Composition and biological activities of Prunus mahaleb L. (Rosaceae) Leaf extract; antibiofilm effect on clinical strains

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Abstract

In this work, leaves of *Prunus mahaleb* L. plant were extracted using methanol solvent. The chemical constituents of the n-hexane fraction of the methanol extract were identified by gas chromatography mass spectrometry (GC-MS) analysis. The amount of phenolic and flavonoid compounds of the extract was measured. The antioxidant and anticancer activities were investigated by 2,2-diphenyl-1-picryl hydrazyl (DPPH) and brine shrimp method, respectively. Then the ability of biofilm inhibition of the extract was evaluated. The results showed that octadecenoic acid (26.13%) and 14-methyl-8-hexadecyn-1-ol (18.54%) were the main constituents of the extract. The extract also contains significant amounts of phenolic and flavonoid compounds (100.31 ± 0.004 and 42.07 ± 0.014 µg/mg, respectively). DPPH test showed that the extract had high antioxidant activity (IC_{50} = 53.09 ± 0.13 µg/ml), but the brine shrimp test showed that the extract had no anticancer effect. The evolution of biofilm inhibition showed that the extract had good inhibitory effect on multidrug resistance *E. coli* strains. This study was performed for the first time on the leaf extract of *P. mahaleb* L. plant (collected from the Marivan, Iran) which showed that the leaves of the plant have significant amounts of phenolic and flavonoid compounds and have good anti-biofilm and antioxidant properties.

1. Introduction

Prunus mahaleb L. belongs to the genus *Prunus* of Rosaceae family, with more than 400 species worldwide. Other names for this plant in English are Mahaleb cherry, Rock cherry and St. Lucie cherry, also known in Persian as cherry blossom, Mahlab and Malham. This species is native to Mediterranean, southern and central Europe, northwest Africa and southwest Asia, such as Pakistan, Turkey, Iraq and Iran (Sabeti, 2008). This plant prefers warm, dry weather and well-drained soils and is resistant to several diseases. The different parts of this species have gardening value, forestry and green space, edible, export, by-products, secondary metabolites and industrial raw materials for pharmaceutical-medical industries, dyeing and cosmetics. It is a transplant base for sour and sweet cherries and a wildlife sanctuary. The edible parts of the plant are fruit and seed. The seeds are used in confectionery, bread production and biscuit making. Dried seeds are also used in Greece, Turkey and Armenia as food additives. However, it should be used with caution because of the hydrogen cyanide present in the leaves and seeds (Kunkel, 1984). In traditional medicine, the plant seeds are used to strengthen the liver, relieve internal pain, expel various internal parasites, remove moisture from the chest and lungs, strengthen the senses, treat suffocation (Arrhythmia) and pain of back and colic. If the grain is poured into the bread, it speeds up the digestion of food. Its wood and leaves are tonics for the body, and it is considered useful for removing bad body odour and insect bites, as well as the incense of the bark to repel insects. If it is boiled in oil with Ruta and Mastic, it is useful for paralysis and tetanus, tremors and joints orally or rubbed on the sore spot (Tonekaboni, 2007).

On the one hand, bacteria became resistant to antibiotics, and on the other hand, new antibiotics are less discovered. For this reason, researchers are looking at different treatments, such as traditional herbal medicine, bacteriophage treatments, and combination therapies (Cheesman, Ilanko, Blonk, & Cock, 2017).

The herbal compounds have antimicrobial effects against pathogens. Moreover, these compounds are also inexpensive, available, and low-risk, so they have always been of interest to researchers (Alanis, Calzada, Cervantes, Torres, & Ceballos, 2005; Bonjar, 2004; Meléndez & Capriles, 2006).

The secondary metabolites of plants have many bioactive and biochemical properties that are used in various pharmaceutical, chemical, cosmetic and especially food industries (Gourine et al., 2010). The phenolic or secondary nitrogen-free compounds have various biological effects, including antibacterial activity (Yadegarinia et al., 2006).

