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# Bacteriological study of *Pantoea agglomerans* isolated from Drinking Water

**Sanaa Rahman Oleiwi**

***Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq***

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**ABSTRACT**

*Pantoea agglomerans* is firstly a plant epiphyte frequently established in various ecological niches, such as soil, aquatic habitats or sediments and food. Four isolates of *P. agglomerans* (4 %) were collected from drinking water samples that were obtained from various sites in Baghdad. Investigation of *P. agglomerans* LPS activity as inducer of DNA fragmentation and its role as antibacterial also anti adhesion agent were studied in the present study. Results of current study revealed that LPS of *P. agglomerans* have an effect on DNA fragmentation of human lymphocyte cells, this effect depends on concentration and exposure time, that suggest the possibility of *P. agglomerans* LPS to play a role as apoptosis inducer, that may make it as anticancer agent. The results showed that *LPS* inhibit the bacterial growth of the tested isolates (*Aeromonas hydrophila, Rahnella aquatilis, Stenotrophomonas maltophilia* and *Burkholderia cepacia*) with a range of inhibition zone between (15-25) mm. The recent results reported the anti adhesion ability of *P.agglomerans LPS* on three different abiotic surfaces, this effect have significant values in cases of polystyrene and glass but not significant value in case of galvanized iron.

***Key words*** **:** *Lipopolysaccharide, DNA fragmentation, Apoptosis, Lymphocyte, Adhesion.*

## Introduction

*P. agglomerans* is firstly a plant epiphyte frequently established in various ecological niches, such as soil, aquatic habitats or sediments and food (Pindi *et al.,* 2013; Nicoletti *et al.,* 2015). It is a rod-shaped gram - ve aerobic bacillus, a yellow -pigmented, that be part of the Enterobacteriaceae family, and has formerly been well called as *Erwinia herbicola* or *Enterobacter agglomerans*) Cheng *et al.,* 2013; Walterson and Stavrinides, 2015. In 1989 reclassified into a new genus (Tindall, 2014), this microorganism is considered as an opportunistic bacteria, caused infection in immuno compromised individuals, also is isolated from hospitals) Dutkiewicz *et al*., 2016). Recently, the useful characteristics of *P. agglomerans* have been suggested, such as its a set of antibiotics producing and the action of the immunepotentiator in the stopping and treating of the human and animals infections or preservation of food versus the tested pathologic effect of *P. agglomerans* (Cheng *et al.,* 2013). Lipopolysaccharide (LPS) of bacteria, a great elements of the outer membrane of gram –ve) Leone *et al.,* 2007), also is familiar as a powerful immunostimulator in animals and humans. Fundamental basis of *P. agglomerans* LPS is alike to other gram-negative species, but several unique properties have also been stated (Cimmino *et al*., 2008). Even though the biological action of *P. agglomerans* LPS is chiefly associated to the constriction of lipid A, several researchers also ascribe an essential action to the system of the O-specific polysaccharide OPS as a determinative of its role) Leone *et al.,* 2007). The goal of this work was investigation of *P. agglomerans* LPS activity as inducer of DNA fragmentation also Its role as antibacterial and anti adhesion agents.

**Corresponding author's email:** sanaeleiwi@yahoo.com

## Materials and Methods

**Isolation and identification**: One handrud drinking water samples obtained from various sites in Baghdad, then applied to microbiological examinations depending on Standing Committee of Analysts, 2017. Isolation was carried out by Membrane Filtration method by using of Reasoner's 2A agar (R2A agar), morphology of isolates were studied by Gram's stain, applied to several biochemical tests and additional verification was achieved by ID 32 mini API system (Nicoletti *et al.,* 2015; Standing Committee of Analysts, 2017).

### Extraction and partial purification of Lipopolysaccharide

Hot phenol-water method was used to extraction Lipopolysaccharide as reported formerly with few modifications (Westphal, Jann, 1965; Bulyhina *et al*., 2020). In short, suspensions of bacteria ($10^8$ CFU / *mL*) were centrifuged at 10,000×*g* for five minutes. Phosphate Buffer Solution (pH=7.2) (0.15 *M*) consist of 0.15 *mM* CaCl2 and 0.5 *mM* MgCl2 was used in rinsing of the pellets twice, then ten milliliters *(ml)* PBS were used in resuspension of pellets. The following stage is adding of an identical amount of hot phenol 90% at (65-70°C) to the mixture then strong shaking at 65-70°C for 15 minutes was performed , ice was used to cooling suspension, also centrifugation at 8500×*g* for 15 minutes was carried out, supernatant was put in fifteen milliliters conical centrifuge tubes and phenol phases were re-extracted by 300 µL distilled water DW. At final concentration (0.5 M) of sodium acetate and ten volumes of ethanol (95%) was added to the extract then was kept at -20 °C overnight to precipitate lipopolysaccharide. Centrifugation at 2000×*g* 4 °C for ten minutes and the pellets were resuspended in one milliliter (*ml)* DW , great dialysis against double DW at 4°C was performed until phenol residual in the liquid phases was completely removed .

