

An *in vitro* study of Anti-bacterial, Anti-Adherence, Anti-Biofilm and Anti-motility activities of the aqueous extracts of fresh and powdered onion (*Allium cepa*) and onion oil

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Abstract

Previous studies showed that extracts of onion possess pharmaceutical properties that render them potential therapies for treatment of neoplastic, metabolic, immunological and infectious diseases. The latter include bacterial, parasitic, fungal as well as viral infections. To investigate the *in vitro* anti-bacterial activities of the aqueous extracts of fresh and powder onion and onion oil on a range of Gram-positive and Gram-negative bacterial isolates. Anti-bacterial activities of onion were studied in light of its effects on the growth, motility, adhesion and biofilm formation in bacterial species of interest.

Aqueous preparations and oil of onion were purchased from a retail food store at Al- Hilla town (2018). The extract of fresh onion was prepared and the powder onion was soaked as 30 gram of powder in 100 ml distilled water and allowed to stand for 72 hr. Then, it is sterilized by filtration (using Millipore 0.45 filter paper). Bacterial isolates (a total of nine Gram-negative and five Gram-positive isolates) were isolated from clinical samples. Agar well diffusion assay, tissue culture plate method (TCP) assay and bacterial ability to adhere to oral epithelial cell were used to evaluate the effects of onion on the growth, motility, adhesion and biofilm formation in bacterial species of interest.

The findings of current study revealed that the aqueous extracts of fresh and powdered onion as well as onion oil have significant inhibitory effects on growth, motility, adherence and biofilm formation in the Gram-positive and Gram-negative bacterial isolated considered in current study.

These preparations could be useful alternatives for commonly utilised synthetic agents for treatment of multi-drug resistant bacteria. However, the exact mechanism(s) behind the observed antimicrobial effects of onion needs further exploration and specification, maybe at the molecular level. In addition, *in vivo* studies involving humans and / or animals are needed to confirm the observed effects of onion.

Keyword: Antimicrobial activity, Anti-adherence, Anti-biofilm and Anti-motility, Extract of Onion, *Allium cepa*.

INTRODUCTION

Onion (*Allium cepa*) is a bulbous plant grown in nearly every country in the five continents [1]. In addition, the reproduction, transport and storage of onions are quite easy. It has been shown that onions have particular medicinal uses both in conventional and modern medicine as they are effective in the treatment of diseases affecting cardiovascular, respiratory as well as musculoskeletal systems [2; 3]. Onions contain carbohydrates, proteins and minerals (such as sodium, potassium and phosphates). In addition, they contain secondary metabolites that exert *in vitro* antibacterial activities such as tannins, terpenoids, alkaloids and flavanoids [3; 4]. Also, onion bulbs contain a wide range of phytochemicals, most of them are hydrocarbons and their derivatives, such as dipropyl disulphide, Allicin, diethyl sulphide, dimethyl disulphide, mercaptopropane or propylmercaptan [5; 6; 7].

Previous studies showed that extracts of onion possess pharmaceutical properties that render them potential therapies for treatment of neoplastic, metabolic, immunological and infectious diseases [8; 9]. The latter includes bacterial, parasitic, fungal as well as viral infections [8; 10-13]. For example, onion extracts showed antibacterial activity against *Vibrio cholera* [1]. Another example, alcoholic and aqueous suspensions of dried onion bulbs exhibited inhibitory effects against the growth of Gram-positive bacteria such as *Staphylococcus aureus* [14] and *Bacillus subtilis* [15]. In addition, the fresh juice of onion (*Allium cepa*) showed significant inhibitory effect against the growth of a range of multi-drug resistant bacteria [16]. Moreover, extracts of onion showed concentration-dependent reduction in the population of bacteria associated with ophthalmic infections [17].

