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Antimicrobial Effect of *Rheum ribes* and Tio2 Nps on Bacterial Biofilm in *Escherichia Coli*

Hind Hussein Obaid[1], Zainab Zamel Khalaf[1], Heba Khaleel Tawfeeq[2], RoaaThaer Sabri[1] and Zeina Abdul-Qader Abdul-Jabbar[1]

[1.] *Department of Biology, College of science, University of Baghdad, Baghdad, Iraq*
[2.] *Central Environmental Laboratory, College of science, University of Baghdad, Baghdad, Iraq*

Abstract: *Rheum ribs reported to have used in traditional medicine system, and also used as antimicrobial agent against different species of bacteria. Tio2 NPs one of the most widely material that investigated for killing or inhibition of bacteria. In order that, this research was came to study the effects of antibiofilm R. ribs extract and Tio2 on uropathogenic E. coli. Furthermore, study the effect of R. ribs and Tio2 combination by using tissue culture plate method. The results showed that R. ribs extract and Tio2 gave inhibition effect against bacterial biofilm in high percentage (60-80%) and (65-76%) respectively, the first effect on (E7-E16) and the second (E9-E16), but the highly effect observed when mix each one of them with others one in percentage (60-78%) against 11 bacterial isolates from (E6-E16). From these results we can concluded that the R. ribs extract and Tio2 may act as an antibacterial agents in thefuture.*

Key words: *Rheum ribs, TiO2 NPs, Biofilm, E.coli.*

I. Introduction

Although most of *Escherichia coli* strains in the gut of humans and animals are bacterial flora, several are excellent opportunistic pathogens [1]**.** *E. coli* extraintestinal infections (urinary tract infections and bloodstream infections) represent a significant public health burden worldwide [2, 3]. Since the 2000s, antimicrobial resistance among *E. coli* isolates has increased contributing to the complexity in management of such infections [4, 5]. *Rheum ribes* (Rhubarb) belongs to the family of polygonaceae and it one of perennial and stout herbs species that chiefly distributed in the countries of Asia, in the temperate and subtropical world regions. The local name of *R. ribes* is Rewas and it grows in the north of Iraq, Iran, Palestine and Turkey [6]. *R. ribes* was used in traditional medicine system, the thick leaf-stalk of the plant used as a vegetable [7]. Roots as well as the powder of leaf-stalk used to relieve gastric illnesses and many disorders like liver, constipation, uterine, headache, bladder and kidney disorders. Also, it used as aperitif and in bile secretion [8]. As for antimicrobial influence, it was used against many of gram negative microbes such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* [9]. Many studies referred to the potential applications of metal oxide nanoparticles in biological systems, therapeutics, diagnostics, surgical devices and antimicrobial uses [10, 11, 12, 13]. Hydroxyl radicals and superoxide ions trigger from titanium dioxide nanoparticles (TiO2) when the late exposed to ultraviolet (UV) light, non-lethal level, and this resulted in decompose of organic compounds and inhibiting the growth of MRSA [14]. This highly efficient TiO_2 NPs oxidizing power is suitable in case of bacteria and other organic substances [16, 17, 18].The increasing application of drugs has resulted in the resistance of pathogenic microorganisms to existing antimicrobial compounds. Hence, exploring and designing alternative drugs from natural products is necessary to combat microbial infections. Plant-based drugs, which probably evolved as a chemical defense against predation or infection, is perceived to have less or no side effect compared with synthetic antibiotics [18, 19]. Biofilm-forming *Escherichia coli* strains are responsible for most infections in patients with indwelling bladder catheters [20]. Biofilm-associated bacteria are often hard to eradicate by antibiotics and can tolerate hundred- or thousand fold Higher doses than the corresponding planktonic bacteria [21]. *E. coli* has also been reported to be able to form intracellular biofilm-like aggregates inside bladder cells, making them hard to reach by both host defense mechanisms and antibiotics [22], sothis study was aimed to investigate the antibiofilm effect of Rhubarb extract and Tio2 NPs against uropathogenic *E. coli* and study the synergistic effect of them against this bacteria.

II. Materials and Methods

2.1 Isolation and identification

Specimens of urine were collected in sterilized containers, In the laboratory within aseptic conditions, the collected specimens were streaked directly on MacConkey and EMB agar (Himedia/India) for identified uropathogenic *E. coli*, then incubated for 24h at 37ºC. Pink colonies were picked and re cultured on another

MacConkey and EMB agar. Further identification tests included the morphological characteristics and biochemical tests were carried out depending on [23]. Finally API E20 system was done.