Costerton described bacterial biofilms in 1978. Biofilm-forming bacteria can be one or more different community types (Lear & Lewis, 2012). In a biofilm, microorganisms produce a matrix for attaching to the surface (Mah & O'Toole, 2001). The interactions between microorganisms with the surface and other microorganisms play an essential role in the development of biofilms. Biofilms are a significant cause of nosocomial infections. However, the external polysaccharides of bacteria were attached to the surface and prevented antibiotics effect against bacteria. The biofilm increases bacterial distribution and pathogenicity. The multidrug resistance bacteria are resistant to various antibiotics; therefore, researchers use herbal extracts to eliminate multidrug-resistant pathogens (Kojic & Darouiche, 2004; Peterson, 2007)

Mohammadi Bazargani et al. applied Peppermint, Anise and Coriander extract for biofilm inhibition of *S. aureus* and *E. coli* clinical strains (Bazargani & Rohloff, 2016). Navasivayam et al. reported biofilm inhibition of *Azadirachta indica*, *Vitex negundu*, *Tridax procumbens*, and *Ocimum tenuiflorumi* extracts against *E. coli* clinical strain (Namasivayam & Roy, 2013).

This study aimed to identify the chemical constituents of the n-hexane fraction of methanol extract of *P. mahaleb* L. leaves. The phenolic and flavonoid compounds of methanol extract were evaluated. Moreover, antioxidant and anticancer activity were investigated. Also, its ability to inhibit biofilm against clinical strains of *E. coli* were determined.

2. Materials and Methods

2.1. Preparation of Plant Extract

P. mahaleb L. was collected from the Marivan area (southeast of Marivan, Iran) in summer 2017 and identified by Dr Toloui. A voucher specimen (UKH 2706) was deposited in the herbarium of the University of Kashan. The leaves were excised from the plant and washed with distilled water twice and dried. The dried leaves were powdered then extracted with methanol solvent (500 ml) using a soxhlet extractor for 8 hours. After extraction, the resulting extract was filtered and evaporated by rotary evaporator (Heidolph, Model RE-801, Germany) to give amorphous solid masses. The methanol extract of the sample (1.5 g) was dissolved in distilled water (150 ml), and the n-hexane fraction was extracted with a decanter then concentrated with a rotary evaporator and dried with a vacuum oven (Memmert, Germany) for 24 hours.

2.2. GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed according to Nasrollahi, et al. (Nasrollahi, Ghoreishi, Ebrahimabadi, & Khoobi, 2019) and components of n-hexane fraction of the methanol extract were identified.

2.3. Antioxidant Activity

The antioxidant activity of the leaf methanolic extract was determined by the DPPH method (Kedare & Singh, 2011). In this method, the ability of sample (as an antioxidant) for transfer electrons to 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical was investigated by changing the colour of the DPPH solution (from violet to yellow). The DPPH radical absorbs at 517 nm, and its decrease absorption is linearly correlated with the amount of antioxidant. Dibutyl hydroxytoluene (BHT) was also used as the standard control. Firstly, the different concentrations (0.8, 0.5, 0.25, 0.1, 0.05, 0.005, 0.0005 mg/ml) of the sample were prepared. Then, each of the concentrations was mixed with 1 ml of the DPPH solution (0.1 mmol/l). The solutions were incubated at room temperature in a dark chamber for 30 min. The absorbance of the solutions was measured at 517 nm using a UV-Visible spectrophotometer (Shimadzu, Model UV1800, Japan). The extract solution without DPPH was prepared as blank. Percentage of DPPH scavenging activity was calculated according to the Eq. 1:

$$\text{Antiradical activity (\%)} = (A_{blank} - A_{sample}) / A_{blank} \times 100 \quad (1)$$

Where A_{blank} is the absorbance of the control reaction, and A_{sample} is the absorbance of the sample or BHT. The IC_{50} value was calculated as the half-maximal inhibitory concentration. The decrease in absorbance of the reaction mixture indicates stronger DPPH radical scavenging activity. The sample was tested three times, and the mean values were calculated.

2.4. Determination of Total Phenolic Content (TPC)

Total phenolic content was analyzed spectrophotometrically using a modified Folin-Ciocalteu colorimetric method (Chu, Sun, Wu, & Liu, 2002). Gallic acid was used as the standard in this method. In brief, 0.02 ml of extract (5 mg/ml) was mixed with 0.1 ml of Folin-Ciocalteu reagent and 3 ml distilled water. After 3 min, 0.3 ml of sodium carbonate (2%) was added. Then, 0.02 ml of dimethyl sulfoxide (DMSO) was also used for the blank. The mixtures were placed for 2 hours at room temperature then the absorbance was measured by UV-Visible spectrophotometer at 760 nm. The calibration curve was calculated using gallic acid. This result was expressed as µg gallic acid equivalent (GAE) per mg of extract. The sample was assayed in triplicate, and mean values were calculated.

