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Study The Effect of Nano-Flavonoid and Flavonoid Compounds Extraction from Bacopa Monnieri L. On The Serratia Marcescens

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ABSTRACT

This study aimed to explore whether extract flavonoid and nano flavonoids can function as an anti-biofilm agent produced by Serratia marcescens . Firstly, flavonoid extraction from the Bacopa monnieri L. and identification by using High Performance Liquid Chromatographic (HPLC) then used casein to manufacture flavonoid-casein nanoparticles by ultrasound method . Several techniques have been used to determine the chemical and physical properties of nanoparticles such as Scanning Electron Microscope (SEM) and Zeta–Potential . S. marcescens isolates were collected and identified by culturing on Blood agar and MacConkey agar medium and confirmed by VITEK 2 system . Flavonoid and nano-flavonoids showed remarkable biofilm inhibitory effect on isolates of multidrug resistant S. marcescens . Strong biofilm producer strains show weak biofilm production After incubation for 24 hours at 37Co with MIC concentration of flavonoid and nano flavonoids .

Keywords: *Serratia marcescens , Bacopa monnieri L. , Flavonoid , Nanoparticles , biofilm .*

1. INTRODUCTION

During thousands of years, the majority of people in the world have been depending on traditional medicine [1]. Herbal substances include a storage of bioactive substances through medicinal characteristics, which has been documented and used by numerous collections of people for the cure of various diseases over time [2]. Bacopa monnieri L. is one of the medicinal plants in medicine. Bacopa monnieri has been shown to possess anticancer, antidiabetic, anti-inflammatory, antimicrobial and antioxidant properties [3]. It is the most important and useful plant in the traditional medicine used as a memory enhancer . Bacopa has been used in various ailments including jaundice, diabetes, cough, leprosy, swelling, diabetes. It is also beneficial for treating skin and blood disorders [4]. pharmacological properties of B. monnieri have been studied extensively and the activities have been attributed mainly to the presence of characteristic Active compounds [5]. Its phytochemical substances are Phenols, alkaloids, flavonoids, glycosides, saponins, and the important constituents such as bacosides, bacopasides, and bacopasaponins, which all of these contribute to its remedial properties [6].

Flavonoids are series of compounds with diphenylpropanes (C6–C3–C6) as the basic skeleton and 2 aromatic rings connected to each other through the central 3-carbon bridge [7]. Flavonoids are the most diverse phenolic compounds which are closely related to flower color formation of plants and play an important role in plant–environment interaction.Flavonoids play a variety of biological activities in plants, animals and bacteria [8]. Flavonoids have inhibitory activity against bacteria [9], against fungal [10] and flavonoids posse Antioxidant activity [11].

Nanotechnology has marked a significant revolution in various areas of science [12]. Nanotechnology has been promising in different field of science and among it is the use of nanoparticles as antibacterial agents [13]. The nanoparticles are of different shape, size and structure. It be spherical, cylindrical, tubular, conical, spiral, flat, etc. or irregular and differ from 1 nm to 100 nm in size [14]. Nanoparticles are made up of carbon, metal, metal oxides or organic matter. The nanoparticles exhibit a unique physical, chemical and biological properties at nanoscale compared to their respective particles at higher scales [15]. Nanomaterials have wide-ranging implications in a variety of areas [16]. It is expected that some of the more immediate applications of nanoparticles will be in medical diagnosis and therapeutics. Exciting examples include detection of genetic disorders using gold nanoparticles and cell transfection for gene therapy and drug delivery [17].

