

Full Length Research Paper

Susceptibility of imipenem-resistant *Pseudomonas aeruginosa* to flavonoid glycosides of date palm (*Phoenix dactylifera* L.) tamar growing in Al Madinah, Saudi Arabia

Samy Selim^{1,2*}, Sahar El Alfy², Meshref Al-Ruwaili¹, Amin Abdo³ and Soad Al Jaouni⁴

¹Microbiology Laboratory, Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Al-Jouf University, P.O. 2014, Sakaka, Saudi Arabia.

²Microbiology Section, Botany Department, Faculty of Science, Suez Canal University, Ismailia, 41522, Egypt.

³Gomohory Hospital, Ministry of Health, Sana'a, Yemen.

⁴Department of Hematology, Faculty of Medicine, King Abdulaziz University, P.O. Box 80215, Jeddah 21589, Saudi Arabia.

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The fruits of the date palm (*Phoenix dactylifera* L.) are consumed throughout the world and are an important part of the diet in the Middle East. Dates at tamar stage contain a wide array of flavones, but little is known about the antimicrobial of flavonoid glycosides compounds in dates. In this study, we evaluated the antimicrobial of flavonoid glycosides extracted from the date fruits grown in Al Madinah, Saudi Arabia at full ripe stage against imipenem-resistant *P. aeruginosa* (IRP). A chloroform fraction prepared from the date showed potent inhibitory activity against IRP. The active compounds were elucidated to be quercetin, luteolin, and apigenin based on their spectral analysis. Flavonoid glycosides showed significant antibacterial activities against IRP. These compounds represent novel leads, and future studies may allow the development of a pharmacologically acceptable antimicrobial agent or class of agents.

Key words: *Phoenix dactylifera*, flavonoid glycosides, imipenem-resistant *P. aeruginosa*.

INTRODUCTION

Flavonoids are ubiquitous in photosynthesising cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity. Moreover,

several groups have demonstrated synergy between active flavonoids as well as between flavonoids and existing chemotherapeutics. Reports of activity in the field of antibacterial flavonoid research are widely conflicting, probably owing to inter- and intra-assay variation in susceptibility testing. However, several high-quality investigations have examined the relationship between flavonoid structure and antibacterial activity and these are in close agreement. In addition, numerous research groups have sought to elucidate the antibacterial mechanisms of action of selected flavonoids (Cushnie and Lamb, 2005).

Pseudomonas aeruginosa is an important nosocomial pathogen with its ability to propagate on medical devices, hospital environment and even in disinfectants. It causes

*Corresponding author. E-mail: sadomm2003@yahoo.com.

high morbidity and mortality in the services of oncology, hematology, surgery, burn and intensive care units (Fluit et al., 2000). Infections due to this virulent organism are difficult to both control and treat because of intrinsic resistance to many antimicrobial agents (Quinn, 1994; Carmeli et al. 1999). Imipenem, a broad-spectrum β -lactam antibiotic, is one of the most effective drugs against *P. aeruginosa*. The incidence of imipenem-resistance is increasing among gram negative bacilli, particularly *P. aeruginosa*, often associated with resistance to other antipseudomonal drugs (Fridkin et al., 1999). The increasing resistance of *P. aeruginosa* is a growing threat to the clinical management of such infections (Deshpande et al. 2004; Zavascki et al. 2005).