2.5. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined by the modified aluminium colorimetric method; also, quercetin used as the standard (Chang, Yang, Wen, & Chern, 2002). Briefly, 0.5 ml of the extract (1 mg/ml) were placed in a test tube then 0.1 ml of aluminium chloride (10%), 0.1 ml of sodium acetate (1 mol/l), 1.5 ml of methanol and 2.8 ml distilled water were added to the tube and then mixed. Then, 0.5 ml of methanol was also used as a blank. Finally, the tubes were incubated at room temperature for 30 min. The

absorbance was measured by UV-Visible spectrophotometer at 415 nm. The calibration curve was calculated using quercetin. The concentration of flavonoid was expressed as µg quercetin equivalent (QE) per mg of extract. The sample was assayed in triplicate, and mean values were calculated.

2.6. Brine Shrimp Lethality Assay (BSLA)

Brine shrimp lethality test was performed according to Meyer et al. (Meyer et al., 1982) method with minor adaptations. Brine shrimp lethality bioassay is widely applied in the bioassay for the bioactive compounds (Zhao, Hui, Rupprecht, McLaughlin, & Wood, 1992). In this study, the brine shrimp, *Artemia salina*, was utilized as a convenient monitor for the screening. The eggs of the brine shrimp (0.5 g) were hatched in 1 litre artificial seawater (3.8% NaCl solution in 9 pH) for 48 hours in a water bath at 30°C with aeration pump and proper lighting. The extract (0.05 g) was dissolved in DMSO (3.2%) plus artificial seawater to attain concentrations of 10 µg/ml, 100 µg/ml, 300 µg/ml, 500 µg/ml, 700 µg/ml, 1000 µg/ml. Vincristine sulfate (VS) and DMSO were utilized as a positive and negative control, respectively. Then matured shrimps (nauplii) were added to each of all test and control vials and were placed in the water bath. After 24 hours, the number of surviving nauplii in each vial were counted. The Eq. 2 calculates the percentage of lethality:

The percentage of lethality = (number of live larvae in the negative control - number of live larvae in the sample) / number of live larvae in the negative control) × 100 (2)

Finally, the chart of the lethality percentage to concentration was drawn. The LC_{50}, which is a concentration of the sample with the lethality (50%), was calculated in µg/ml. Experiments on sample and control solutions were repeated three times, and mean values were calculated.

2.7. Biofilm Inhibition Assay

2.7.1. Clinical Strains

Multidrug resistance strains were isolated and collected from different clinical specimens, including urine and lung, etc., from patients in different care units of Qom (Iran) hospitals. The characteristics obtained from each strain are given in Table 1. The clinical strains were cultured overnight at 37°C in nutrient agar (NA) medium.

Table 1
Selected clinical strains

No.	Bacteria	Name	Code	No.	Bacteria	Name	Code
1	*E. coli*	N23	3056	10	*E. coli*	N32	4828
2	*E. coli*	N3	5149	11	*E. coli*	G3	159
3	*E. coli*	G29	885	12	*E. coli*	N24	3069
4	*E. coli*	N21	2422	13	*E. coli*	G17	228
5	*E. coli*	N37	4469	14	*E. coli*	G15	726
6	*E. coli*	N31	4745	15	*Pseudomonas aeruginosa*	N19	3316
7	*E. coli*	N30	4701	16	*Klebsiella pneumonia*	N9	14
8	*E. coli*	G13	572	17	*Pseudomonas aeruginosa*	N34	4935
9	*E. coli*	N29	4221	18	*Staphylococcus aureus*	G23	48629

In this study, we applied nalidixic acid, vancomycin, amikacin, cefotaxime, ceftriaxone, cefixime, imipenem, trimethoprim-sulfamethoxazole, nitrofurantoin, ciprofloxacin and gentamicin.

2.7.2. Biofilm Formation Assay

Biofilm formation assay was performed using the microtiter plate method. The bacterial suspension was diluted 1:100 with tryptic soy broth (TSB) medium, and 0.2 ml of dilution was added into each well of the sterile plate. 0.2 ml TSB medium without bacteria was used as a negative control. The plate was incubated at 37°C for 24 hours; then, the wells were washed with sterilized phosphate-buffered saline (PBS) to remove loosely adhered cells. Briefly, 0.2 ml of methanol was added to each well for 20 minutes and allowed to dry at room temperature. The wells were stained with 0.2 ml of crystal violet (1%) at room temperature for 10 minutes. Then the wells were washed with sterile deionized water, and the stains were removed. After drying the wells, 0.2 ml acetic acid was added to each well. After 15 minutes, the optical density of the plate was measured at 570 nm with an ELISA (Enzyme-linked immunosorbent assay) microplate reader (BioTek, Epoch Microplate Spectrophotometer, USA). The biofilm formation of the strains was calculated using the method of Stepanovic et al. (Stepanović, Vuković, Dakić, Savić, & Švabić-Vlahović, 2000).