### Human peripheral blood lymphocyte isolation

Human peripheral blood lymphocytes were isolated as reported by previous studies (Oleiwi, 2015), four concentrations of LPS (0 (control), 15, 20, 25, 30 µg/ml) were tested at three incubation periods (exposure times) 24, 48 and 72 hr.

**DNA fragmentation test :** DNA fragments of isolated lymphocyte cells were obtained depending on the procedure reported previously (Fernandez-Botran, Vetvicka, 2000; Oleiwi, 2015) as explained in the coming stages:

1. At the end of incubation periods (24, 48 and 72 hr.), the tubes were centrifuged one thousand rpm for ten minutes, these tubes were defined as letter A.
2. The supernatants were discharged to another tubes, these tubes were defined as letter B.
3. One ml of triton x-100 tris EDTA (TTE) solution was added to pellet in the A tubes and with strong shaking.
4. The A tubes were centrifuged at 14000 rpm for ten minutes in 4°C.
5. The supernatant of A tubes were discharged into another tubes and were defined as letter C.
6. One milliliter of TritonX-100 Tris EDTA (TTE) solution was added to the pellet in A tubes .
7. One milliliter of Trichloro Acetic Acid TCA 25% was added to the (A, B, C) tubes with strong shaking, the tubes were incubated overnight at 4 °C
8. At the incubation period end, the tubes were centrifuged at 14000 rpm for ten minutes to precipitate of DNA, the supernatant was discarded.
9. A volume of 320 µL 5% of Trichloro acetic acid TCA was added to the tubes and were placed in water bath at 90°C for fifteen minutes
11. At water bath period end, 640 µl of Diphenylamine reagent (DPA) were added, shaked the tubes softly and incubated them over night at 37°C, the color must alter to blue.
12. Optical density OD was measured at 600 nm.
13. The percentage of DNA fragmentation was determined as formula:

$$F\% = \frac{B + C}{A + B + C} \times 100$$

F%: Percentage of DNA fragmentation

A: O.D of A tube -B: O.D of B tube - C: O.D of C tube

**Antibacterial activity of LPS:** Antibacterial activity of LPS was tested against several bacterial species were isolated from drinking water (*Aeromonas hydrophila, Rahnella aquatilis, stenotrophomonas maltophilia and burkholderia cepacia*) that achieved by agar well diffusion method (Prescott *et al.,* 2002).

### Study the effect of *P. agglomerans* LPS as anti adhesion agent

*Investigation the anti adhesion property of P.*

*agglomerans* LPS on abiotic surfaces was achieved according to (Adetunji and Isola, 2011; Oleiwi *et al.,* 2020), three types of surfaces were used (polystyrene, glass and galvanized iron) after preparation them as described by (Mafu *et al.,* 2011), control was prepared without adding of LPS. Anti adhesion ability of LPS was tested by using drinking water *Aeromonas hydrophila* isolate , the concentration of crystal violet was detected by reading of optical density of de-staining solution at 492 nm (OD 492 value - C value).

**Statistical Analysis:** Detection of the effect of difference factors in parameters was achieved by the statistical analysis system- SAS 2012) program, least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means.

## Results and Discussion

**Isolation and Identification :** Four isolates of P. agglomerans (4 %) were collected from drinking water samples. Current results came in agreement with former results (Pindi *et al.,* 2013; Al-Bayatti, 2013; Nicoletti *et al.,* 2015).

*P. agglomerans* is usually isolated from surfaces of plant, fruit seeds, water and human or animal feces ( Stockwell *et al*., 2002 ; Nicoletti *et al.,* 2015) in addition *P. agglomerans* can grow symbiotically with different edible plants ( Kobayashi *et al*., 2018).

**DNA fragmentation test:** Results of current study revealed that LPS of *Pantoea* have an effect on DNA fragmentation of human lymphocyte cells , this effect depend on concentration and exposure time. DNA fragmentation was determenated by reading of optical density at 600 nm under treatment with different concentrations (15, 20, 25, 30 µg/ml) of LPS at three exposure time (24, 48, 72 hr.), results were summarized in (Table 1).

Depending on LPS concentrations, the results suggested there was significant increasing of DNA fragmentation percentage under all LPS concentrations compared to control, but depending on exposure time (24, 48, 72 hr), DNA fragmentation percentage showed significant increasing at (25, 30 µg/ml) only. The results of recent study suggest the possibility that *P. agglomerans* LPS play a role as apoptosis inducer, that may make it as anticancer agent. Previous study conducted by (Hiroi *et al*., 1998) found that LPS induce apoptosis of human peripheral blood polymorphonuclear leukocytes, this researcher reported that LPS lightly increase the production of DNA fragments, also (Hirai *et al*., 2003) studied effect of gram negative bacteria LPS on apoptosis. In addition, *P. agglomerans* gave a positive result by induction the formation of materials useful for the therapy of cancer and other diseases (Dutkiewicz *et al*., 2016; Morishima and Inagawa, 2016). At the chemical structure level, the biological action of *P. agglomerans* LPS is chiefly associated with structure of lipid A.

