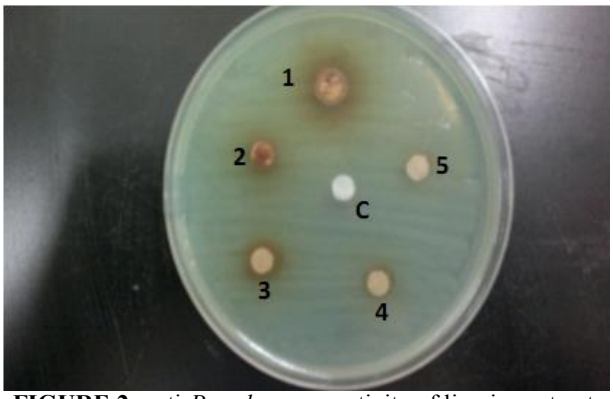

FIGURE 2: anti-*Pseudomonas* activity of licorice extract by disc diffusion method
1,2,3,4,5 :concentration of licorice (500,400,300,200,100)mg /ml respectively C:control

Investigated olibanum extract antibacterial activity was shown in Table (2), extract exhibited higher inhibition zone (20) mm against *Pseudomonas* with high significant differences $p \leq 0.001$, on the other hand, olibanum extract showed moderate significant differences $p \leq 0.01$ against *E. coli* with inhibition zone (7) mm, while *Acinetobacter* and *Klebseilla* did not have any result for sensitive, so they showed the lower significant differences among other bacteria.

TABLE 2: Antibacterial activity of olibanum extract by disc and well diffusion

Plant con. mg/ml	*E.coli*		*Klebseilla*		*Acinetobacter*		*Pseudomonas*	
	DD	WD	DD	WD	DD	*WD*	DD	WD
500	R	7	R	R	R	R	R	20
400	R	7	R	R	R	R	R	15
300	R	R	R	R	R	R	R	14
200	R	R	R	R	R	R	R	9
100	R	R	R	R	R	R	R	7

DD,WD= Disc dif fusion, Well diffusion respectively

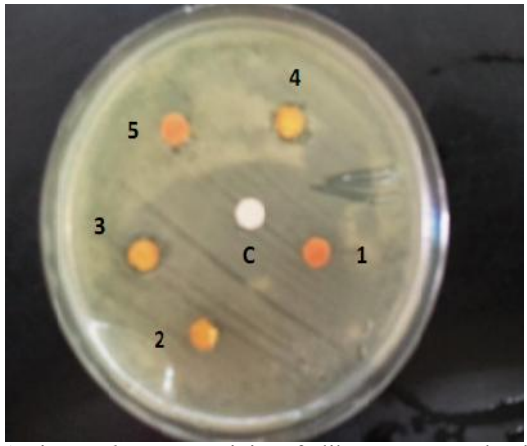

FIGURE 3: Anti-*Pseudomonas* activity of olibanum extract by disc diffusion
1,2,3,4,5 :concentration of olibanum (500,400,300,200,100)mg /ml respectively C: control

These results in our study were in parallel with the findings of previously reported studies that *E. coli* and *Pseudomonas* was sensitive to olibanum extract compared to other bacteria., because of olibanum substances can effect on this bacteria by specific mechanisms including: attacking the phospholipids bilayer of the cell membrane, disrupting forming fatty acid hydro peroxidase caused by oxygenation of unsaturated fatty acids [19]. The patterns of inhibition to same plant vary with the bacteria {the role of bacteria type remembered above} and the solvent used for extraction (eqeous or coholic), these observations may be attributed to two reasons. Firstly, the nature of photochemical active components (alkaloids, anthraquinone, saponins, tannins and others could be

enhanced in presence of alcohol extract., Secondly, the stronger extraction capacity of alcohol may have produced greater active constituents responsible for antibacterial activity., for example Resin of some *Boswellia* is reported to contain about 5%-9% essential oil, 65%-85% at alcohol-soluble resin [20]. In addition to that Tajkarimi *et al*. (2010) found that ethanol extract is a group of antibacterial components because of these major components can constitute up to 85% and other components are usually at trace levels, this result agree with our study in alcoholic extract activity.

Turmeric extract revealed the lowest activity among other extracts against bacteria, it could not inhibit any growth of them in DD and WD method except *Klebseilla* that submitted to sensitivity towards turmeric extract with all its concentration in WD method, so the inhibition zones ranged between (15-8) mm and exhibited high significant differences $p \leq 0.001$ compared to other bacteria Table (3).

