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RESEARCH ARTICLE

Inhibitory effects of Garlic extract on uropathogenic *Escherichia coli*; *Proteus mirabilis* and *Trichomonas vaginalis* isolated from urogenital tract cases

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ABSTRACT:

Since various and numerous pathogenic microorganisms can infect and dwell human's urogenital tract, and because the ability of bacteria to develop resistance to antibiotics, in addition to the side effects of some of these antibiotics in treatment of infectious diseases, therefore we need to alternative herbal and non-antibiotic therapy for treating the urogenital tract infections such as Garlic (*Allium sativum* L.). The aim of the study is to investigate the antimicrobial effects of Garlic extract on local uropathogenic species of Escherichia coli, Proteus mirabilis and *Trichomonas vaginalis* in cases of urogenital tract infections. The tested urogenital microorganisms were isolated and identified according to diagnostic features as golden of morphology and biophysiology for Escherichia coli, Proteus mirabilis and *Trichomonas vaginalis*. Fresh garlic bulbs were obtained from local markets of Iraq. The aqueous garlic extract (AGE) was performed according to standard method. The antibacterial activity of AGE was done against motility, adherence and biofilm formation, and compared with some UTI antibiotics. The antiparasitic activity of AGE was done against growth rate, motility and viability of cultured *vaginalis* in vitro by use medium. current study suggest that the AGE has antibacterial effects against E. coli and Pr. mirabilis. The diameter of the inhibition zone was ranged from 27.0 to 28.0 mm for garlic extract, as compared with 12.0 to 18.0 mm for Ciprofloxacin against both genera; E. coli and Proteus mirabilis, respectively. The efficacy of adherence and biofilm formation by *E. coli* and Pr. Mirabilis was much reduced. The motility of these bacteria was completely inhibited following AGE exposure to 30%, and temporally inhibited by 20%. Furthermore, the growth rate, viability and motility of the studied protozoan parasite " *vaginalis*" were completely inhibited following exposure to crude extract of AGE post 1 hour, completely inhibited following AGE exposure of 30% post 24 hour, completely inhibited by 20% post 48 hour, completely inhibited by 10% post 72 hour and temporally inhibited by 5% post 96 hour, respectively.

KEYWORDS: Garlic, *Allium sativum*, antibacterial, antiprotozoal, urogenital, alternative herb, UTI, E. coli , Proteus mirabilis and *T. vaginalis*.

1. INTRODUCTION:

The garlic plant (*Allium sativum* Linn.) is belonged to the Liliaceae family and fall within the group of onion, such as mountain onion and shallot, and has antimicroial action against many common pathogenic microorganisms [1, 2]. The soap plant (Garlic) has a traditional dietary and medicinal uses as an anti-infective mediator. In vitro, there is an evidence of the antimicrobial activity of fresh and freeze-dried garlic extracts for various microorganisms like : bacteria ,

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fungi , parasites , and the viruses supports the fact of these applications[3,4 ,5]. Urinary tract infections (UTIs) are important health concern with approximately 150 million cases of UTIs occurring each year on a global basis with correspondingly significant morbidity and associated healthcare costs estimated at $1.6 billion[6]. The urinary tract is one of the most common sites of bacterial infections in humans. Eighty percent of urinary tract infections (UTIs) in humans are caused by Gram-negative, rod-shaped, flagellated and facultative anaerobic bacteria of the family Enterobacteriaceae with name Escherichia coli and Proteus species[7].

In patients with a recurrent urinary tract infection, there is a long-term of antimicrobial treatment is formulated. And this method is operative but can cause contrary reactions and can give rise to increase emergence of antimicrobial resistance. So, the need for alternative therapies for UTI prophylaxis is evident. Treatment by herb is one of the non-antibiotic alternative therapy[8, 9]. The main of the work was to study the antimicrobial effects of aqueous garlic extract on *Escherichia coli* , *Proteus mirabilis* and *vaginalis* isolated from urogenital tract patients, and to evaluate its effect on bacterial motility, adherence to uroepithelial cells, biofilm formation and other virulence that have important role in pathogenesis of these studied microorganisms in genitourinary tract. Furthermore, the present study was tried to plains the inhibitory effects of AGE on study parasite's motility ,viability and growth rate, so that ,there is a good chance can be mediated from the utilization of AGE for further use as a potential natural sources for the development of novel alternative herb.