Bacterial motility is a feature of Gram-negative as well as Gram-positive bacteria [18; 19]. It is an activity in which bacteria quickly move on wet surfaces in a synchronized way, however, it does not begin unless bacteria attain certain population. The development of this activity includes the detection of a suitable physical and chemical signals from the surrounding with the resultant production of elongated (due to

inhibition of bacterial division) and flagellated cells, synchronized progression of these cells and consolidation to vegetative cells and beginning of a fresh cycle [18; 19]. Bacterial motility is genetically controlled and associated with their biofilm formation with subsequent resistance to antibacterial agents [20-22]. In addition, it is influenced by availability of exterior nutrients, humidity of the surface and presence or absence of chemical agents such as surfactants and antiseptics.

On the other hand, biofilms are means by which bacteria can resist environmental challenges and stay alive for a long time by being firmly attached to the host surfaces [23; 24]. In these biofilms, bacteria find accommodation in a milieu composed of sugars, proteins and nucleic acids. Biofilms formation is a feature of chronic bacterial infections where they (bacteria) become multi-drug resistant and can not be attacked by immune cells of the host with subsequent augmentation of economic burden due to frequent admissions to hospital [8]. For example, catheter-related infection of the urinary tract and the subsequent formation of biofilm by *Proteus* species are triggered by swarming activity of these microorganisms [20]. Moreover, many antibacterial drugs are virtually ineffective against biofilm cells as compared to the more sensitive planktonic cells. Bacterial cells are capable of producing multi-layer biofilms with the greater numbers of cells in the upper and lower layers, while water channels (in between) are to convey nutritional elements and waste products [25].

Moreover, there are two cell-surface attached organelles responsible for bacterial motility and biofilm formation. These are the flagella and type IV pili (26). They have been shown to facilitate bacterial association with host surfaces [26]. In addition, when environmental factors are optimal, bacteria will continue their progression as well as cellular division and eventually formation of horizontal and homogeneous biofilm; otherwise the latter will be consisting of cellular collections [27]. As a result, bacterial swarming and biofilm formation are inter-related and, therefore, pharmaceuticals that can antagonize the former will indirectly prevent formation of biofilms and prevent the development of multi-drug resistant bacteria [28].

One of the powerful means by which bacteria gain access to host cells is appearance of a wide range of adhesions at different stages of the infectious process. These adhesions are critical for virulence of bacteria and, hence, antimicrobial agents that interrupt bacterial adhesion activity can render them non-virulent [29]. Moreover, the production of virulent elements by bacteria is genetically-regulated by the quorum system which is controlled by small messengers known autoinducers. Therefore, blockade of the quorum system may represent a novel approach for fighting bacterial virulence [29].

Despite the number of studies that had investigated anti-bacterial effects of onion extracts, the mechanism(s) underlying these effects is still uncertain and/or not established. Therefore, the broad aim of current study was to investigate the *in vitro* anti-bacterial activities of the aqueous extracts of fresh and powdered onion and onion oil on a range of Gram-positive (G^{+ve}) and Gram-negative (G^{-ve}) bacterial isolates. Anti-bacterial activities of onion were studied in light of its effects on growth, motility, adhesion and biofilm formation in the bacterial species of interest.

MATERIALS AND METHODS

Preparation of Extracts

Aqueous preparation of onion and oil of onion were collected from a retail food store (Al- Hilla) 2018. The extracts of fresh onion was prepared and the powder onion was soaked 30g of powder by 100ml distilled water, and allowed to stand for 72 hr, and sterilized by filtration (using Millipore 0.45 filter [30]).

Bacterial Isolates

A total of 9 Gram-negative, and 5 Gram-positive isolates (isolated from clinical samples) were used in this study. The bacterial isolates represented by; *S. aureus*, *S. epidermidis*, *S. pyogenes*, *E. faecalis*, *S. pneumoniae*, *P. aeruginosa*, *P. fluorescens*, *E. coli*, *S. typhi*, *E. aeruginosa*, *K. pneumoniae*, *Proteus mirabilis*, *P. vulgaris*, *Acinetobacter*. These bacteria were activated and cloned three successive times in nutrient agar and stored on nutrient agar slant at 4 °C. The identification of these organisms was confirmed by using conventional biochemical tests [31].