Date palms (*P. dactylifera* L., Palmae) have been cultivated in the Middle East over at least 6000 years ago (Al Farsi and Lee, 2008). For the natives in this region, dates are considered a staple carbohydrate food (Al-Shahib and Marshall, 2003). Date fruits are also used in the production of local beverages and spirits. The fruits which are the most commonly used part are an important source of nutrition, especially in the arid regions where due to the extreme conditions, very few plants can grow. Date fruits are a good source of low cost food and are an integral part of Arabian diet. Besides nutritional value, date fruits are rich in phenolic compounds possessing antioxidant activity. The pollen grains of date palm have been used in Egyptian local practices to improve fertility in women, and in some locations in Arabia, date pits are roasted and used in lieu of coffee as a hot beverage. Relatively few pharmacological studies have been conducted on dates. For example, it has been shown that, depending on the type of extract used, date fruit and pit extracts significantly increase or decrease gastrointestinal transit (GIT) in mice (Al-Qarawi et al., 2003), and that date fruit extract has strong antioxidant and antimutagenic properties (Vayalil, 2002). For Muslims all over the world, dates are of religious importance and are mentioned in many places in the Quran. In view of the wide consumption of dates in our region, the fact that dates are anecdotally reputed to be useful against peptic ulcers, and the fact that Muslims customarily consume more of the dates during the fasting month of Ramadan (Al Farsi and Lee, 2008; Al-Shahib and Marshall, 2003), possibly to protect the gastric mucosa from the damaging effect of gastric acid, and because of the scarcity of information on the pharmacological properties of date fruits and pits, we considered undertaking this study to assess the influence of date extracts on the incidence and severity of ethanol-induced gastric ulceration. In fact, Muslims believe that "He who eats seven dates every morning will not be affected by poison or magic on the day he eats them".

From the current literature, there is not much data concerning the antimicrobial effect of date palm. This present investigation was carried out to evaluate the antimicrobial of flavones extracted from the date fruits grown in Al Madinah, Saudi Arabia at full ripe stage in the

ripening against IRP.

MATERIALS AND METHODS

Date material

The dates used for this study were procured from a supermarket in Al Madinah, Saudi Arabia. Ripe fruits of uniform size, free of physical damage and injury from insects and fungal infection, were selected and used for all experiments. Upon arrival at the laboratory, the samples (100 to 150 g portions) were packed in polyethylene bags, sealed, and stored at -20°C until used.

Chemicals

All chemicals and solvents were obtained from Sigma Aldrich Co. Ltd (St. Louis, France).

Extraction

Air-dried of date was extracted with MeOH for three days at room temperature to give an extract (129 g) which was suspended in H₂O and partitioned with n-hexane, CHCl₃ and EtOAc, successively. The active CHCl₃ fraction (23 g) was subjected to silica gel (Merck, Kieselgel 60; 0.063–0.2 mm particle size; 11×100 cm) column chromatography. The fraction was eluted with n-hexane/EtOAc: 20:80 (10×250 ml), followed by MeOH. Fractions of similar composition determined by thin layer chromatography (TLC) analysis were pooled.

Determination of total flavonoid

Flavonoid content in the methanolic extract of plant was determined by aluminum chloride calorimetric method (Chang et al., 2002). Briefly, 0.50 ml of methanolic extract of sample was diluted with 1.50 ml of distilled water and 0.50 ml of 10% (w/v) aluminum chloride^{was} added along with 0.10 ml of 1 M potassium acetate and 2.80 ml of distilled water. This mixture was incubated at room temperature for 30 min. The absorbance of resulting reaction mixture was measured at 415 nm UV spectrophotometer (Hitachi U-2001). Quantification of flavonoids was done on the basis of standard curve of quercetin prepared in 80% methanol and results were expressed in milligram quercetin equivalent (QE) per 100 g fw of fruits.

Extraction of flavonoid glycosides

The pitted fruit (5 g) was homogenized in a polytron (2 min on ice) with 10 ml of extraction solution (4 mM NaF in methanol to inactivate polyphenoloxidases and prevent phenolic degradation due to browning). Homogenates were kept in ice until centrifuged (15 min, 2 to 5°C at 16000 g). The supernatant was recovered and filtered through 0.45 mm filters (Osmonics/MSI Cameo Nylon Filters, Fisher Scientific, Los Angeles, CA) and directly analyzed by high performance liquid chromatography (HPLC) after a period not exceeding 24 h (Tomas-Barberan et al., 2001). Extracts were separated using HPLC on a 250 mm X 2.0 mm i.d., 5 μ m phenomenex prodigy, ODS column monitoring at 370 nm. The structures of the compounds were determined using spectroscopic techniques including UV, mass spectrometry, ¹H and ¹³C nuclear magnetic resonance (NMR), DEPT, heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple-quantum correlation (HMQC) spectrometry.