2.MATERIALSAND METHODS:

2.1Aqueous Garlic extraction:

The fresh Garlic (*Allium sativum* L.) bulbs were collected from the local markets of Hilla City/Iraq, and authenticated as *Allium sativum* (University of Babylon, College of Science). Then, peeling garlic bulbs should be done, weighed (50g), clean garlic should be taken and using ethanol to sterile surface. Evaporating of ethanol was accomplished in a sterile laminar flow chamber.

The AGE was prepared according to methods previously reported by Hinidi *et al*., (2014). By use a sterile mortar and pestle the 50g of garlic was homogenized, and added to 100 ml of distilled water, then allowed it to stand for a time of 72 hr, The sterilization of the mixture was performed by filtration through a Millipore filter paper 0.45. The prepared AGE was regarded as a concentration of 50% from the extract. The aqueous solution was stored in a sterile bottles and be kept at 4ºC until further use for screening of antimicrobial activity[10].

2.2 Microorganisms used:

Two types of microorganisms which are *T. vaginalis* and bacterial isolates that include five isolates from each *E. coli* and *Proteus mirabilis* were detected and isolated from clinical cases of UTI patients at Babylon Hospital for Maternity and Children / Iraq, during the period June-October 2015. The clinical bacterial isolates were identified to species level based using standard microbiological methods including conventional biochemical tests [11,12] and commercial API20E system at college of Nursing/ Babylon University, Iraq.

2.2.1 Antibacterial activity of AGE:

To preparation bacterial suspension, five colonies from a young cultures was diluted in 5 ml of normal saline and incubated at 37C for 5 hours. Then adjusting the turbidity and comparing it with the standard tube (Number 0.5 of McFarland) for yielding a uniform suspension containing 1.5×108 CFU/ml. Dipping a cotton swab into adjustment suspension and streaking the entire Mueller-Hinton agar surface of plates and they were left for 15 minutes at the room temperature to be dried. The study bacterial media were cut into four wells (5mm of diameter) by use a cork borer and add 0.1ml of the prepared AGE. The plates were incubated at 37C° for overnight. The size of zone of inhibition was measured from edge of well to the edge of inhibition of growth[8].

2.2.2 Antibiotics susceptibility testing:

The antibacterial activity of some antibiotics was determined by using the agar disc diffusion test(DDT). Virtually, the agar plates were inoculated with 0.1ml of broth culture of the study microorganisms. The antibiotics disks (Hi-Comb, India) of Ciprofloxacin(5µ g) were added in the center of agar plate (study plates were performed in a triplicates). All plates of the tested microorganisms were then allowed to be incubated at 37°C. After incubation period for18 hr, the MIC values for each antibiotic disc were determined by using Hi-comb strip (Hi-media, India) and compared with break-points which recommended by CLSI [13].

2.2.3 Antibacterial adherence of AGE:

Modified method [14] was used for detection of ability of bacteria for adherence to epithelial cells. The epithelial cells of bladder cavity were collected and transferred directly into sterile tubes contain PBS (PH 7) after that wash the epithelial cells by PBS, and centrifugation (at 5000 rpm for 10 minute) for three times. The filtrated epithelial cells treated with standard bacterial suspension and then with AGE (1.2500 mg/ml) for different incubation times, 1-10 hr. at 37C. The mixture was washed by PBS to remove unadherent bacteria. The epithelial cells were fixed by ethanol for 15 minutes, and stained with Giemsa stain (30%) for 20 minutes. The cells were then examined under a light

microscope, and the mean of tissue cells which bound more than 10 bacteria per cell was calculated.

2.2.4 Anti-biofilm formation:

In the current study, the isolates of both tested bacteria were filtered for their ability to form biofilm by use a tissue culture plate (TCP)method as described in references [15, 16] with a modification in duration of incubation which was extended to 24 hours according to producer that described by Nakao, *et al*.(2012).