This bacteria have essential rol in biological controlling and prevention of bacterial diseases on fruits (Nunes *et al*., 2001), there are previous studies

**Table 2.** Action of *P. agglomerans* LPS as antibacterial agent

| Inhibition zone diameter (mm) | Bacteria |
|---|---|
| 15 ± 0.62 c | *Aeromonas hydrophila* |
| 17 ± 0.71 bc | *Burkholderia cepacia* |
| 20 ± 0.76 b | *Stenotrophomonas maltophilia* |
| 25 ± 1.04 a | *Rahnella aquatilis* |
| 4.091 * | LSD value |

Means having with the different letters in column differed significantly. * (P ≤ 0.05).

**Table 1.** Effect of *P. agglomerans* LPS on DNA fragmentation (three concentrations and three exposure times)

| Concentration of LPS (µg/ml) | Mean ± SE | | | LSD value |
|---|---|---|---|---|
| | 24 hr. | 48 hr. | 72 hr. | |
| Control | 58.00 ± 2.19C [a] | 59.00 ± 1.98C [a] | 57.00 ± 1.75B a | 2.71 NS |
| 15 | 69.285± 2.77B [a] | 71.21± 2.63B [a] | 71.99± 3.09A a | 4.59 NS |
| 20 | 73.012± 3.05AB [a] | 70.92± 2.08B [a] | 70.52± 2.66A a | 4.08 NS |
| 25 | 72.011± 2.82AB [ab] | 75.233± 3.16AB [a] | 70.021± 2.62A b | 4.79 * |
| 30 | 75.532± 2.94A [a] | 77.378± 2.86A [a] | 69.381± 2.61A b | 4.93 * |
| LSD value | 5.28 * | 6.04 * | 4.62 * | —- |

Means having with the different big letters in same column and small letters in same row differed significantly. * (P ≤ 0.05).

of beneficial effect of *P. agglomerans* LPS such as oral administration is related to some essential health advantages like stopping of diabetes (Iguchi *et al.,* 1992), atopic dermatitis (Wakame *et al.,* 2015) and development of influenza virus vaccine efficiency (Fukasaka *et al*., 2015). Kobayashi *et al*., 2018 investigated the ability of P. *agglomerans* LPS in inflammatory - oxidative responses to stopping atherosclerosis and associated metabolic diseases.

**Antibacterial activity of LPS :** The results showed that *LPS* inhibit the bacterial growth of the tested isolates with a range of inhibition zone between (15-25) mm. The results of recent study found that bacteria *Rahnella aquatilis* was the most affected bacteria by LPS of *P. agglomerans* (25 mm), followed by *Stenotrophomonas maltophilia* (20 mm ), *Burkholderia cepacia* (17 mm) and *Aeromonas hydrophila* (15 mm)(Table 2).

Giddens and Bean, (2007) investigated antibacterial activity of one of *P. agglomerans* products is D-alanylgriseoluteic acid (AGA) considered as a powerful antimicrobial phenazine compound that have antibacterial property against range of bacteria.

**Table 3.** Effect LPS as anti adhesion agent on polystyrene (OD at 492)

| Mean ± SE | Group |
|---|---|
| 0.478 ± 0.03 b | Polystyrene |
| 0.591 ± 0.02 a | Control |
| 0.091 * | T-test |

* (P≤0.05).

**Table 4.** Effect of LPS as anti adhesion agent on glass (OD at 492)

| Mean ± SE | Group |
|---|---|
| 0.221 ± 0.02 b | Glass |
| 0.345 ± 0.04 a | Control |
| 0.105 * | T-test |

* (P≤0.05).

**Table 5.** Effect of LPS as anti adhesion agent on galvanized iron (OD at 492)

| Mean ± SE | Group |
|---|---|
| 0.429 ± 0.05 | Galvanized iron |
| 0.570 ± 0.03 | Control |
| 0.163 NS | T-test |

NS: Non-Significant.

**Study the effect of P.agglomerans LPS as anti adhesion agent**

This test is useful to investigation of adhesion on abiotic surfaces, the recent results suggested the anti adhesion effect of *P.agglomerans LPS* on three different surface, but in different degrees. This effect have significant value in case of polystyrene and glass but in case of galvanized iron the effect was not significant, the explanation of these results is the roughness of surfaces, surface with more roughness is considered to be difficult to remove it's biofilm (Tables 3, 4, 5). Other researchers reported that *P.agglomerans LPS* have ability to reduced the adhesion (Zdorovenko *et al*., 2017; Bulyhina *et al*., 2020).

## Conclusion

Current study investigated the role of drinking water *P.agglomerans LPS* isolates as inducer of DNA fragmentation, that may make it as anticancer agent. Also possible act as antibacterial and anti adhesion factor on abiotic surfaces that may have potential applications in clinical and environmental fields.

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