Antimicrobial tests

Bacterial strains

The agar diffusion assay was performed according to modified Kirby-Bauer disc diffusion method (Robert et al., 2003). One loopful of each test organism was suspended in 3 ml 0.9% NaCl solution separately. All *P. aeruginosa* were isolated prospectively in the microbiology laboratory of Microbiology Section, Faculty of Science, Suez Canal University, Egypt. Identification and antimicrobial susceptibility of the isolated strains were done by VITEK automated system (BioMerieux, Marcy l'Etoile, France). The isolates (12) with minimum inhibitory concentration (MIC) value ≥ 8 $\mu\text{g/ml}$ were recorded as IRP. *P. aeruginosa* ATCC 15442 strain was used in all the experiments as a control.

Determination of anti- imipenem-resistant *P. aeruginosa* activity

The MIC and the minimum bactericidal concentrations (MBC) were determined using the agar dilution method. All strains were grown in cetrимide broth for 24 h at 37°C. After incubation, they were diluted with the same medium to give a concentration of approximately 10^8 colony forming units (cfu/ml). The isolated compounds were dissolved in dimethyl sulfoxide (DMSO) and two-fold serial dilutions were made before adding the same to Cetrимide agar plates. Bacterial cell suspensions were inoculated onto the plates using a bacteria planter (5 μl). The final inoculum concentration of cfu inoculated onto the agar plates was 5×10^5 for all strains. An agar plate containing only DMSO served as the control. All assays were carried out in triplicate.

Time-kill studies

Time-kill studies were performed for *P. aeruginosa* in McCartney bottles using a method based on the European standard quantitative suspension test (European Standard EN 1040; European Committee for Standardization, 1997). An initial inoculum of 5×10^8 cfu ml^{-1} was prepared as described previously for isolate for use in time-kill studies by diluting an actively growing culture in cetrимide broth with the inoculum used for each isolate verified by a total viable count. Samples (1 ml) of the initial inoculum were then added to 9 ml sterile water containing either date extract and Tween 80 (test) or Tween 80 only (control). The final concentrations of date extract and Tween 80 were 5%. The McCartney bottles for all isolates were shaken (100 rpm) at 37°C and samples (1 ml) were taken in triplicate from 0 to 120 min and serial tenfold dilution were made and plated on cetrимide agar (Oxoid, UK). The total viable count was determined after overnight incubation at 37°C.

Biofilm susceptibility

Biofilm susceptibility tests were performed for 12 IRP isolates. An initial inoculum of 5×10^8 cfu. ml^{-1} was prepared for each isolate for use in biofilm susceptibility studies by diluting an actively growing culture in cetrимide broth as described above. Samples of the initial inoculum (0.05 ml) for each isolate were inoculated onto the surface of six glass discs, which were dried at 37°C in an incubator for 1 h. After the discs had been washed gently with sterile phosphate buffered saline (PBS) to remove any non-adherent bacteria, they were placed in two sterile Petri dishes (three discs per dish) containing 20 ml cetrимide broth and incubated at 37°C for 24 h. After gently washing with sterile PBS to remove any non-adherent bacteria, the discs were transferred to separate conical flasks containing 10 ml of test (5% date extract with 0.5% Tween 80 in

sterile distilled water) or control (0.5% Tween 80 in sterile distilled water) suspension. The flasks were shaken (100 rpm) at 37°C in an orbital incubator for 1 h. Following incubation, the discs were washed and placed in 5 ml PBS in sterile McCartney bottles and bacteria retained on the surface were dislodged by rapid vortex mixing (30 s). Serial tenfold dilution was performed and total viable counts determined as described above for the time-kill studies. All experiments were performed in triplicate with three discs tested with tamer extract suspension and three discs tested with control suspension for each isolate.