2.2.5 Anti-bacterial motilit:

Iwalokun, *et al*., (2004) describe procedure in reference [17] was used for detection of AGE effect on bacterial motility: AGE was prepared and added separately in concentration ranging (0.0-10 mg/ml) into bacterial culture media; nutrient agar and soft agar plates. All media were cultured with bacterial isolates, and then be incubated at 25°C for 18 hr. After that, the effect of AGE on motility activity of *Pr. mirabilis* was determined by measuring swarming diameter of and compared with swarming of positive control, whereas *E. coli* motility was determined by inhibition of growth dissemination out line of stabbing. The hanging-drop method for demonstration of motility in *E. coli* isolates also was done.

2.3 Culturing of *T. vaginalis* parasite *in vitro*:

Clinical samples; vaginal swabs and secretions; were collected from 86 female patients(two samples from each patient: the first sample for direct wet-mount examination of vaginal secretions by use saline suspension and the second sample for culturing and control purposes) of childbearing age attending the Maternity and Children Hilla Hospital / Iraq , during the period from June to october 2015. By using a sterile swab containing stuart's transport media "via magent 77/6, Rho (MI) Italy". The samples were transported immediately to the laboratory of the hospital to be examined by direct wet-mount and then these samples would be removed (positive cases) from the transport media and used to inoculate medium no. 2 (oxoid) within a few hours after collection them to the laboratory of Science College/ Babylon University. The cultured-samples were incubated at 37°C and checked daily for the parasite's growth rate, viability and motility under the microscope field for a total of five days [18].

2.3.1 Counting of *T. vaginalis in vitro*

For counting the protozoan parasite of present study, we have follow (with a modifying manner) a counting procedure that used previously by Philip, *et al*., (1987) for counting *T. vaginalis* parasite [19] .Virtually, we have taken two samples "in duplicate manner" from the study cultured medium and then put it into a levy double counting chamber hemocytometer and counted at x100 and then at x400 magnification powers , respectively. In addition, un appropriate dilutions were made from medium to be inspected for obtaining an initial concentration of 250 organisms per ml [19]. In fact, all *T. vaginalis*-positive samples that detected by wet mount were succeed to grow in medium , a duplicate counts were made for each sample for obtaining more accurate results. Samples were taken for counting after mechanical agitation for at least 10 seconds using a tipped Pasteur pipettes to sample bottom of the cultured tubes every hour for the first few hours and then for twelve hours for five days, the tubes should be canned and capped tightly and disclosed only for sampling purposes [20].

2.3.2 Anti-*T. vaginalis* activity of AGE :

To exhibit the inhibitory effect of aqueous garlic extract (AGE) in the growth rate of *T. vaginalis* parasite, our present study have prepared a crude garlic extract (as mentioned previously in the present study for bacterial processes) and making serial dilutions of concentrations ranged from 5%,10% , 20% and 30% , respectively[10]. The garlic extract treated cultured- samples (sixteen positive samples) were incubated at 37°C and viewed daily for the growth rate of *T. vaginalis* under the microscope field for a long of time of the used incubation period. Actually, the culture was considered negative if no growth was seen post five days of inoculation with incubation [18].

2.3.3 Anti-*T. vaginalis* motility *in vitro*:

Samples were taken from *T. vaginalis* positive culture and examined under microscopic field (in a duplicate manner), the specimen was placed in 1.0 ml of physiological saline. Firstly, be examined at x100 and then at x400 of magnification power. Examination was thorough "the cultured tubes were not centrifuged prior to sampling ", with over whole field of slide examined at x 100. Only motile *T. vaginalis* parasite was considered positive [21].

2.3.4 Anti-*T. vaginalis* viability *in vitro*:

For investigating the viability of parasite in the present study, we have made duplicate for each tested sample from the AGE treated-culture. Also, in relation to the culture incubation time used(in days), in relation to results of microscopic field to the motile parasite and according to the AGE concentration treated culture used, we have interpretative study-obtained results. Culture was considered negative if no growth was seen post five days of incubation period [22].

2.4 Statistical Analysis:

In fact, most obtained results of the present study were numbered and analyzed statistically as percentages, rates and means.