Serratia marcescens is Gram-negative , rod-shaped with rounded ends bacteria. Serratia is facultative anaerobic and belonging to the Enterobacterales order [18, 19] . Recent studies have emphasized the threat posed by this species that has emerged as opportunistic pathogen, prominently reported in nosocomial outbreaks in neonatal intensive care units (NICUs), intensive care units (ICUs), and various hospital settings. This strain demonstrates remarkable pathogenic versatility, capable

of eliciting a broad spectrum of clinical pathologies, which include wounds, urinary tract infections, central nervous system infections, bloodstream infections, pneumonia, and keratitis [20]. There are high morbidity rates regarding S. marcescens bacteremia [21]. The incidence of infections attributed to S. marcescens is linked to the expression of numerous virulence factors and the emergence of antibiotic resistance [22]. The clinical management of S. marcescens infections is challenging due to its intrinsic resistance to different classes of antibiotics such as ampicillin, first and second generation cephalosporins, macrolides, and cationic antimicrobial peptides (CAPs) [23, 24].

2. MATERIALS AND METHODS

Collection and Preparation the plant for extraction

The plant (*Bacopa monniere* L.) was collected from the banks of the Shatt al-Arab in Basra city on 2023/3/19 . The plant was Prepared in the plant extracts laboratory in the Department of Biology / College of Science for Girls / University of Baghdad, after the aerial part (leaf , stem , flower) of the plant was separated and washed well with water several times, dried at room temperature (27-30) °C and then grinding the plant using electric blender.

Extraction and identification of flavonoids

Twenty fiveg of dried powder were extracted with 250mL of 80% methanol at 25°C, for 24 hours. The extract was filtrated by a separatory funnel, and the precipitate treated with 25mL acetone and 30mL concentrated HCl. The mixture was then filtered and the filtered was dried by rotary evaporator [25]. By HPLC, flavonoids were identified and measured its quantities. The conditions of HPLC were the column separation was C18-OSD (250 mm , 4.6mm) . The column temperature was 30ºC the gradient elution method, with eluent (methanol) and eluent (1%) formic acid in water (70 : 30 v/v) at flow-rate of 0.7 mL/min. The injected volume of samples 100 µL and standards was 100 µL and it was done automatically using autosampler. The spectra were acquired in the 280 nm [26] . The total flavonoid content of crude extract was determined by the aluminum chloride colorimetric method [27].

Synthesis of Flavonoids - Casein nanoparticle

1 g of casein was dissolved in 50 ml of millipore deionized water and filtered from 0.11µm microfilter to remove any impurities (sterilizing process) . To the above mentioned solution Flavonoids mixture was added. The mixture was refluxed for 4 hours at 80 °C . Then the mixture was left to cool own to room temperture and then the product was evaporated on stem bath to remove maxiumam volume of solvent . Thereafter, the gel-like mixture was collected by centrifuge at 12000 rpm for 5 min in cooling centrifuge at 10 °C. 1 g of flavonoids- Casein was added to 50 ml of sterilized deionized water and sonicated for 5 min using ultrasound probe (750 watt) in open pulse process in an ice bath to make the temperature inside the flavonoids - Casein system does not exceed 30 ˚C to prevent protein dissociation in the high temperature that resulted from the ultrasound waves. This solution was used freshly for further steps [28].

Characterization of Flavonoids - Casein nanoparticles

Many methods were used to determine nanoparticles characteristics such as :

Scanning Electron Microscope (SEM) used to characterize and visualize surface morphology, particle size distribution, particle shape and agglomeration of nanoparticles

Zeta–Potential of the nanoparticles was determined by Zeta-potential analyzer to study the stability of the prepared nanoparticles [29].

Serratia marcescens collection , Isolation and identification

A total of 30 blood samples were collected from patients then identified primarily by culturing on Blood agar and MacConkey agar medium at 37°C for 18-24 h and confirmed by VITEK 2 system.

Biofilm formation assay for *Serratia marcescens* isolates

In the current study, 30 clinical isolates of , *Serratia marcescens* .were screened for their capability to form biofilm via microtitration plate's method as stated by Zhang *et al*. [30] with some modification.

1- *Serratia marcescens* isolated from fresh agar plates were inoculated in 5 mL of brain heart infusion (BHI) with 2% sucrose, the broth was incubated for 24 h at 37ºC [31].