Scanning electron microscopy

Stationary phase cultures of *P. aeruginosa* were prepared by inoculating 10 ml volumes of cetrимide broth and incubating overnight. Organisms were harvested by centrifugation for 15 min at 4000 *g* and the pellets resuspended in PBS-T. Suspensions of *P. aeruginosa* were treated with 4% (v/v) date extract for 30 and 60 min. Untreated controls in PBS were left on the bench for 60 min. All treatments were performed at room temperature. After treatment, cell suspensions were centrifuged for 15 min at 4000 *g*, and pellets were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Fixed microbial pellets were processed in graded alcohols, propylene oxide and araldite, and cured for 48 h at 60°C. Samples were then coated with gold and observed using JOEL Scanning Electron Microscope at 15 kV.

Statistical analysis

The variations between experiments were estimated by standard deviations and the statistical significance of changes was estimated using the student's *t*-test. Only the probability $P \leq 5\%$ was regarded as indicative of statistical significance.

RESULTS AND DISCUSSION

Flavonoids present in plants possess diverse health benefits, which includes antioxidant and radical scavenging activities, reduction of certain chronic diseases, prevention of some cardiovascular disorders and certain kinds of cancerous processes (Tapas et al., 2008). Hong et al. (2006) assessed the flavonoid content in the Deglet Noor variety during the Khalal stage of maturity and identified 13 flavonoid glycosides of luteolin, quercetin and apigenin. Quercetin and luteolin formed primarily O-glycosidic linkages whereas apigenin was present as the C-glycoside. As of today, dates also have the unique distinction of being the only food to contain flavonoid sulfates (Hong et al., 2006). Chaira et al. (2009) recently reported that among the famous Tunisian dates, the highest content of flavonoids was present in the Korkobbi variety (54.46 quercetin equivalents/100 g fresh weight). In our study, Al Madinah date showed very high total flavonoid contents; 159 mg QE/100 gram fw. The HPLC chromatogram demonstrates the presence of peaks absorbed at 370 nm, which are possible conjugates arising from quercetin, luteolin and apigenin (Figure 1) based upon calculated masses. Previously published work (Tomas-Lorente and Ferrerres, 1998; Hong et al., 2006) demonstrates that luteolin and chrysoeriol (luteolin

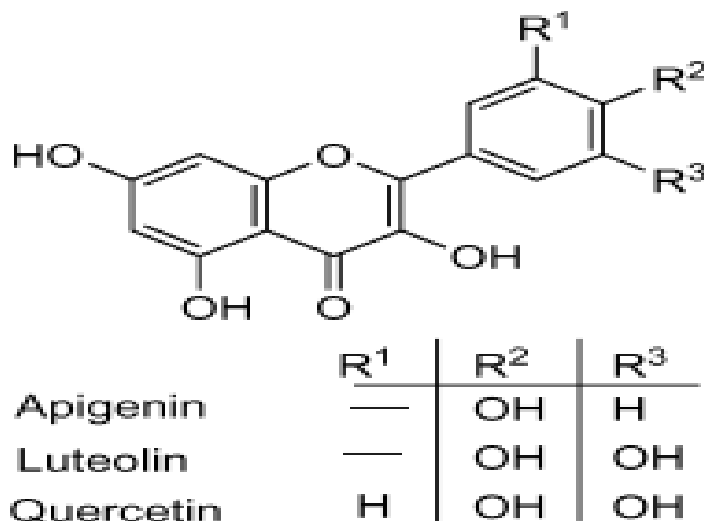


Figure 1. Representative structure of apigenin, luteolin and quercetin.

Table 1. Antibacterial activity of methanol extract and solvent soluble fractions from the date palm against *P. aeruginosa* ATCC 15442.

Sample	MIC (mg/ml)	MBC (mg/ml)
MeOH extract	0.05	2
n-Hexane fraction	2	4
CHCl ₃ fraction	0.05	2
EtOAc fraction	4	16

3'-methyl ether) glycosides present in dates are linked at position 7 of the flavone molecule, the most common linkage for these flavone glycosides. The UV spectrum of the compounds present in dates in this study agrees with this kind of substitution; therefore, we tentatively suggest that the luteolin and chrysoeriol (methyl luteolin) glycosides characterized in the present work are 7-glycosides. In the case of the flavonol glycosides of both quercetin and isorhamnetin (quercetin 3'-methyl ether), the UV spectrum indicates that the sugar residues are linked to the hydroxyl at the 3-position of the flavonol molecule, as the UV maximum for band I is always below 355 nm, which is indicative of substitution of the hydroxyl at the 3-position. Tomas-Lorente and Ferrerres (1998) also described nonsulfated and sulfated flavonoid glycoside conjugates of luteolin, quercetin, chrysoeriol and isorhamnetin.