2.2 Plant

2.2.1 Plant collection and preparation of alcoholic extract

Local markets from Karbalaa and Baghdad were visited to collect Rhizomes of *R. ribes* in October 2013.These markets Authenticated, according to taxonomic method, by Herbarium of Iraqi Health Ministry. As for extraction, 100 gm of *R. ribes* rhizomes needed to prepare 94% methanol extract by using percolation. Time of extraction was 24h. Whatman No.1 filter paper used to filtrate the extract. Sterilized by 0.22 µ micro filters [24].

2.2.2 GC-MS analysis

Modified method [26] was used for Gas Chromatography – Mass Spectrometry GC-MS analysis based on [25]. Two agilents (6890GC system) and (5973N MSD) were coupled and operating at 70 eV, using 200 °C lit temperature; temperature of ion source was 200 °C; volume of (1 µl) used for split injection (50:1 split ratio); Capillary column (Agilent J & W, USA) with HP-5MS 30 m x 0.25 mm ID x 0.25 µm film was used; 100 °C/min oven; 275 °C for 20 min at 10 °C/ min; temperature of transfer line: 220 °C. Helium carrier gas; flow rate (constant) 1 ml/ min; Agilent GC/MSD ChemStation Version (D.02.00) was used for data acquisition

2.3 Nanoparticles

2.2.1 Preparation of TiO2 NPs suspension

The preparation was done according to[27],TiO2 NPs (100 mg) was added to sterile D.W (10 ml) and then vigorously shaken. Ultrasound (40 kHz) was focused on the suspending solution for 30 min, after that, suspending solution was autoclaved (121 ◦C for 20 min) and left to cool at room temp.

2.4 Biofilm assay

Method described by [28] was followed to achieve biofilm formation:

To study the ability of adherence, BHI broth was used to grow (16) *E. coli* isolates. The broth contained 1% glucose in 96 – well polystyrene tissue culture plates. Incubation was then done at 37 ◦C for 24 h under aerobic conditions. After got on planktonic cells, the late washed three times with deionized water. In each well, the adhering cells fixed with 200 µl absolute methanol for a period of 20 min. The plates left for drying overnight after they emptied. To stain the adhering cells, 200 µl of 0.1 % crystal violet was used for 15 min and the excess stain was then rinsed off. The plates left to air dried overnight after they washed with distilled water. To dissolve the crystal violate, 1 ml of 96 % ethanol was used per well and the absorbance of the plates were then recorded at 490 nm using a spectrophotometer. The experiment proceeds in triplicates. The wells with sterile TSB recorded as a negative control. The result calculate as in table below.

Table: Classification of bacterial adherence by tissue culture plate method (28)

OD values	Adherence	Biofilm formation
< OD c	Non	Non
OD < OD ≤ 2*ODc	Weakly	Weak
2*ODc<ODt≤ 2*ODc	Moderately	Moderate
4ODc<OD t	Strong	High

2.5 Detection the antibiofilm activity

2.5.1 Detection the antibiofilm activity of *R. ribes* extract against *E. coli*

The same protocol was used to produce biofilm as mentioned earlier but the different concentrations of plant extract were added with bacterial suspension in the wells as triplicate for each concentration, incubated for 24 hr at 37 ºC, after that all wells were washed, stained, and read O.D at 490 nm.

2.5.2 Detection the antibiofilm activity of Tio2 against *E. coli*

The same protocol mentioned in the study the effect of *R. ribes* extract was followed but the different concentrations of Tio2 NPs were added with bacterial suspension in the wells as triplicate for each concentration, incubated for 24 hr at 37 ºC, after that all wells were washed, stained, and read O.D at 490 nm.

2.5.3 Detection the synergistic antibiofilm activity of Tio2 and *R. ribes* extract against *E. coli*

To investigate the anti-biofilm activity of Tio2NPs in combination with *R. ribes* extract, a TCP assay was also performed. 100 µl of the bacterial suspension, at 10 CFU, was added to the individual wells of sterile, polystyrene, 96 – well, flat bottomed TCPs after that, Tio2 NPs in combination with Rhubarb extract was added (50 µl from each one of them) with the final concentration being the MIC (the MIC was mentioned in other study under press). The Tio2 NPs were replaced by deionized water in the control well. The TCPs incubated at 37 °C for a period of (8) hours. The wells were then stained with crystal violate (0.1 %) after they washed three

times with distilled water. The stain was rinsed off, resolubilized with ethanol and absorbance was measured at 490 nm .The control was considered to represent 100% of biofilm formation. The following equation was used to calculate biofilm inhibition percentage [29]:

Biofilm inhibition (%) = (Control OD- Test OD / Control OD) ×100

III. Results and Discussion

3.1 Isolation and identification

A total no. of 30 urine samples was processed. Out of these samples, 16 isolates were confirmed as uropathogenic *E .coli* by Gram's staining, culture characterization and biochemical tests. API E20 system was also done as confirmation test.