2.7.3. Effect of Methanol Extract on Biofilm Inhibition

The anti-biofilm activity of the extract was assessed against clinical strains. At first, 0.1 ml of extract solution)2 µg/ml) and 0.1 ml of bacterial suspension adjusted to 0.5 Mcfarland were added to each well. Additionally, bacterial suspension and TSB medium in the absence of extract were served as a positive and negative control, respectively. The plate was incubated at 37°C for 24 hours. The staining method was the same as the biofilm formation, and the optical density (OD) of the plate was measured at 570 nm with the ELISA microplate reader. The percentage of biofilm inhibition was calculated using Eq. 3:

The percentage of biofilm inhibition = $\frac{(C-B)-(T-B)}{(C-B)} \times 100$ (3)

Where C is OD_{570} of positive control, B is OD_{570} of negative control, and T is OD_{570} of cells with the extract (Nikolić, Vasić, Đurđević, Stefanović, & Čomić, 2014).

3. Results and Discussion

3.1. GC-MS Analysis

The chemical components of the extract were identified using gas chromatography-mass spectrometry. The extract compounds were identified based on the available spectra and comparing their inhibition index with the standard compounds. The chemical compositions of the n-hexane fraction are listed in Table 2.

Table 2
Chemical composition of *P. mahaleb* L. leaf extract

No.	Component	RI (min)	KI	Composition (%)
1	Stearic acid (Octadecanoic acid)	0.23	900>	1.21
2	Isopentyl propionate (Isoamyl propionate)	3.16	966	0.64
3	α-Pinene	6.32	939	0.06
4	Eucalyptol (1,8-Cineole)	8.64	1031	0.06
5	Geranyl myristate	21.90	1620	0.12
6	Neophytadiene	30.26	1836	0.74
7	14-Methyl-8-hexadecyn-1-ol	42.29	2232	18.54
8	Octadecenoic acid (1-methylethyl ester)	42.92	2179	26.13

Eight compounds include octadecenoic acid (26.13%) and 14-methyl-8-hexadecyn-1-ol (18.54%), were recorded as main components of the leaf extract. In general, the results indicated the presence of compounds such as fatty acids, esters, alcohols, alkanes (alkyl and alkyne) and aromatics. Also, the extraction of partial yields of methanol extract and n-hexane fraction of *P. mahaleb* L. leaf is shown in Table 3.

Table 3
Extraction partial yield of *P. mahaleb*

Leaf extract fractions	Yield (W/W %)
Methanol extract	20.73
n-Hexane extract	6.11

In another research, volatile compounds of leaves, flowers, stem bark and wood of *P. mahaleb* L. have consisted hexadecanoic acid (17.8%) and phytol (5.1%) in leaves; coumarin (34.1%) and, hexadecanoic acid (9.3%) in stem skin and normal alkanes, heneicosane (22.1%) in flowers. Moreover, octacosane (13%) was the major constituent. Dodecanoic, tetradecanoic and linoleic acids were also among the main compounds of all samples (Mastelić, Jerković, & Mesić, 2006).

3.2. Antioxidant Activity

In this test, the antioxidant ability was calculated by the colorless purple of the DPPH solution. The electrons transfer from the sample to the DPPH solution, and the solution becomes colorless. However, the method is not dependent on the polarity of the sample. The percentages of inhibition versus the negative logarithm of sample concentration and standard (BHT synthetic antioxidant) were plotted. The IC_{50} value of the sample and BHT was calculated as reported in Table 4.

Table 4
IC_{50} values of extract and standard

Sample	IC_{50} (µg/ml)
Methanol extract	53.09 ± 0.13
BHT	17.78 ± 0.12

A comparison of IC_{50} of the extract with BHT showed that the extract has high antioxidant activity (see Table 4).

According to previous research by Mariod et al., IC_{50} of different fractions of *P. mahaleb* L. seed extract was 2.9 to 5.6 µg/ml compared to ascorbic acid standard with an IC_{50} value of 0.017 µg/ml. Their results showed that the total methanol extract of plant seeds had the highest antioxidant activity compared to other fractions (Mariod, Ibrahim, Ismail, & Ismail, 2010).