2- Twenty microliter of bacterial suspension from each isolates (equivalent to 0.5 Mcfarland standard) was add and used to inoculate microtiter wells containing 180 µl from the Brain Heart Infusion Broth to each well of the microplate, which was cover and incubated at 37 °C for 24 hours.

3- After incubation, the plate was washed three times with normal saline to eliminate non-adherent cells.

4- To fix the adhered cells, 200 µl of 99% methanol per well was add for 15 minutes. The plate was dried for 30 minutes at

room temperature .

5- Then 200 µl of 1% crystal violet was added for 15 minutes.

6- After eliminating the dye solution and washing with sterile distill water, the attached dye was solubilized with 96% ethanol, and the optical density was appointed in a micro-titer plate reader at 630 nm .The biofilm formation was evaluated as described in Table (1).

Table 1: Evaluation of biofilm formation by microtiter plate method.

Optical density	**Adherence**
OD ≤ ODc	**Non–adherent**
2ODc > OD > ODc	**Weak**
4 ODc > OD > 2ODc	**Moderate**
OD >4 ODc	**Strong**
Cut off value (ODc) = average OD of negative control + (3 *Standard Deviation).	

Determination of flavonoid extract and nano flavonoid MIC

Double serial dilutions (1-1024 µg / ml) of flavonoid extract and nano flavonoid where prepared frome a stock (10mg/1ml) in a micro titer plate using MuellerHinton broth as diluent. All wells were inoculated with 20µl of bacterial suspension comparable to McFarland standard no. 0.5 (1.5×108CFU/ml) except for the negative control wells. Microtiter plates were incubated at 37°C for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all of the wells and incubated for 2h to observe any color changes. The MIC Concentrations were determined visually in broth micro dilutions as the lowest concentrations at which color changed from blue to pink in the resazurin broth assay [32].

Antibiofilm activity of flavonoid extract and nano flavonoid MIC against *Serratia marcescens*

The same protocol was used for the biofilm formation assay according to Mahdi *et al*. [33]. After prepared sterilized Brain Heart Infusion broth with 2% sucrose ,180µl of BHIB that treated with flavonoid extract and nano flavonoid MIC was added to each well, 20 µl of *Serratia marcescens* suspension was introduced compared to 0.5 McFarland ,whereas the control contained just 180µl and 20µl of *Serratia marcescens* suspension, after incubation, the medium was taken from the wells and washed three times with sterile Phosphate buffer saline to remove the unattached *Serratia marcescens* cells and left to dry for 15 minutes at room temperature, the wells were then filled with 200µl of crystal violet (0.1%) and allowed to sit for 20 minutes .To remove unbound dye, the stained wells were washed three times with Phosphate buffer saline (PH 7.2) and allowed to dry at room temperature for 15 minutes, finally, 200µl of 95% ethanol was poured to each well, and the optical density was measured using an ElISA reader at 630 nm.

Statistical Analysis

The Statistical Packages of Social Sciences-SPSS [34] program was used to detect the effect of difference groups (samples) in study parameters. Least significant difference-LSD was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

3. RESULTS AND DISCUSSION

Quantification of flavonoids by using reversed phase HPLC analysis :

HPLC analysis for flavonoids extract , total flavonoid content was determine and some flavonoids were identified, such as (Myricetin, Hesperidin, Rutin, Qurcetine and Kaempferol) in Table (2) and and Fig (1) .

The total amount of flavonoids is 98.0 (mg / 100 gm) and Major flavonoids found in B. monnieri in this study are Qurcetine 70.2 (µg / gm) followed by Rutin 69.8 (µg / gm).