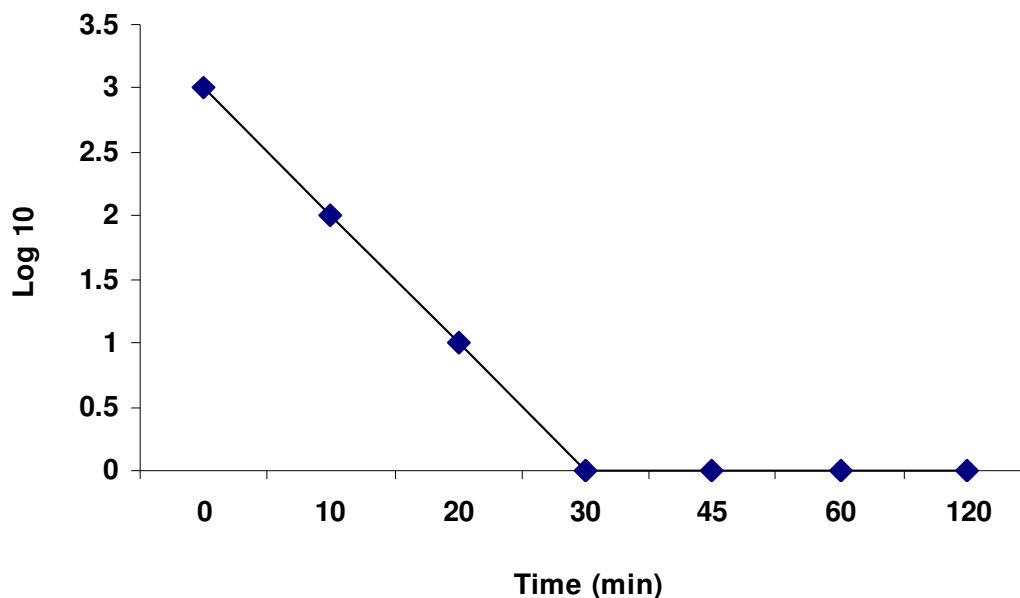
To obtain the active component related to the antibacterial activity, date was extracted with MeOH and fractionated successively with n-hexane, CHCl₃ and EtOAc. Antibacterial activities of these fractions against *P. aeruginosa* ATCC 15442 were tested (Table 1). The MeOH and CHCl₃ fraction showed significant MIC and MBC values in comparison with the other fractions. Therefore, the CHCl₃ fraction was employed for further purification. Out of the first 10 fractions obtained by silica

gel column chromatography of the CHCl₃ fraction, flavonoid glycosides showed potent inhibitory activity (MIC/MBC: 0.5/2 mg/ml) against *P. aeruginosa* ATCC 15442. The MIC/MBC values of the isolated compounds against IRP are summarized in Table 2. The MICs and MBCs for the 12 isolates, as determined by the broth micro dilution method, ranged from 0.5 to 1 mg/ml. Based on the MIC and MBC results, none of the isolates could be considered resistant. Each of the clinically isolated strains was significantly resistant to imipenem and showed MIC/MBC values similar to or higher than 128/256 mg/ml. Flavonoid glycosides compounds showed very potent inhibitory activity against clinical isolates of IRP. The effect on the cell viabilities of IRP demonstrated that exposure of date extract at 5% concentration had a potential antibacterial effect on the viabilities of strains. The exposure times of extract for the complete inhibition of cell viability of IRP were found to be 5% at 30 min, respectively (Figure 2).

Biofilms formed by 12 IRP isolates were completely eradicated following exposure to 5% extract for 1 h (Table 3). The possible antimicrobial mechanism of action is related to disruption of hydrophobic structures within phospholipid bilayers of the bacterial cell. Further studies using an animal model of skin colonization would be useful for *in vivo* confirmation of the differences in

Table 2. Antibacterial activities of flavonoid glycosides isolated from date palm against clinical isolates of IRP strains.