3.2 Determination of chemical compounds in *R. ribs* extract GC-MS analyses

Nine compounds were found in this method. Pattern of fragmentation of compound's peaks compared to that of library compounds. Nine compounds were identified using this method (Table1), (Fig.1). The major components present was 1'H-Cholesta-2,5-dieno[3,2-b]indole, 1'-(phenylmethyl)-(17.094%).

Table (1): Composition of *R. ribes* rhizomes methanolic extracts.

No.	Compound	Retention time (min)	Amount (%)	Chemical Formula	Molecular weight	Synonyms
1	Oxalic acid, cyclohexylnonyl ester	4.102	3.772%	$C_{17}H_{30}O_4$	298	no synonyms
2	Benzenepropanoic acid, α-(hydroxyimino)-	4.8	2.521%	$C_9H_9NO_3$	179	1.(2Z)-2-(Hydroxyimino)-3-phenylpropanoic acid #
3	4H-Pyran-4-one 2,3-dihydro-3,5-dihydroxy-6-methyl-	4.857	2.823%	$C_6H_8O_4$	144	1.3,5- Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one 2.2,3- Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one 3. Pyranone 4.2,3-Dihydro-3,5-dihydroxy-6-methyl-4-pyrone 5.3-Hydroxy-2,3-dihydromaltol
4	1,3-Diazacyclooctane-2-thione	6.377	3.178%	$C_6H_{12}N_2S$	144	1.1,3-Diazocane-2-thione #
5	[1,1'-Biphenyl]-4,4'-diamine,	8.857	33.208%	$C_{38}H_{32}N_2$	516	no synonyms
6	Bis(diiodoarsino)methane	13.798	14.622%	$CH_2As_2I_4$	672	no synonyms.
7	Silane	15.024	14.622%	$C_{35}H_{74}O_2Si$	554	no synonyms.
8	1'H-Cholesta-2,5-dieno[3,2-b]indole,	17.222	12.557%	: $C_{40}H_{53}N$	547	1.7-Benzyl-1-(1,5-dimethylhexyl)-12a,14a-dimethyl-1,2,3,3a,3b,4,6,7,12,12a,12b,13,14,14a-tetradecahydrocyclopenta[5,6]naphtho[2,1-b]carbazole
9	4,6-Bis(4-chloro-3-(trifluoromethyl)phenoxy)-2-pyrimidinol tbdms	20.471	7.237%	$C_{24}H_{22}Cl_2F_6N_2O_3Si$	598	1.Cl-1077 tbdms

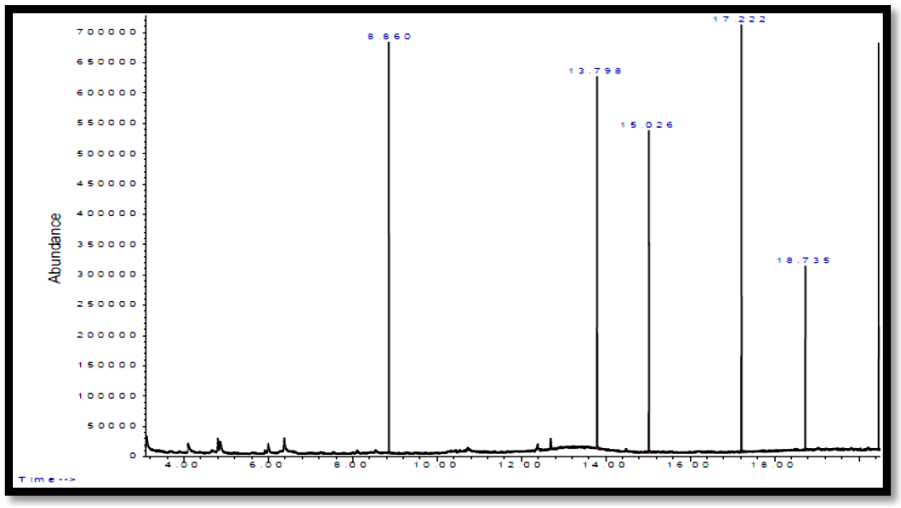

Figure (1): Chromatogram of *R. ribes* rhizomes methanolic extracts by GC-MS.