TABLE 3: Antibacterial activity of turmeric extract by disc and well diffusion

Plant con. mg/ml	*E.coli*		*Klebseilla*		*Acinetobacter*		*Pseudomonas*	
	DD	WD	DD	WD	DD	*WD*	DD	WD
500	R	R	R	15	R	R	R	R
400	R	R	R	12	R	R	R	R
300	R	R	R	10	R	R	R	R
200	R	R	R	8	R	R	R	R
100	R	R	R	8	R	R	R	R

DD,WD= Disc dif fusion, Well diffusion respectively

A comparison between the anti- Gram negative and positive bacteria activity of turmeric extract ,indicate that Gram negative bacteria are lower sensitive to this extract , this may be related to a difference in the structure of their cell wall , gram negative bacteria have an outer membrane and the cell wall of Gram positive bacteria are made up of twenty times as much peptidoglycan than the walls of Gram negative , in addition antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane resulting in leakage of the cytoplasm [21].These causes may be explain inability turmeric extract to inhibit most test Gram negative bacteria. In the same time ,the antibacterial activity of turmeric that appeared in *Klebseilla* can be due to presence of curcumin {The more important part in turmeric} and its derivatives in the molecule , which have efficacy against bacteria generally[22,23].

The mechanism of antibacterial action of turmeric is not yet clear , but hypothesis have been proposed different workers which involve :hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, followed by perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes ,destruction of electrons transport systems and cell wall perturbation [24].

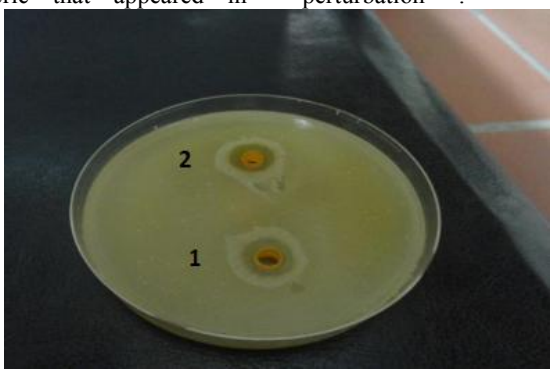

FIGURE 4: Anti-*Klebseilla* activity of turmeric extract by well diffusion
1, 2= (500,400) mg/ml turmeric extract concentrations respectively

The MIC and MBC values obtained for extracts against the bacterial strains varied among the three extracts, The MIC values corresponded well to the MBC values in licorice and olibanum extract; there were low and moderate significant differences between these values (MIC and MBC) against *Pseudomonas* and *E. coli* respectively and high significant differences against *Klebseilla* Table (4).

TABLE 4: Minimum inhibition and bactericidal concentration (MIC and MBC) of plants extract against tested bacteria except *Acinetobacter*

Plant extract	*E.coli*		*Klebseilla*		*Pseudomonas*	
	MIC	MBC	MIC	MBC	MIC	MBC
Licorice	300	500	-	-	200	400
Olibanum	400	500	-	-	200	500
Turmrric	-	-	100	500	-	-

MIC, MBC : mg/ml concentration

In the other hand, antibiotic sensitivity test showed that *Pseudomonas*was the morest resistance among residue, it was resistant to all antibiotics used, whereas *E. coli* was resistant to Nitrofurantion, levofloxacin (LE) and *Acineto bacter* was resistant to (Amoxicillin, Ampicillin) while *Klebsiella*alone was resistant to one antibiotic is (Levofloxacin) Table (5).

TABLE 5: Bacterial sensitivity to antibiotics

Antibiotics	Symbol	*E.coli*	*Klebsiella*	*Acinetobacter*	*Pseudomonas*
Amoxicillin	AMX	9	6	R	R
Nitrofurantion	F	R	15	6	R
Levofloxacin	LF	R	R	13	R
Ampicillin	AMP	7	9	R	R

The bacterial isolate resistant (*Acinetobacter* and *Klebsiella*) to the extract are explained by many option like: the nature of bacteria itself, physiological structure, genetic mutation and the plant extract target sites other than those used by antibiotic, this is also explain why the sensitive bacteria to the extract activity (like *E. coli*) was resistant to some antibiotic and sensitive to other and in the same time it was sensitive to all extract concentration (19). DD method in our study was more practical then WD method , most results were revealed by this method ,but this is opposite opinion to others, which remembered that (DD method is not possible to demonstrate the antibacterial effect in the paper disc assay, probably because the paper disc retains the active component and does not allow it to diffuse into the Muller Hinton Agar) while others agreed with us that (DD method is considered as a successful quick measure of the inhibitory effect of various plant extracts[25]. The synergistic effect results of three plants extract LOT (Licorice, Olibanum, Turmeric) revealed that *Acinetobacter* was the more sensitive (20) mm to LOT with high significant differences $p \leq 0.001$ in spite of its resistance to LOT when they used alone. In WD method, *E.coli and Acinetobacter* had moderate significant differences $p \leq 0.01$, they were inhibited in (9-8) respectively, but *Klebseilla* and *Pseudomonas* did not response to LOT in any assay Table (6). Synergism was happened in our study when LOT effected on *E.coli and Acinetobacter* because of (the combined effect of three plants substances was higher than the sum of the individual effects) this is synergism. This result was not equaled with result when LOT used as a combination against other bacteria, antagonism could happen [26].