In vitro Antimicrobial activity testing using Agar well diffusion assay [32]

Loopfull growths from bacterial isolates were inoculated into nutrient broth incubated at 37 °C for 18 hours. The bacterial suspensions were diluted with normal saline. Adjust the turbidity and compare with standard tube (McFarland number 0.5) to yield a uniform suspension containing 1.5×10^8 CFU / ml. Dip cotton swab into adjustment suspension and streak the entire Mueller-Hinton agar and sabouraud dextrose agar surface of plates and the plates were left for one 5 -15 minutes at room temperature to dry. Media were cut into four wells (5mm diameter) by cork borer and add 0.1ml of the extracts. The plates were incubated at 37°C for overnight. The size of zone of inhibition was measured from edge of well to the edge of inhibition of growth.

Biofilm Formation Assay

Tissue culture plate method (TCP) assay (also called semi quantitative microtiter plate test (biofilm assay) described by Christensen et al. [33] was most widely used and was considered as standard test for detection of biofilm formation as follow:

1. Isolates from fresh agar plates were inoculated in TSB containing 1% glucose and incubated for 18 hour at 37°C and then diluted 1:100 with fresh TSB.
2. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates wells were filled with 150µl aliquots of the diluted cultures and only broth served as control to check

non-specific binding of media. Each isolate was inoculated in triplicate.

3. The tissue culture plates were incubated for 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria.
4. Biofilms formed by adherent 'sessile' organisms and plant extracts in plate were fixed by placing in oven at 37°C for 30min.
5. All wells stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying.
6. 150µl of acetone/ethanol (20:80, v/v) mixture were added to dissolve bounded crystal violet. The optical density (O.D.) at 630 nm were recorded and the results were interpreted according to the table [2].
7. This method was repeated and modified by adding the extracts in stage 2 to inhibit the biofilm formation by extracts.

Adherence test

The ability of bacteria to adhere to oral epithelial cell is one of important virulence properties of this bacteria and detected as following steps:-

1. Prepare the bacterial broth and incubated for 72 hrs.
2. Prepare dilution of bacterial broth by use phosphate buffer (PBS) then take 10^8 (CFU/ cm³).
3. Take the oral epithelial cells by swabbing the epithelial layers of oral cavity by cotton swabs, then transferred directly in to sterile tubes contain PBS (PH 7) after that wash the epithelial cells by PBS by using centrifuge (5000 rpm for 10 minute) for three times.
4. Filtered the PBS contain epithelial cells by filter paper, then place the epithelial cells on cover slide by press the cover on surface of filter paper then lifted to be dry.
5. Place the cover slides the cover slide on sterile glass plate then add 5ml of previously prepared bacterial broth and extract, then place the plate contains the epithelial cells and bacterial broth on incubator for 1hr at 37c.
6. Wash the cover slides by PBS to remove un adherent bacteria.
7. Fixed the epithelial cells by ethanol for 15 minutes.
8. Stain with giemsa stain(30%) for 20 minutes then wash the cover slides by DW and lifted to dry by air.
9. Place the cover slides on glass slides by inverted position, then tested under light microscope (34; 35).
10. This method was repeated and modified by adding the extracts in stage 3 to inhibit the biofilm formation by extracts.

Inhibition of motility by plant extract

The method of Iwalokun *et al.*, [36], which contain the following steps was used:

1. Bacterial isolate was cultured on nutrient agar incubated at 37°C for 24 hr. as a control.
2. Plant extract was added separately in concentration of (10%, 20%, 30%) respectively and incubated at 37°C for 24 hr.
3. Few drops of 90% ethanol was added on petri dish cover and cultured with bacterial isolate incubated at 37°C for 24 hr.
4. After that determined the effect of plant extract on swarming activity by measuring swarming diameter.