Strain	Flavonoid glycoside		Imipenem	
	MIC(mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
<i>P. aeruginosa</i> ATCC 15442	0.5	2	64	128
<i>P. aeruginosa</i> 1	0.5	1	128	256
<i>P. aeruginosa</i> 2	0.5	1	128	256
<i>P. aeruginosa</i> 3	0.5	1	128	256
<i>P. aeruginosa</i> 4	0.5	1	128	256
<i>P. aeruginosa</i> 5	0.5	1	128	256
<i>P. aeruginosa</i> 6	0.5	1	128	256
<i>P. aeruginosa</i> 7	0.5	1	128	256
<i>P. aeruginosa</i> 8	0.5	1	128	256
<i>P. aeruginosa</i> 9	0.5	1	128	256
<i>P. aeruginosa</i> 10	0.5	1	128	256
<i>P. aeruginosa</i> 11	0.5	1	128	256
<i>P. aeruginosa</i> 12	0.5	1	128	256

**Figure 2.** Effect of date extract (5%) on viability of *P. aeruginosa*. Values are the average of three individual replicates (means \pm S.D). Differences between samples were determined by the Student's t-test and were considered to be significant when $p \leq 0.05$.

biofilm susceptibility that are apparent *in vitro* between IRP isolates. We further studied the possible mode of action of flavonoid glycosides against *P. aeruginosa* by observing the bacteria cell morphology through scanning electron microscopy (SEM) at 60 min after applying flavonoid glycosides. From the SEM results (Figure 3), it was clearly indicated that the cell morphology of *P. aeruginosa* when treated with flavonoid glycosides, started to deform at 30 min onward and at 60 min, most

cells were completely deformed whose results was correlated to their strong antibacterial activity. Therefore, it can be suggested that flavonoid glycosides compounds may interact with or damage the cell wall of *P. aeruginosa* as seen by the formation of pores on the cell wall of *P. aeruginosa* (Figure 3).

These results indicate the possibility of exploitation of flavonoid glycosides originated from date palm (*P. dactylifera* L.) growing in Al Madinah, Saudi Arabia as

Table 3. Effect of treatment with 5% date extract on biofilm grown IRP isolates.

Isolate	Extract	Control
<i>P. aeruginosa</i> 1	0	$2.63 \times 10^4 \pm 1.58 \times 10^4$
<i>P. aeruginosa</i> 2	0	$1.07 \times 10^3 \pm 3.10 \times 10^3$
<i>P. aeruginosa</i> 3	0	$1.24 \times 10^5 \pm 1.04 \times 10^5$
<i>P. aeruginosa</i> 4	0	$2.36 \times 10^4 \pm 2.12 \times 10^4$
<i>P. aeruginosa</i> 5	0	$2.11 \times 10^3 \pm 1.37 \times 10^3$
<i>P. aeruginosa</i> 6	0	$3.60 \times 10^3 \pm 6.25 \times 10^3$
<i>P. aeruginosa</i> 7	0	$1.12 \times 10^5 \pm 2.45 \times 10^5$
<i>P. aeruginosa</i> 8	0	$4.12 \times 10^4 \pm 2.33 \times 10^4$
<i>P. aeruginosa</i> 9	0	$5.36 \times 10^6 \pm 6.12 \times 10^6$
<i>P. aeruginosa</i> 10	0	$7.25 \times 10^5 \pm 3.91 \times 10^5$
<i>P. aeruginosa</i> 11	0	$5.33 \times 10^5 \pm 7.50 \times 10^5$
<i>P. aeruginosa</i> 12	0	$3.22 \times 10^5 \pm 2.58 \times 10^5$

Values are numbers of adherent bacteria (cfu cm⁻²) following treatment [mean (±SD)].