3. RESULTS AND DISCUSSION:

Antibacterial effects of AGE:

The findings of present study suggest that AGE have antibacterial properties against gram negative bacteria isolated from UTI cases caused by *E. coli* and *Proteus mirabilis*. The diameter of the inhibition zone was ranged from 27.0 to 28.0 mm, for garlic extract, as compared with 12.0 to 18.0 mm for Ciprofloxacin against both genera; *E. coli* and *Proteus mirabilis*, respectively. Our results indicate a considerable antibacterial activity of aqueous garlic extract which is more than the antibacterial activity of ciprofloxacin against tested organisms, as shown in fig.1 and fig.2, respectively .Indeed, the therapeutic effect of garlic is possible because of its high contents of organ sulfur compounds, which are responsible for a typical odor and a well-known flavor of garlic. Hence, thiosulfates compounds play an important role in the antibiotic activity of garlic. The Hsieh, *et al*.,(2001) were showed that the antimicrobial activity of garlic is completely obliterated when the thiosulfates (as in allicin) are abolished from the garlic extract[23]. In addition, the reduction of allicin to diallyl disulfide, the antibacterial activity of AGE is abolished greatly.

Durairaj, *et al*.,(2010) showed that the allicin can exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action[24]. The difference in the structural bacterial strains may also play an effective role in the bacterial susceptibility to garlic components .For those individuals with a recurrent infections, the prolong uses of antibiotics may cause severe side effects and resistance in bacteria against antibiotics[25]. As the resistance of antibiotic elicit an increasing level, the use of alternative strategies such as garlic consumption become more important use for preventing such infections [26].

Anti-adhesion effects of AGE:

The results of our study have concentrated on understanding how AGE effect on the adherence of *E. coli* and *Pr. mirabilis* in the epithelial cells, and focusing on their beneficial effects in preventing urinary tract infections The ability of both genera to binding to epithelial cells was decreased when exposed to AGE during in vitro experiment, Table-1 below .

Table (1): Anti bio film and anti-adherence activity of aquatic extract against *E. coli* and *P. mirabilis*.

Bacterial isolates	Biofilm formation	Adherence
P. mirabilis	High reduced	High reduced
E. cloi	High reduced	High reduced

The adhering of bacterial species to the uroepithelial cells in the urinary tract due to special receptor is found on surface of target cell. Garlic extract may have compounds similar structure in the receptors of the uroepithelial cells and block the receptors of uroepithelial cell. Hence the *E.coli* and *Pr. mirabilis* will not able to adhere to receptors of uroepithelial cells and they are killed by the antibiotics and flush out when patient consume the glass of water [24,26].

Effect of AGE on biofilm formation:

The biofilm experiment was done in triplicate and repeated three times when treated with 0.2 ml of 0.5 mg /ml AGE solution. The data were then averaged, and the results were interpreted according to Mathur and co-workers method [27]. Result of present study about effect of AGE on formation of bacterial biofilm showing below in Table-1. The results appear that the biofilm formation of *E. coli* and *Pr. mirabilis* was much reduced.

Anti-motility effect of AGE:

In vitro, the effect of AGE on motility of *E. coli* and *Pr. Mirabilis* was investigated. The media plates that used for bacterial motility were supplemented with different concentration of aqueous garlic extract solution as in table-2 .

Table (2):Anti-motility activity of aquatic extract against *E. coli* and *Pr. Mirabilis*.

Bacterial isolates	Concentration of aquatic extract		
	10%	20%	30%
P. mirabilis	Motile	Weak motile	Non motile
E. coli	Motile	Weak motile	Nonmotile

The phenomenon of *Pr. Mirabilis* swarming and motility of *E. coli* were inhibited following AGE exposure. The results show that exposure to 30% concentration of AGE completely blocked motility of both genera, even after 18 hr of incubation. AGE solution at concentration 20% cause a temporal decrease in the motility .The effect of AGE on bacterial motility is interpreted by it contains chemical compounds, which have been shown to inhibit the motility of several uropathogenes [24,25,26].