Antibacterial activity assay

The antibacterial activity was determined by agar disc diffusion [31]. Agar plates were inoculated with 0.1ml broth culture of tested organisms and was spreader with sterile an L-shaped rod glass spreader. The antibiotics disks of ciprofloxacin were add in the center of agar plate. (The plates were performed in triplicates).

All plate of the tested organisms was then allowed to incubate at 37°C for overnight. After 24 h of incubation, each extract was noted for zone of inhibition for all isolates. The diameters of the zone of inhibitions were measured by measuring scale in millimeter (mm).

Data analysis

Descriptive statistics (bar charts and tables) were use for data presentation. The latter were expressed as Mean±SEM. For variables with two factors, independent *t*-tests with Welch's correction were used for comparisons. For variables with more than two factors a series of one-way Analyses of Variance (ANOVA followed by Tukey's multiple comparison test) were used. Probability values less than .05 were considered statistically significant.

RESULTS AND DISCUSSION

There is growing interest in studying the potential pharmaceutical properties and therapeutic applications, especially antimicrobial activities, of onion [38].

The findings of current study showed that the aqueous extracts of fresh and powdered onion as well as onion oil exert powerful inhibitory effects on growth of a range of Gram-positive (G⁺) and Gram-negative (G⁻) bacteria (Figures 1, 2 and 3). In addition, current study reported that Gram-positive and Gram-negative bacteria considered in the study showed comparable sensitivity to onion preparations employed in current study (Table 3). Also, current study revealed comparable minimal inhibitory concentrations (MIC) for onion preparations in question (Table 4).

Moreover, anti-bacterial activities of onion preparations investigated in current study (measured by zone of inhibition of bacterial growth) seemed to be more powerful than that of ciproflaxacin (Table 3). In addition, the aqueous extracts of fresh and powdered onion exhibited more powerful inhibitory effects than onion oil on the growth of both Gram-positive and Gram-negative bacteria considered in current study (Table 3). Also, the aqueous extract of onion powder showed more powerful antibacterial effects than the aqueous extract of fresh onion, especially on Gram-negative bacteria considered in current study (Table 3).

Furthermore, aqueous extracts of fresh onion and onion powder as well as onion oil, employed in current study, exhibited significant inhibitory effects on the growth of some Gram-negative isolates that were not affected by the synthetic anti-bacterial agent, ciproflaxacin (Figures 1, 2, 3 and 4). Biofilm formation by bacteria is a mechanism by which they become multi-drug resistant with the subsequent chronicity of infections [8]. However, the effects of onion on bacterial biofilms formation, adhesion and motility did not receive adequate interest.

Therefore, one of the salient finding of current study was that the aqueous extracts of fresh and powdered onion as well as onion oil showed moderate and high antagonistic effects on biofilm formation and adherence activities of Gram-negative bacteria in question (Table 5). In addition, onion preparations employed in this study exhibited concentration-dependent inhibition of motility in Gram-negative bacteria considered in this study (Tables 6, 7 and 8). No apparent inhibition of bacterial motility was seen at 10% concentration, however, bacterial sensitivity to anti-motility effects of onion preparations became noticeable at 15% concentration of the latter (Table 7). Complete inhibition of motility was reported at 20% concentration of onion preparations employed in current study (Table 8).

On the other hand, formation of adhesions is an essential virulent factor for

concentration-dependent inhibition of bacterial motility (Tables 6,7 and 8).

The inhibitory effects of onion preparations on bacterial growth reported in current study were similarly reported by numerous previous studies that had investigated the effects of onion and its extraxts on the growth of a wide range of bacteria, fungi, viruses and parasites (1; 14; 15; 16; 17; 38; 39; 41; 42; 43; 44).

One of the studies that investigated the effects of some compounds on bacterial biofilm formation is that investigated the inhibitory effect of a small (9-amino acid) peptide on biofilm formation by Gram-negative and Gram-positive bacteria [45]. The study reported that this short peptide produced morphological and physical changes in bacterial biofilms and decreased bacterial population in these biofilms.