Table 2: Quantification of flavonoids from *Bacopa monnieri* L. by HPLC

No.	**Name of flavonoids**	**Concentration**	**Retention Time (min)**
1	**Total flavonoid content (mg / 100 gm)**	**98.0**	**-------**

2	**Myricetin (µg / gm)**	**38.9**	**2.90**
3	**Hesperidin (µg / gm)**	**41.0**	**4.01**
4	**Rutin (µg / gm)**	**69.8**	**4.81**
5	**Qurcetine (µg / gm)**	**70.2**	**6.05**
6	**Kaempferol (µg / gm)**	**55.4**	**7.85**

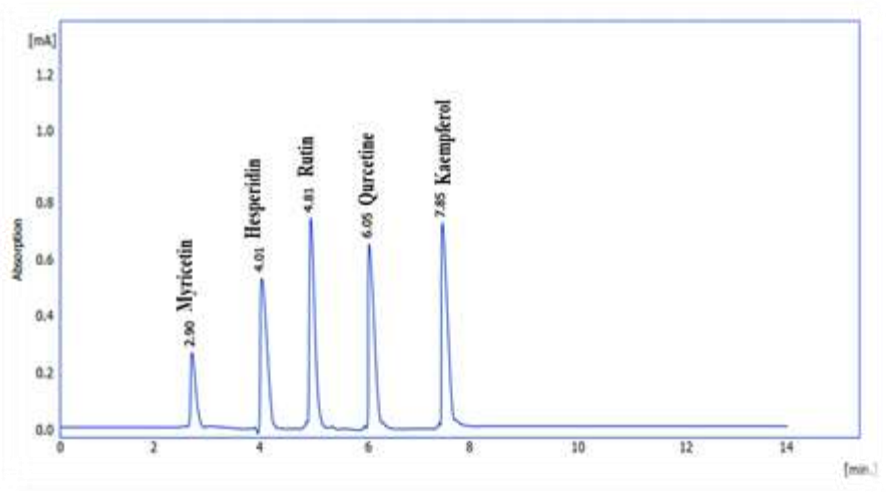

Figure 1. HPLC analysis of flavonoids to *Bacopa monnieri* L.

Characterization of Flavonoid -casein nanoparticles

FESEM

The FESEM measurement of flavonoid -casein (Figure 2) showed the presence of an irregular structure consisting of agglomerates whose surfaces are covered with spherical particles whose dimensions are in the range of 85-88 nm, in addition to sheet-like structures whose thickness does not exceed 35 nm. These structures, which differ from the structure of casein (usually geometric structures resembling sphere) and within the nanoscale range, confirm the transformation of the substance into the nanoform by the addition of flavonoid. This effect can be attributed to the flavonoids playing the role of dispersants after being attached to the surface of the casein by stacking forces via the aromatic system. According to [35] the size of the NPs has an impact on their activity, with tiny particles being generally recognized as being more effective than large ones.

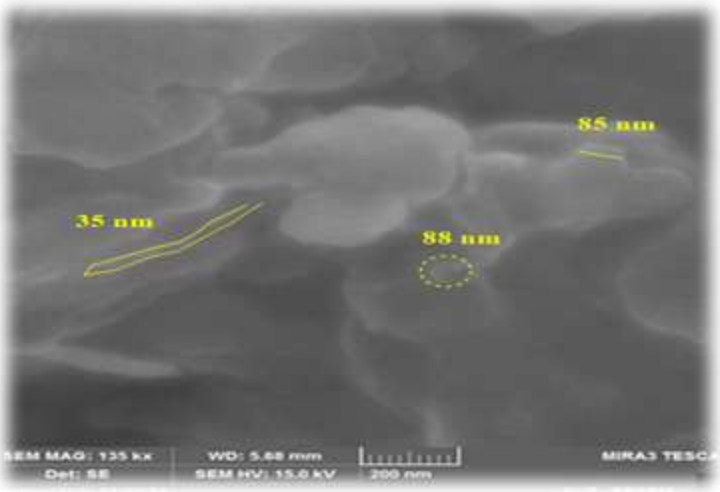

Figure 2 . FESEM of flavonoid-casein NPs .

Zeta potential

This measurement was used to study the stability of the prepared nanocomposite (flavonoid -casein), where the measurement demonstrated the presence of a broad peak that begins at 88 millivolts and ends at 145 millivolts, with a peak centered at 127 millivolts, indicates the high stability of the substance. In addition, the measurement shows a shoulder peak at 100 millivolts, which is evidence of the presence of two substances, this result from physical bonding. If the material was chemically bonded, the measurement would show a single sharp peak without an accompanying shoulder peak, as in Figure (3).