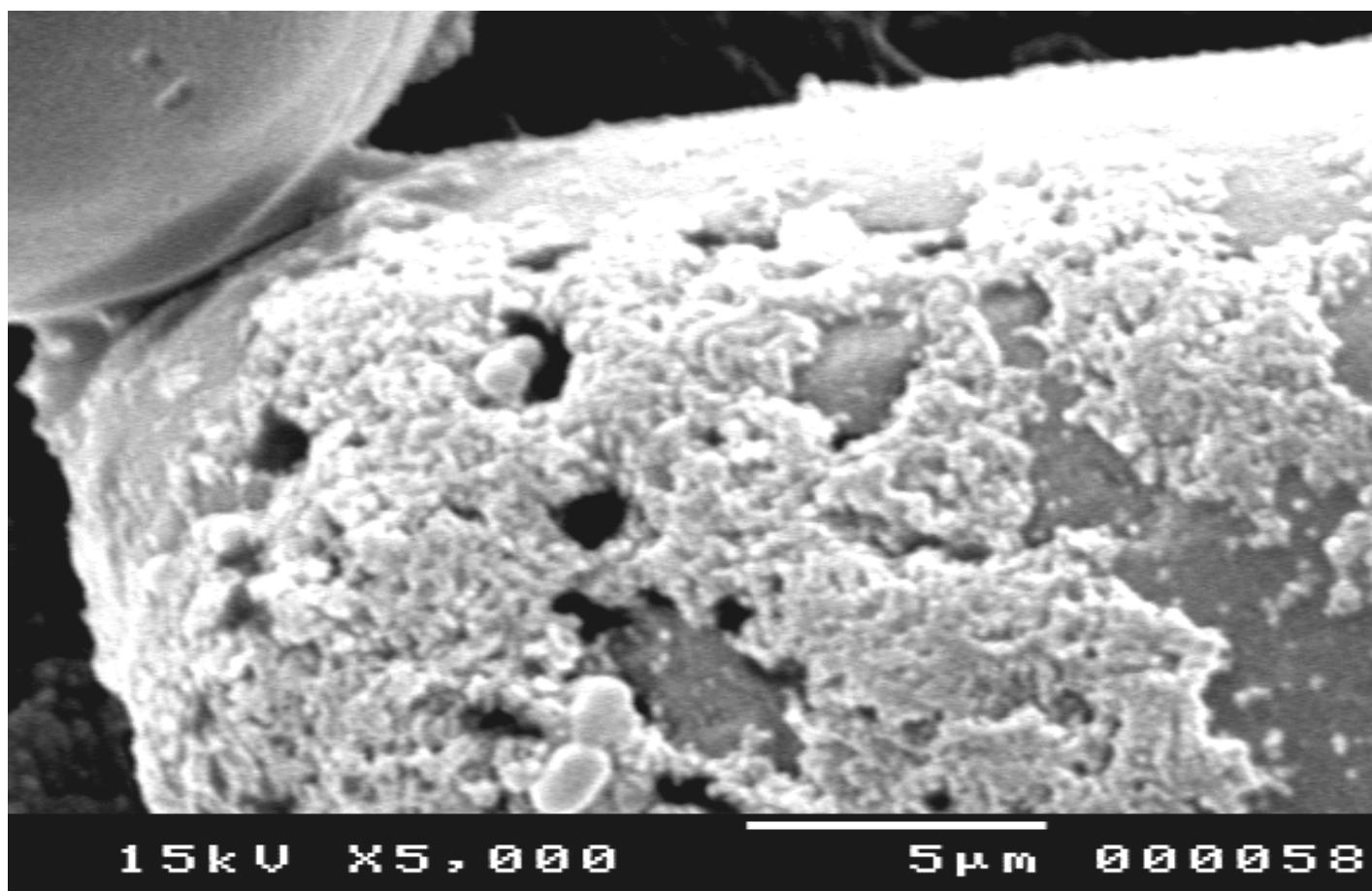


Figure 3. Scanning electron microscopy of cell morphology of *P. aeruginosa* treated with flavonoid glycosides at 60 min showing damage of the cell wall.

effective inhibitors of IRP. This is the first report on the anti-IRP activities of flavonoid glycosides in the literature.

The isolated compounds are expected to be useful for the study of anti-IRP agents in the future.

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