Moreover, Abbas [46] examined the effects of Egyptian Clover Honey on motility and biofilm formation of *Proteus mirabilis*. The researcher concluded that clover honey can be employed for treatment of diabetic foot infections due to *P. mirabilis*, because it (honey) exerted potent inhibitory effects on these virulent factors of the bacterium in question. These findings were supported by those obtained by Kazemian and co-workers [47] who reported concentration-dependent anti-biofilm effect for the phytochemical Chamaemelum nobile on *P. aeruginosa*.

Of much relevance to current study is the work of Hindi et al. [48] who showed that the vinegar and aquatic extracts of black raisins have significant anti-motilit and anti-biofilm properties against a range of bacteria (both Gram-positive and Gram-negative) and fungi that are able to colonize oral cavity.

As long as no solvents have been utilised in the preparation of onion extracts in question, antimicrobial effects of onion reported in current study could be exclusively attributed to its ingredients of phytonutrients (especially polyphenols, flavanoids and allicin) as they are known to have a broad spectrum of antimicrobial activities [6; 49; 50].

Nonetheless, the more noticeable anti-bacterial effects of the aqueous extract of powdered onion, reported in current study, need and in-depth investigation and specification in a future study.

In light of the conclusions drawn in previous studies [1; 14; 15; 16; 17; 38; 39; 41; 42; 43; 44; 45; 46; 47; 49], the anti-bacterial effects of onion preparations reported in current study could be explained by one or more of the following mechanisms:

1. The phytochemicals in onion might have created hostile ecology for bacteria that had intimidated their growth.
2. These compounds have inhibited, in a concentration-dependent manner, flagellum-regulated bacterial motility through inhibiting formation or function of flagella and/ or cellular differentiation.
3. The effects of onion on biofilm formation might have stemmed from its ability to reduce bacterial population reaching the surface.
4. Onion preparations used in current study might have caused disintegration of biofilm ingredients.
5. It is possible that onion has induced dysregulation of genes controlling formation of bacterial flagella.
6. Onion preparations might have blocked the effects of environmental physical and chemical factors that are known to trigger motility in bacteria.
7. The phytonutrients polyphenols have inhibitory effect on glucosyltransferase, an enzyme required for colonization and adhesion by bacteria.
8. Phytonutrients in onion might have inhibitory impact on the quorum system of bacterial isolates considered in current study.

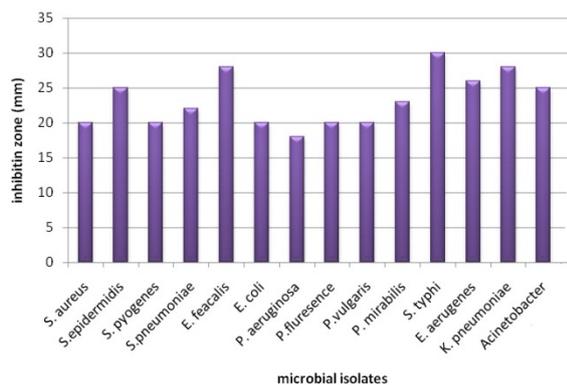


Figure 1: Anti-bacterial activity of aqueous extract of fresh onion by agar well method.

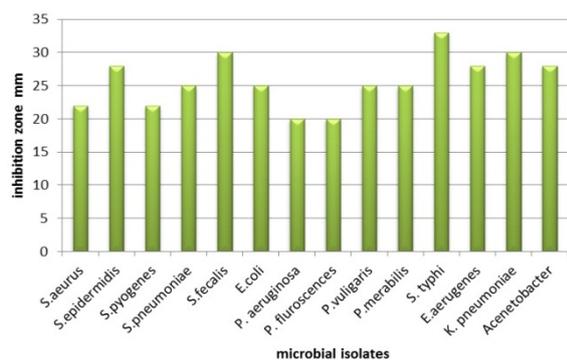


Figure 2: Anti-bacterial activity of aqueous extract of powdered onion by agar well method.