The method of GC – MS was given nine compounds. There are a pharmaceutical uses of theses determined compounds. As for oxalic acid, the element in Human blood which it essential for immune system in conditions of many diseases such as, bacterial, viral, cancer and vascular conditions. And when level of oxalic acid drops below its normal range this can be causes reduce immune system efficiency in fighting of many diseases [30]. Also, Bis-(diiodarsino) methane compound have important applications particularly in induce apoptosis of human cancer cells and equalize of abnormal cell growth in cervical dysplasia [31].

3.3 Biofilm assay

The results of present study showed that 16 bacterial isolates (93.75%) gave strong biofilm, while only one isolates (6.25 %) gave moderate biofilm, that's mean all uropathogenic *E. coli* in this study were biofilm producers.(see table (2)).

The biofilm production by microtitre plate assay has been performed for numerous bacteria including *E. coli* [32].

The differences in biofilm thickness resulted from different reasons such as differences in isolates capacity to form biofilm. Perhaps, the primary number of cells that succeeded in adherence and the differences of quality and quantity of auto inducers (quorum sensing signaling molecules) that produced from each isolate play an essential role [33, 34].

The prevalence and detection of biofilm producers have been showed to be dependent upon various factors such as the method, media and incubation period used [35]. In the previous study such as Mohamed *et al*.[36] , he was found that biofilm producing isolates in percentage (53.0%) who used

Luria broth incubated for 18 h and fixed the bacteria with Bouin fixative prior to the rinsing step with phosphate buffered saline (PBS) and Merendez-arancibia*et al*. [37] (78%) who used minimal glucose medium (M63) and incubated for 24 h.

Table (2): Biofilm producing by *E. coli* before treatment, usingmicrotiter plate assay

Isolates	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
O.D	**0.231**	**0.277**	**0.273**	**0.252**	**0.281**	**0.282**	**0.328**	**0.365**
Biofilm producing	**Moderate**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**
Isolates	**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**15**	**16**
O.D	**0.335**	**0.286**	**0.299**	**0.339**	**0.282**	**0.279**	**0.287**	**0.262**
Biofilm producing	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**	**Strong**

Other findings by[38] who found a rate of 67.5% and[39] who found a rate of 92.0%, which shows that there is an increased prevalence of biofilm formation by uropathogenic*E. coli* and females are in most cases at higher risks of acquiring *E. coli* induced UTIs.

3.4 Detection the antibiofilm activity

3.4.1 Detection the antibiofilm activity of *R. ribes* methanolic extract

Rhubarb extract gave antimicrobial effect against Planktonic form of uropathogenic *E. coli* in all its concentrations, these results reported in other research [40].There are little studies about the effect of Rhubarb extract on bacterial biofilm, while several studies conducted on its effect against bacteria in a planktonic form. The present research interested in study of effect of this extract on uropathogenic *E. coli* biofilm, the results indicated that Rhubarb extract was effective against all bacterial isolates and the percentage of effectiveness was higher than from these that appeared by Tio2 NPs, the higher effect against bacterial isolates (E7- E16), in percentage of inhibition (60% - 80%), while lower effect on isolates (E1- E6) in percentage (32%- 51%) as mentioned in table (3). Abdulla, K.K. [41]in your study showed that the *R. ribes* ethanol and aqueous extracts exhibit a broad spectrum of activity against *E. coli, S. aureus, P. aeruginosa* and *P. mirabilis.*The antibacterial effects of rhubarb are believed to have been caused by its inhibition of enzymes in the mitochondrial electron +transport system [42].

Table (3): The antibiofilm effect of Rhubarb *R. ribes* methanolic extract against *E.coli*

Isolates	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
O.D	**0.132**	**0.152**	**0.135**	**0.172**	**0.144**	**0.146**	**0.093**	**0.145**
Percentage of inhibition	**44%**	**45%**	**51%**	**32%**	**49%**	**48%**	**72%**	**72%**
Isolates	**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**15**	**16**
O.D	**0.101**	**0.06**	**0.073**	**0.070**	**0.079**	**0.079**	**0.071**	**0.061**
Percentage of inhibition	**60%**	**70%**	**76%**	**80%**	**72%**	**72%**	**75%**	**77%**