3.3. Determination of Total Phenol and Flavonoid Content

Total phenolic and flavonoid contents of the extract were evaluated using gallic acid and quercetin standards, respectively, as shown in Table 5.

Table 5
Values of total phenolic and flavonoid content

Extract	TPC (µg/mg)	TFC (µg/mg)
Methanol extract	100.31 ± 0.004	42.07 ± 0.014

The total amount of phenolic, and flavonoid compounds in leaf methanol extract were calculated using gallic acid and quercetin calibration curves with the linear regression Equations 4 and 5, respectively:

$Y = 0.0009X + 0.0319$ $R^2 = 0.9576$ (4)

$Y = 0.0079X + 0.0206$ $R^2 = 0.9992$ (5)

Where Y is measured absorption and X is the gallic acid and quercetin concentration (µg/ml), respectively.

The results indicated a significant amount of phenolic and flavonoid compounds in the leaf methanolic extract, which is part of the phenolic compounds belonging to flavonoids. The results of this experiment are in agreement with the results of the DPPH assay. Usually, the number of phenolic compounds in the plant will be inversely correlated with the IC_{50} value obtained in DPPH. Furthermore, the antioxidant power of the plant extract is due to the phenolic compounds.

Oskoueian et al. found that the total amount of phenolic and flavonoid compounds in methanol extract of *P. mahaleb* L. seed were 75.7 mg/g gallic acid and 28.5 mg/g rutin, respectively and also, these values in the hexane extract were 34.6 mg/g gallic acid and 16.2 mg/g rutin, respectively (Oskoueian, Haghighi, Ebrahimi, & Oskoueian, 2012).

3.4. Brine Shrimp Lethality Assay (BSLA)

The anticancer activity of the leaf methanol extract was investigated using the brine shrimp test. In this experiment, the LC_{50} value of the extract and VS was > 1000 and 0.625 µg/ml, respectively. According to the previous report, three levels of high, moderate and week toxicity were indicated in the ranged of LC_{50} (0-100 µg/ml), (100–500 µg/ml), and (500–1000 µg/ml) respectively. Moreover, the LC_{50} above 1000 µg/ml is due to the lack of toxicity of the sample against *Artemia salina* (Padmaja et al., 2002). Therefore, the extract had no cytotoxic activity.

In the present study, the antimutagenicity effect of samples with three levels of weak, medium and strong was considered to be less than 25%, between 25% and 40% and above 40%, respectively.

Karchesi et al. reported that leaf methanol extract of *Prunus* spp. (Cherry) with LC_{50} > 1000 µg/ml had no cytotoxicity and anticancer properties (Karchesy, Kelsey, Constantine, & Karchesy, 2016).

3.5. Biofilm Inhibition Assay

The results of biofilm formation using 18 clinical strains are shown in Table 6. According to the results, the selected strains were able to form three groups of strong, medium and weak biofilms.

Table 6
Results of biofilm formation power of clinical strains

No.	Bacteria	Code	Biofilm formation power	No.	Bacteria	Code	Biofilm formation power
1	*E. coli*	3056	Medium	10	*E. coli*	4828	Weak
2	*E. coli*	5149	Weak	11	*E. coli*	159	Weak
3	*E. coli*	885	Strong	12	*E. coli*	3069	Weak
4	*E. coli*	2422	Strong	13	*E. coli*	228	Strong
5	*Pseudomonas aeruginosa*	3316	Medium	14	*E. coli*	726	Strong
6	*Klebsiella pneumonia*	14	Medium	15	*E. coli*	4469	Weak
7	*Pseudomonas aeruginosa*	4935	Medium	16	*E. coli*	4745	Strong
8	*Staphylococcus aureus*	48629	Weak	17	*E. coli*	4701	Strong
9	*E. coli*	4221	Strong	18	*E. coli*	572	Strong

The percentages of biofilms inhibition formed using leaf methanol extract are shown in Fig. 1.

According to the results, the leaf methanol extract showed good biofilm inhibition effect on the clinical strains include *E. coli* (3056), *E. coli* (4221), *E. coli* (885), *E. coli* (4701) and *E. coli* (4745). The highest percentage of biofilm inhibition was against *E. coli* (885) with 85.7 ± 0.48%, and the lowest was against *E. coli* (726) with 0.56 ± 0.39%.