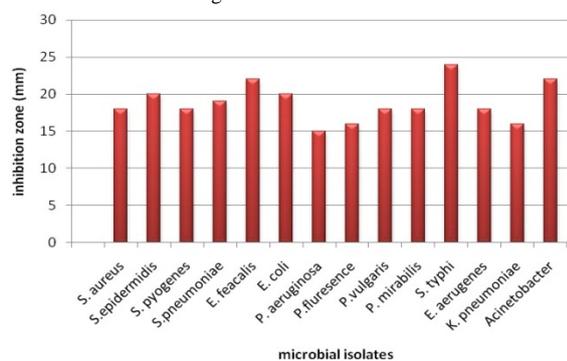


Figure 3: Anti-bacterial activity of onion oil by agar well method.

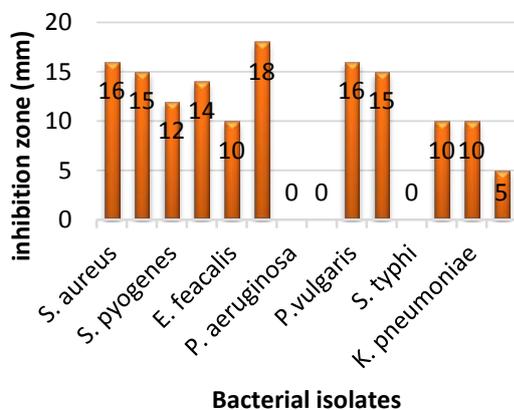


Figure 4: Anti-bacterial effects of Ciprofloxacin by agar well ,method.

Table 1: Classification of bacterial adherence and biofilm formation by TCP method [37]

Mean of OD value at 630nm	Adherence	Biofilms formation
<0.120	None	None
0.120-0.240	Moderate	Moderate
>0.240	Strong	High

Table 2: Classification of bacterial adherence by TCP method

Mean OD value	Adherence	Biofilms formation
<0.120	None	None / Weak
0.120 – 0.240	Moderately	Moderate
>0.240	Strong	High

Table 3: Effects of fresh and powdered onion extracts and onion oil on growth of G⁺ and G⁻ bacteria

Onion extracts	Zone of inhibition of bacterial growth (Mean±SE) / mm		P value
	G ⁺ (n=5)	G ⁻ (n=9)	
Aqueous of fresh onion	23±1.5* ¹	23.3±1.4* [†]	0.88
Aqueous extract of Powder ed onion	24.8±1.7* ²	25.7±1.3* ³ [†]	0.68
Oil	19.4±0.7*	18.1±1.2*	0.36
Control (Ciproflouxacin)	13.4±1.1	9.3±2.5	0.16

* Significant difference from control (P≤0.001).

¹ Significant difference from onion oil (P≤0.01).

² Significant difference from onion oil (P≤0.001).

³ Significant difference from Aqueous extract (P≤0.05).

[†]Significant difference from onion oil (P≤0.001).

Table 4: Effects of fresh and powdered onion extracts and onion oil on bacterial isolates by determination of MIC of the extract.

Microorganism	Aqueous extract of fresh Onion	Aqueous extract of powdered Onion	Onion oil
	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
<i>S. aureus</i>	1280≥	1280≥	1280≥
<i>S.epidermidis</i>	1280≥	1280≥	1280≥
<i>S. pyogenes</i>	1280≥	1280≥	1280≥
<i>S.pneumoniae</i>	1280≥	1280≥	1280≥
<i>E. faecalis</i>	1280≥	1280≥	1280≥
<i>E. coli</i>	2560≥	2560≥	2560≥
<i>P. aeruginosa</i>	2560≥	2560≥	2560≥
<i>P. flurescence</i>	2560≥	2560≥	2560≥
<i>P.vulgaris</i>	2560≥	2560≥	2560≥
<i>P. mirabilis</i>	2560≥	2560≥	2560≥
<i>S. typhi</i>	2560≥	2560≥	2560≥
<i>E. aerugenes</i>	5120≥	5120≥	5120≥
<i>K. pneumoniae</i>	2560≥	2560≥	2560≥
<i>Acinetobacter</i>	2560≥	2560≥	2560≥