3.4.2 Detection the antibiofilm activity of TiO2 NPs

Well diffusion method by other study [40] was used to investigate the antimicrobial activity of TiO2 NPs against uropathogenic *E. coli*. photocatalytic antimicrobial activity of TiO2 NPs was the most studied among various NPs [43]. In a study by Roy *et al.* [44], they suggested that the TiO2 NPs alone was unable to act as antibacterial, But when it combined with antibiotics they will exhibit antimicrobial activity. But here TiO2 NPs without any type of combination was able to inhibit uropathogenic*E.coli.*The results of present study revealed that Tio2 NPs were affected against all bacterial isolates but highly effect against isolates from (E9 – E16) in the percentage of inhibition (65- 76%), as show in table (4). There are also studies on bactericidal activity of nitrogen-doped metal oxide nano catalysts on *E. coli* biofilms and on the photocatalytic oxidation of biofilm components on TiO2- coated surfaces [45]. In conclusion, the use of TiO2 photocatalysts as alternative means of self-disinfecting contaminated surfaces by further development may provide potent disinfecting solutions for prevention of biofilm formation. TiO2 photo catalysts can be used as effective biofilm disinfectant in food processing industries [46, 47]. Suspensions containing TiO2 are effective at killing Escherichia coli. This has led to the development of photo catalytic methods for the killing of bacteria and viruses using TiO2 in aqueous media [48, 49].

Table (4): The antibiofilm effect of Tio2 against *E.coli*

Isolates	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
O.D	**0.179**	**0.173**	**0.138**	**0.144**	**0.14**	**0.121**	**0.127**	**0.126**
Percentage of inhibition	**23%**	**38%**	**50%**	**43%**	**50%**	**57%**	**61%**	**61%**
Isolates	**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**15**	**16**
O.D	**0.082**	**0.066**	**0.076**	**0.092**	**0.079**	**0.083**	**0.083**	**0.080**
Percentage of inhibition	**65%**	**76%**	**76%**	**73%**	**72%**	**70%**	**71%**	**69%**

3.4.3 synergiticantibiofilm activity of *R. ribes* methanolic extract and Tio2 NPs **against *E.coli*** In other experiment, study the combination effect of Rhubarb extract and Tio2 was done, the results showed that the antibiofilm effects by*Rheum ribs* and Tio2 together were better than from use each one of them only. All bacteril biofilm was inhibited in percentage from (45%- 78%), the highly effects against isolates (E6- E16) with percentage (60- 78), wheares lower effects against isolates (E1- E5), in percentage of inhibition (45- 59%), see table (5).

Table (5): synergiticantibiofilm activity of *R. ribes*methanolic extract and Tio2 NPs against*E.coli*

Isolates	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
O.D	**0.166**	**0.152**	**0.111**	**0.116**	**0.138**	**0.105**	**0.13**	**0.142**
Percentage of inhibition	**50%**	**45%**	**59%**	**54%**	**51%**	**63%**	**60%**	**60%**
Isolates	**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**15**	**16**
O.D	**0.084**	**0.053**	**0.068**	**0.08**	**0.061**	**0.08**	**0.077**	**0.07**
Percentage of inhibition	**61%**	**75%**	**77%**	**76%**	**78%**	**71%**	**73%**	**70%**

The efficacy of natural products as antimicrobials accompanied by slight or lack of side effect is most likely depends on the compound's structure that interacts with the toxin or pathogen and not with molecules of the host meaning that their effect is specific. This approach has become the rationale for natural drug design studies as a new field of research [50]. When we are made comparision between the three treatment against bacterial biofilm it can be noticed that the effect of *R.ribs* extract higher than from TiO2 NPs treatment for the bacterial isolates (E1- E8), but is nearly similar against bacterial isolates from (E9- E16). It can be concluded also that synergitic effect of *R.ribs* with TiO2 on bacterial isolates gave the best inhibitory effect for biofilm of all isolates at high proportion in compare with other treatments, see table (6).

Table (6): Comparetive between Percentages of inhibition of antibiofilm activity for three treatments against *E.coli*

Isolates number		**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
Percentage of inhibition	***R. ribes***	**44%**	**45%**	**51%**	**32%**	**49%**	**48%**	**72%**	**72%**
	Tio2 NPs	**23%**	**38%**	**50%**	**43%**	**50%**	**57%**	**61%**	**61%**
	***R. ribes*+Tio2** NPs	**50%**	**45%**	**59%**	**54%**	**51%**	**63%**	**60%**	**60%**
Isolates number		**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**15**	**16**
Percentage of inhibition	***R. ribes***	**60%**	**70%**	**76%**	**80%**	**72%**	**72%**	**75%**	**77%**
	Tio2 NPs	**65%**	**76%**	**76%**	**73%**	**72%**	**70%**	**71%**	**69%**
	***R. ribes*+Tio2** NPs	**61%**	**75%**	**77%**	**76%**	**78%**	**71%**	**73%**	**70%**

IV. Conclusion

In conclusion, we report a strong synergistic efficiency of *R.ribs* TiO2 against *E. coli* biofilm.

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