Culturing of *T. vaginalis* parasite *in vitro*:

After daily checking to the incubated-cultured samples for the parasite's growth rate under microscope field for a total of incubation period with the comparison of the obtained results with that found in control groups [18,28], we have interpretive our study obtained results. Virtually, all incubated-cultured samples (16 samples) were positive in growth rate "in a control groups" because the cultured samples were untreated with AGE to be affected by AGE activity *in vitro*. Whereas, in AGE treated-cultured samples, the growth rate and other physiological processes of the studied parasite were affected accordingly.

The explanation for such obtained results may be related to the action of inhibitory effects of AGE components especially the organ sulfur compounds mainly the Allicin[20]. In fact, many studies were carried out on the chemical composition of the A. sativum plant and showed that the most important constituents of this plant are organosulfur compounds such as allicin, S-allylcysteine, diallyldisulphide, and diallyltrisulfide [5,9,30,31].

Counting of *T. vaginalis in vitro*:

After following "a modified method" counting procedure that used previously by Philip, *et al*., (1987) for counting *T. vaginalis in vitro*[19], we have obtained and started with an initial concentration of 250 organisms per ml. Furthermore, all positive specimens that detected by wet mount were succeed to grow in medium ,a duplicate counts were made for each sample for obtaining more accurate results .The obtained results were showed that a detectable decreasing in the number of treated-cultured parasite oppose increasing in AGE concentration with increasing in the incubation period to the cultured samples, as shown in table-3 .

Table (3): Number of *T. vaginalis* parasite in treated and in an untreated-AGE culturing media *in vitro.*

T. vaginalis AGE Concentration	Post incubation period	Surviving number in treated culture-media/ml	%	Surviving number in an untreated culture-media (control group)/ml	%
Crude 100%	1 hour	0.00	0.00	250	100
30%	24 hours	0.00	0.00	226	92
20%	48 hours	0.00	0.00	212	85
10%	72 hours	0.00	0.00	195	78
5%	96 hours	25	10	175	70

The result of present study were in constitute with other studies [19,20,22]. The action of AGE can bring about a strong inhibitory and lethal effects on several vital processes of AGE-treated microorganisms such as: decrease in growth rate or surviving; decrease in protein synthesis; inducing apoptosis through the mitochondrial pathways; inhibiting enzymes synthesis and inducing cytopathic effects in microorganisms [32,33,34,35.]

Anti-*T. vaginalis* activity of AGE:

The obtained results of present study exhibit that AGE have antiparasitic effect against protozoan parasite isolated from urogenital tract cases caused by *T. vaginalis* .The growth rate of the study cultured parasite in medium was ranged from normal progressive growth rate to slower ones during the first day of incubation till fourth day of incubation, respectively, with the respect to administered AGE concentration and comparison with results of control groups Virtually, all concentrations of AGE were exhibit notable effects on the growth rate of the studied parasite for five days of incubation with regarding to AGE concentration and incubation period in days.

The AGE crude concentration was with high inhibitory effect on the growth rate of the study parasite, since the growth rate parasite was inhibited completely post one hour of incubation. Also, the growth rate of parasite was inhibited completely following the administered AGE concentrations, which were: 30% of AGE post one day, 20% of AGE post two days, 10% of AGE post three days and temporally inhibited by 5% post four days, as shown in both tables:3 and 4, respectively.

The explanation for such obtained results can be attributed to the fact that documented the allicin, ajoene, and organosulfides from garlic are effective antiprotozoal compounds [36,37]. Another studies were showed similar results [30,33,34].

Anti-*T. vaginalis* motility *in vitro*:

The same prepared AGE concentrations for investigating the growth rate of the present study parasite, were used for determining motility of *T. vaginalis in vitro*. All concentrations of AGE were exhibited a notable inhibitory effects against motility of study parasite with regarding to time of incubation and with comparison of control group.

Immediately, the crude concentration of AGE was with high inhibitory effect against the motility of parasite *in vitro* "when compared with the study control group", whereas, it was graduated subsequently in other concentrations, as depicted in table-4.

Table (4):Determining the inhibitory effect of AGE on the motility of *T. vaginalis* parasite *in vitro*.