In another research, Abreu et al. have studied the biofilm inhibition effect of methanol extract of *P. domestica*, *P. avium* and *P. persica* on *S. aureus* (CECT976) strain. They found that these extracts have no apparent effect on biofilm inhibition. In contrast, the extracts exhibited inhibitory potency at concentrations above 4 g/l (Abreu et al., 2016).

4. Conclusion

In this study, the GC-MS analysis indicated that octadecenoic acid and 14-methyl-8-hexadecyn-1-ol were the main constituents in n-hexane fraction of the methanol extract of leaves of *P. mahaleb* L.

Moreover, the plant leaf has significant amounts of phenolic and flavonoid compounds. According to the results, the plant has an apparent effect on inhibition biofilm. This plant can be used as a good antioxidant and also be useful in the treatment of infectious diseases caused by some multidrug resistance strains that form biofilms. So it can be used in the food, pharmaceutical and cosmetics industries.

Abbreviations

GC-MS: Gas chromatography-mass spectrometry, DPPH: 2,2-diphenyl-1-picryl hydrazyl, BHT: Dibutyl hydroxytoluene, IC50: The half maximal inhibitory concentration, TPC: Determination of total phenolic content, DMSO: Dimethyl sulfoxide, GAE: Gallic acid equivalent, TFC: Determination of total flavonoid content, QE: Quercetin equivalent, BSLA: Brine shrimp lethality assay, VS: Vincristine sulfate, LC50: Lethal concentration 50, NA: Nutrient agar, TSB: Tryptic soy broth, PBS: Phosphate-buffered saline, ELISA: Enzyme-linked immunosorbent assay, OD: Optical density.

Declarations

Author Contributions

Zahra Dahtizadeh: MSc student carried out the experiment, data analysis and write the manuscript. Fereshteh Jookar Kashi and Zeinab Toluei: Supervisor conceived and planned the experiment.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Figures

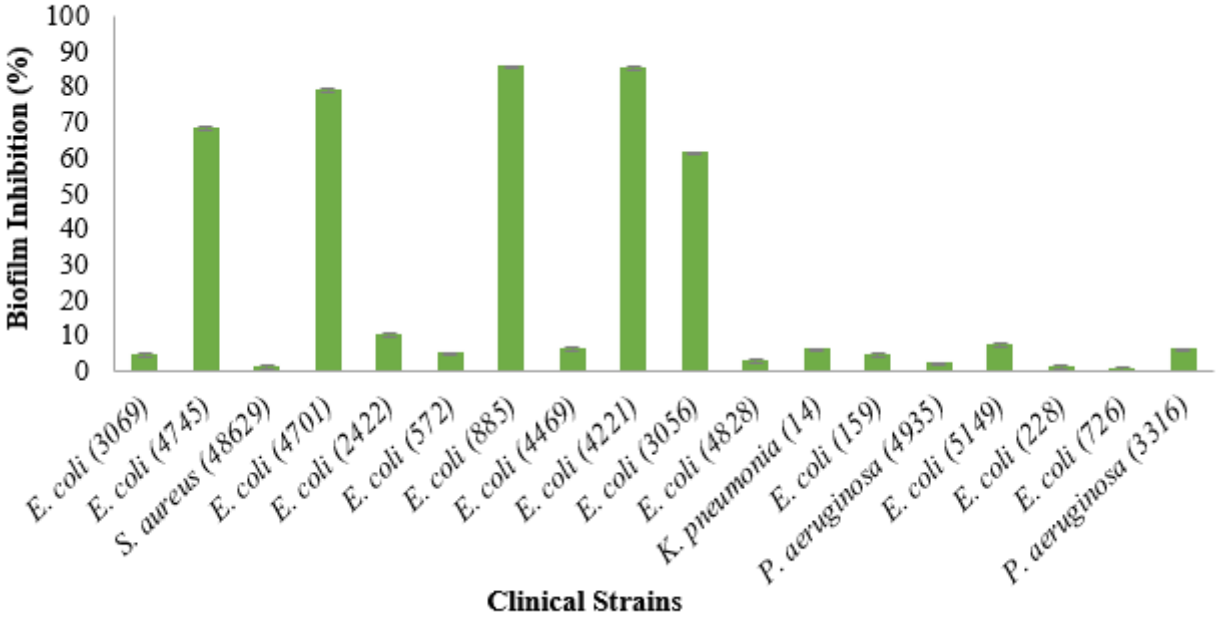

Figure 1

Biofilm inhibition percentage in the present of leaf methanol extract

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