Table 5: Anti biofilm and anti-adherence activities of the aqueous extracts of fresh and powdered onion and onion oil against Gram negative bacteria

Bacteria	Biofilm formation			Adherence		
	Aqueous extract of fresh Onion	Onion oil	Aqueous extract of powdered Onion	Aqueous extract of fresh Onion	Aqueous extract of powdered Onion	Onion oil
<i>S. typhi</i>	*Moderate	High*	High	Moderate	High	High
<i>P. aeruginosa</i>	Moderate	High	High	Moderate	High	High
<i>P. fluorescense</i>	Moderate	High	High	Moderate	High	High
<i>P. vulgaris</i>	Moderate	High	High	Moderate	High	High
<i>P. mirabilis</i>	Moderate	High	High	Moderate	High	High
<i>K. pneumoniae</i>	Moderate	High	High	Moderate	High	High
<i>E. aerogenes</i>	Moderate	High	High	Moderate	High	High
<i>Acinetobacter</i>	Moderate	High	High	Moderate	High	High
<i>E. coli</i>	Moderate	High	High	Moderate	High	High

*Moderately (0.120-0.240)

** High (>0.240)

Table 6: Anti-motility activity of the aqueous extracts of fresh and powdered onion and onion oil against Gram negative bacteria at 10% concentration

Bacteria	10%		
	aqueous extract of fresh Onion	Aqueous extract of powdered Onion	Onion oil
<i>S. typhi</i>	motile	motile	motile
<i>P. aeruginosa</i>	motile	motile	motile
<i>P. fluorescense</i>	motile	motile	motile
<i>P. vulgaris</i>	motile	motile	motile
<i>P. mirabilis</i>	motile	motile	motile
<i>Acinetobacter</i>	motile	motile	motile
<i>E. coli</i>	motile	motile	motile

Table 7: Anti-motility activity of the aqueous extracts of fresh and powdered onion and onion oil against Gram negative bacteria at 15% concentration

Bacteria	15%		
	aqueous extract of fresh Onion	Aqueous extract of powdered Onion	crude Oil g Onion
<i>S. typhi</i>	Non motile	Non motile	Non motile
<i>P. aeruginosa</i>	motile	motile	motile
<i>P. fluorescense</i>	motile	motile	motile
<i>P. vulgaris</i>	motile	motile	motile
<i>P. mirabilis</i>	motile	motile	motile
<i>Acinetobacter</i>	Non motile	Non motile	Non motile
<i>E. coli</i>	Non motile	Non motile	Non motile

Table 8: Anti-motility activity of fresh and powdered onion extracts and onion oil against Gram negative bacteria at 20% concentration

Bacteria	20%		
	Aqueous extract of fresh Onion	Aqueous extract of powdered Onion	Onion oil
<i>S. typhi</i>	Non motile	Non motile	Non motile
<i>P. aeruginosa</i>	Non motile	Non motile	Non motile
<i>P. fluorescense</i>	Non motile	Non motile	Non motile
<i>P. vulgaris</i>	Non motile	Non motile	Non motile
<i>P. mirabilis</i>	Non motile	Non motile	Non motile
<i>Acinetobacter</i>	Non motile	Non motile	Non motile
<i>E. coli</i>	Non motile	Non motile	Non motile

CONCLUSION

The significant finding of our study was that onion preparations considered in the study have anti-adhesion, anti-biofilm formation and anti-motility in the bacterial isolates of interest. Nonetheless, these preparations could be useful alternatives for commonly utilised synthetic agents for treatment of multi-drug resistant bacteria. However, the exact mechanism(s) behind the observed antimicrobial effects of onion needs further exploration and specification, maybe at the molecular level. In addition, in vivo studies involving humans and / or animals are needed to confirm the observed effects of onion.

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