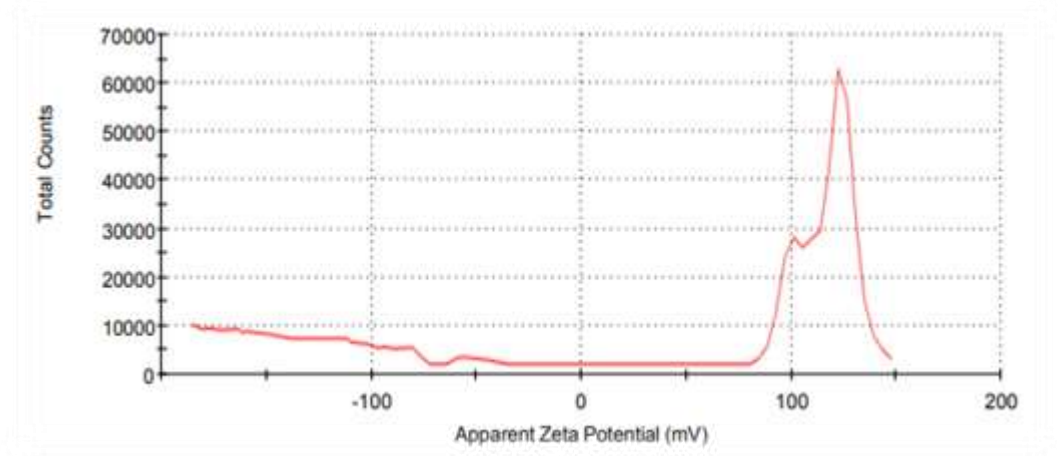

Figure 3. Zeta potential of flavonoid- casein NPs .

***Serratia marcescens* Isolation and identification**

Isolates were collected from males and female patients were confirmed as S. *marcescens* , the conformation was carried out by VITEK 2 system that considered a reliable identification technique, in addition to swarming on blood agar they developed as bacterial colonies after 24 hours of incubation the majority of bacterial isolates generated hemolysis as shown in figure (4- A) . Shimuta *et al*. [36] noticed that S. *marcescens* produces hemolysis on human blood agar and the S. *marcescens* hemolytic a factor is the hemolysin ShlA. However, this study indicating presence another S.*marcescens* hemolytic factor is a phospholipase A (PhlA) affected cell membranes resulting in hemolysis and cell death. S. *marcescens* colonies on the MacConkey agar medium appeared to be pale resulting from non-lactose fermentation in the medium as shown in figure (4-B). S. *marcescens* have ability to produce red non-soluble pigment which is called prodigiosin , in the same manner Srimathi *et al*. [37] mentioned that non- lactose fermenting is the reason where S. *marcescens* appeared in pale color on the MacConkey agar.