Synergism effect of LOT extracts, have been shown in some studies [27, 28, 29]. Generally, the results obtained by above mentioned methods confirmed that antibacterial activity of three extract was not significantly greater than other studies[30, 31], another one was parallel with our study that showed the extracts effectiveness not on all strains and they are more active on Gram positive than negative bacteria[32,33,34]. In the same table it was interesting to note that all test bacteria showed greater sensitive to *Bifidobacterium* filtrate (CFS) in both matter, once and twice concentrated, CFS1 and CFS2 respectively. CFS1 gave moderate significant differences represented with range (8-17) mm inhibition zone whereas there was range (9-22) mm inhibition zone with high significant differences when CFS2 was used compared with the low significant differences in LOT using.

TABLE 6: The antibacterial activity of three plants extract and *Bifidobacterium* CFS1 and CFS2.

Antimicrobia	*E.coli*		*Klebseilla*		*Acinetobacter*		*Pseudomonas*	
l agent	DD	WD	DD	WD	DD	*WD*	DD	WD
LOT	9	R	R	R	8	20	R	R
CFS1	9.5	8	10	9	9	17	9	7
CFS2	10	12	13	9	10	22	10	12

LOT: Licorice, Olibanum, Turmeric.

CFS1, CFS2 : Cell Free Supernatant concentrated once ,twice respectively

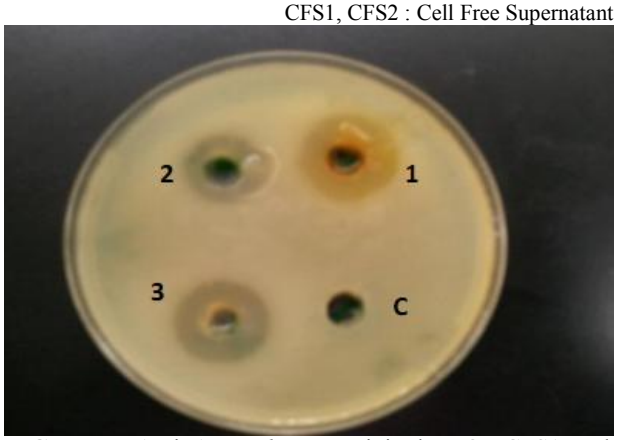

FIGURE 5: Anti-*Acinetobacter* activity by LOT,CFS1 and CFS2

1:LOT-extract 2:CFC1 3:CFC2 C:Control

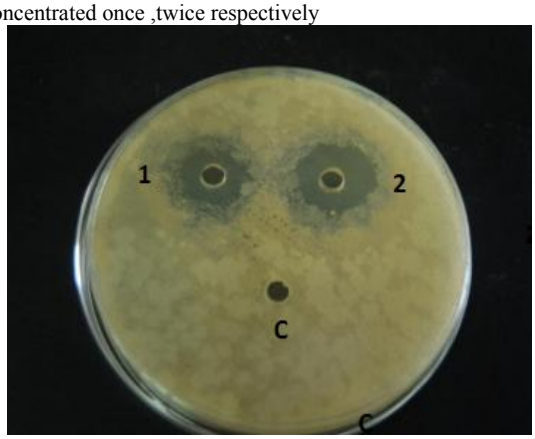

FIGURE 6: Anti-*E.coli* activity by CFS1 and CFS2

1: CFS1 2:CFS2 C:Control

The mechanisms underlying *Bifidobacterium* inhibition of test bacteria may be due to presence many fraction containing proteins with a molecular mass below 5.000 Da and the finding of in vitro pointed to the peptideic nature of the *Bifidobacterium* linked to bacteria inhibition[6]. However, there are also reports of compounds of proteinaceous nature with antagonistic activity against all bacteria (these proteinaceous inhibitors target the cell membrane and depolarize it, and also inhibit synthesis of the cell wall, there are one of those peptides were characterized as Bacteriocin called Bifidocin B [34]. Diacetyl, hydrogen peroxide {H_2O_2 can have a strong oxidizing effect on membrane lipids and cellular proteins}, organic acids such as lactic acid, acetic and propionic acids, the most documented kind of metabolites. The antagonistic actions of acids are believed to be: 1. interference with the maintenance of cell membrane potential, 2.inhibition of active transport, 3. Reduction of intracellular pH and 4.inhibition of various metabolites functions .They have a broad mode of action and inhibit both Gram-negative and positive bacteria as well as yeasts and molds[35,36]. Besides the production of inhibitory compounds *Bifidobacterium* have ability to compete with the pathogens for nutrients during the growth, the combined influence of large numbers of competing *Bifidobacterium* and the resulting decrease in pH produce an unfavorable environment for many pathogens such as UT pathogens[37]. All of the reasons above give clear reasons for the high activity that obtained in our results.

CONCLUSION

The study plants do not have the complete affiance to all microorganisms. Omni drug (LOT extract) don't recommended to UTI just for virus. Mixed 2 plants or more don't always lead to synergism effect .opposite, may be appear antagonistic effect. Positive inhibition is limited by (type of method, test organisms, plant type, extract solvent). Finally, *Bifidobacterium* isolate conferred that it is the status of a probiotic bacterium with activity against UTI pathogen in vitro.

RECOMMENDATION

Further studies, like purification active compounds and limit their targets in pathogen cell. Make these probiotic as drugs (capsule or tap) as alternative treatment from chemical drugs. Test these natural drug on UTI infection.

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