T. vaginalis AGE-Concentration	Post incubation period	Type of parasite motility in an untreated culture	Type of parasite motility in treated culture
Crude(100%(	1hour	Rapid and jerky	Absent/Immotile
30%	24 hours	Rapid and jerky	Absent/Immotile
20%	48 hours	jerky	Absent/Immotile
10%	72 hours	Sluggish	Sluggish
5%	96 hours	Weakened	Too twitch

The interpretation for such obtained results may be due to high drastic influenced concentration of AGE during incubation period, since the inhibitory effect of plant extract components will increase with the increasing of extract concentration repeatedly [30,34,37].

In the present study, only motile *T. vaginalis* parasite was considered survived "unaffected yet", whereas the immotile parasite was considered positive "be affected drastically." The results of present study weren't differed with other studies[38,39].

Anti-*T. vaginalis* viability *in vitro*:

According to obtained results of present study" as shown in the preceded tables 3 and 4", the interpretative explanation for such obtained results was: absent, sluggish and twitch in parasite motility opposite increasing in AGE concentration, decrease in parasite surviving opposite prolonged incubation time, and decrease in parasite growth rate opposite both increasing in AGE concentration and extended time of incubation). Virtually, all treated cultured samples were considered positive if no growth was seen till and post incubation period for five days of incubation [22,39]. Many other studies were referred to and explained such results [22,30,40,41].

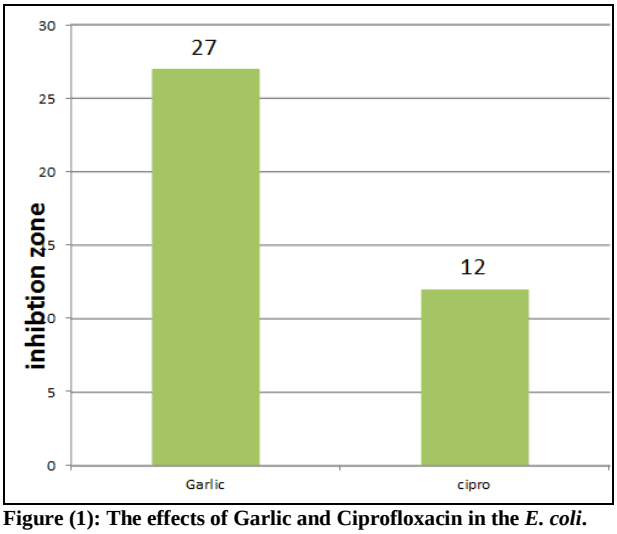

Figure (1): The effects of Garlic and Ciprofloxacin in the *E. coli*.

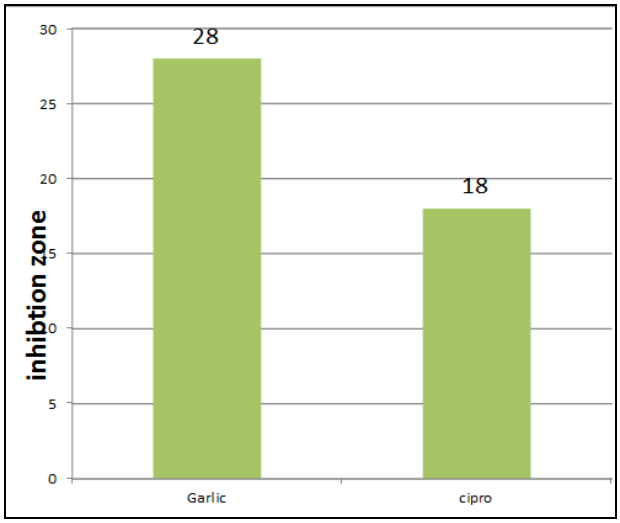

Figure (2): The effects of Garlic and Ciprofloxacin in the*Proteus mirabilis*.

CONCLUSION:

The aqueous garlic extract (AGE) has potential antimicrobial activity against urogenital pathogens; *coli*, *Pr. mirabilis* and *T. vaginalis*, and it can be used for preventing a numerous urogential tract infections.

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