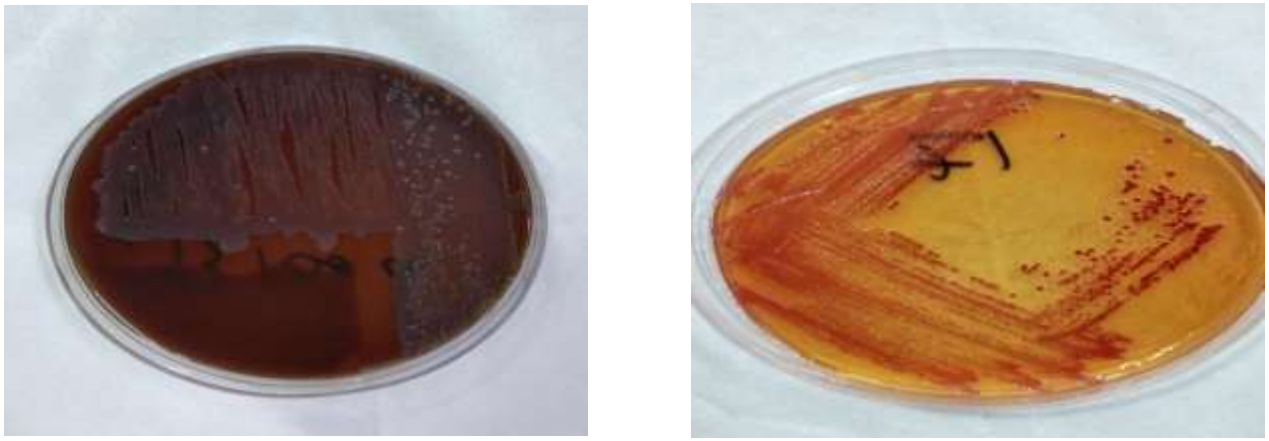

Figure 4 . Growth *Serratia marcescens* isolates on (A) Blood agar (B) MacConkey agar

Biofilm formation by *Serratia marcescens*:

Biofilm formation is considered as a marker of virulence. In this study, the ability of S. *marcescens* biofilm producing isolates was evaluated by means of pre-sterilized 96-well polystyrene microtiter plates, which considered as a standard test for the detection of biofilm biomass by ELISA reader [38]. The results were categorized into four groups (non-biofilm producer,

weak, moderate, and strong) based on limits. The present study declared that out of 30 S. *marcescens*, 10 isolates formed a weak biofilm at a ratio of about 33.3% , 12 isolates formed a moderate biofilm at a ratio of 40% , whereas 8 isolates formed a strong biofilm at a ratio of about 26.7% . The differences in biofilm thickness might be due to several reasons; differences of isolates capacity to form biofilm or perhaps differences in primary number of cells that succeeded in adherence and differences of quality and quantity of quorum sensing signaling molecules that produced from each isolate play important roles [39,40]. This result is an agreement with other studies investigated S. *marcescens* biofilm formation. Mun *et al.* [41] revealed that 22.2% and 55.5% of S. *marcescens* isolates were strong biofilm producers and moderate biofilm producers respectively. Al-Fayyadh *et al*. [42] discovered all S. *marcescens* isolates produced biofilm. The biofilm intensity based on estimated cutoff value of S. *marcescens* isolates summarized in Table (3).

Table 3 : Biofilm intensity based on estimated cutoff value*of *Serratia marcescens* isolates

ID Biofilm	Intensity	OD630 Limits number of isolates	Number of isolates	percentage %
1	**Non-biofilm producer**	**< 0.0651**	**0**	**0**
2	**Weak**	**0.0651-0.130**	**10**	**33.3%**
3	**Moderate**	**0.130- 0.260**	**12**	**40%**
4	**Strong**	**≥ 0.260**	**8**	**26.7%**

***cutoff value = 0.065 (defined as the Mean of control OD630 plus 3* Standard deviation).**

Determination of flavonoid extract and nano flavonoid MIC :

The broth microdilution method was used to determine the MIC of flavonoid extract and nano flavonoid in 96-well microtiter plate. The susceptibility of the eight isolates S. *marcescens* with higher biofilm formation proberity against flavonoid extract and nano flavonoid were tested by determining the MIC using microtiter plate. The results showed that the MIC of flavonoid extract was ranged from 64-512 µg/ml . Also the results showed that the MIC of nano flavonoid was ranged from 8-64 µg/ml .The MIC of flavonoid extract and nano flavonoid which can inhibit to bacteria growth shown in table (4).

Table 4: The MIC of flavonoid extract and nano flavonoid against *Serratia marcescens* isolates.

S. *marcescens* Isolates	Flavonoid extract (µg/ml)	Nano flavonoid (µg/ml)
1	**64**	**16**
2	**512**	**64**
3	**256**	**32**
4	**512**	**32**
5	**64**	**8**
6	**64**	**32**
7	**128**	**16**
8	**128**	**16**

The effect of MIC of flavonoid extract and nano flavonoid on biofilm formation:

The inhibitory effect of flavonoid extract and nano flavonoid was studied at in MIC concentration on S. *marcescens* biofilm formation. After completing the treatment of the bacterial isolates with MIC of flavonoid extract and nano flavonoid the result show bacterial sensitivity to flavonoid extract and nano flavonoid and the biofilm formation was reduced. The OD of the biofilm before treatment is significantly higher in the eight S. *marcescens* isolates (1.30 ±0.05, 1.61 ±0.24 , 0.695 ±0.03 , 0.470 ±0.04 , 0.686 ±0.04 , 0.630 ±0.02 , 0.738 ±0.02 and 0.491 ±0.03) as compared with flavonoid extract (0.442 ±0.03 , 0.442 ±0.03 , 0.377 ±0.02 , 0.230 ±0.02 , 0.325 ±0.02 , 0.347 ±0.01 , 0.314 ±0.01 and 0.195 ±0.02 respectively) and nano

flavonoid (0.107 ±0.01 , 0.165 ±0.02 , 0.096 ±0.002 , 0.112 ±0.01 , 0.157 ±0.01 , 0.099 ±0.01 , 0.093 ±0.01 and 0.089 ±0.01 respectively) . as shown in Table (5).

Interestingly the Nano compounds (flavonoid) inhibits the biofilm formation more than flavonoid extract. The highest inhibition of biofilm formation of S.*marcescens* shown in nano flavonoid compound compared to other compounds. Flavonoids have been identified as compounds capable of exerting antibacterial activities via various mechanisms of action. Flavonoids have also been found to reduce adhesion and biofilm formation, porin on the cell membrane, membrane permeability, and pathogenicity, all of which are crucial for bacterial growth [43,44,45]. Pandey *et al*. [46] reported that the flavonid exhibited potent antibacterial effects with a low minimum inhibitory concentration by inducing intracellular reactive oxygen species.

Table 5 : Antibiofilm activity of phenolic extract and flavonoid extract and nano phenolic and nano flavonoid.

***S.marcescens* Isolates**	**Before Treatment**	**After Treatment (Anti-biofilm)**		**L.S.D.**
	Biofilm	**Flavonoid extract**	**Nano flavonoid**	
1	**1.30 ±0.05 a**	**0.442 ±0.03 b**	**0.107 ±0.01 c**	**0.148 ****
2	**1.61 ±0.24 a**	**0.442 ±0.03 b**	**0.165 ±0.02 c**	**0.155 ****
3	**0.695 ±0.03 a**	**0.377 ±0.02 b**	**0.096 ±0.002 c**	**0.109 ****
4	**0.470 ±0.04 a**	**0.230 ±0.02 c**	**0.112 ±0.01 d**	**0.097 ***
5	**0.686 ±0.04 a**	**0.325 ±0.02 c**	**0.157 ±0.01 d**	**0.106 ***
6	**0.630 ±0.02 a**	**0.347 ±0.01 b**	**0.099 ±0.01 d**	**0.095 ***
7	**0.738 ±0.02 a**	**0.314 ±0.01 c**	**0.093 ±0.01d**	**0.112 ***
8	**0.491 ±0.03 a**	**0.195 ±0.02 c**	**0.089 ±0.01 d**	**0.087 ***

Means having with the different letters in same row differed significantly.

*** (P≤0.05), ** (P≤0.01).**

4. CONCLUSION

The results of the study showed flavonoids and nano-flavonoids can inhibit biofilm formation by affecting vital processes within bacterial cells. On the other hand, nano-flavonoids are more effective than flavonoids due to their ability to enter bacterial cells faster and more efficiently due to their smaller size . These properties make nano-flavonoids more capable of interacting with biofilms, leading to reduction in their ability to form biofilms. Therefore, flavonoids and nano-flavonoids are promising candidates as inhibitors for *Serratia marcescens*, which is known for its ability to resist antibiotics

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